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Insight into a Unique Carbon Resource Partitioning Mechanism in *Aggregatibacter actinomycetemcomitans*

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Insight into a Unique Carbon Resource Partitioning Mechanism in *Aggregatibacter actinomycetemcomitans*

by

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Dedication

This work is dedicated my friends and family.

Acknowledgements

I would like to acknowledge everyone I have worked with in the Whiteley lab. To Marvin: Thank you for all of your support and guidance. Thank you to everyone in the lab for being my extended family. To Brad: You are an amazing husband. Thank you for your love, support, and unparalleled patience.

Insight into a Unique Carbon Resource Partitioning Mechanism in *Aggregatibacter actinomycetemcomitans*

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Aggregatibacter actinomycetemcomitans is a Gram negative bacterium found exclusively in the mammalian oral cavity where it resides in the gingival crevice, the space between the tooth and gum tissue. Though it has historically been considered a common commensal organism, it is now appreciated that *A. actinomycetemcomitans* is an opportunistic pathogen associated with the diseases periodontitis and endocarditis. To cause infection, *A. actinomycetemcomitans* must interact and compete with neighboring bacteria for space and nutrients, though little is known about the physiology it employs within the gingival crevice. Using *A. actinomycetemcomitans* grown in a chemically defined medium containing carbon sources found in vivo, I use transcriptome analyses and growth studies to show that *A. actinomycetemcomitans* preferentially utilizes lactate over the phosphotransferase system (PTS) sugars glucose and fructose. Additionally, the presence of lactate or pyruvate inhibits the transport and metabolism of these sugars in a post-transcriptionally controlled process I have termed PTS substrate

exclusion. Since lactate is an energetically inferior carbon source, PTS substrate exclusion appears to be a carbon resource partitioning mechanism that allows *A. actinomycetemcomitans* to avoid competition for energetically favorable sugars with other species, and I propose a model to describe this phenomenon. To begin to understand the mechanism of PTS substrate exclusion, I examine the first step of the proposed model by purifying and characterizing the L-lactate dehydrogenase (LctD) from *A. actinomycetemcomitans.* I demonstrate that, unlike other studied lactate dehydrogenases, the LctD from *A. actinomycetemcomitans* does not exhibit feedback inhibition in the presence of physiologically relevant concentrations of pyruvate, which supports my hypothesis that elevated intracellular pyruvate levels inhibit the PTS. The results of my studies provide insight into a new regulatory mechanism governing carbon utilization in this bacterium.

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1.1 Importance of studying *A. actinomycetemcomitans*

1.1.1 Overview of *A. actinomycetemcomitans*

Aggregatibacter (Actinobacillus) actinomycetemcomitans is a Gram negative, non-motile, facultative anaerobe found exclusively in the mammalian oral cavity (71). *A. actinomycetemcomitans* is a member of the oral flora in approximately 25 to 50% (55, 70) of the human population and is considered to be both a common commensal organism and an opportunistic pathogen. It is found transiently in children as young as 20 days old (55), eventually becoming a stable member of the oral flora in adults with dental disease (21). *A. actinomycetemcomitans* is best known as a putative causative agent of periodontitis, a disease characterized by tissue destruction and tooth loss that most commonly results from poor dental hygiene (100, 123). In addition to periodontitis, *A. actinomycetemcomitans* is associated with the heart disease infective endocarditis and, more rarely, extra-oral abscesses (82, 83, 122).

1.1.2 *A. actinomycetemcomitans*-associated endocarditis

The diagnosis of *A. actinomycetemcomitans*-related endocarditis was first made in 1964 with a positive blood culture (66). Since that time, the association between *A. actinomycetemcomitans* and endocarditis has been made more apparent with modern molecular techniques. In 2000, PCR was used to detect the presence of *A. actinomycetemcomitans* DNA in atheromatous plaques (40), and, more recently, viable *A. actinomycetemcomitans* has been cultured from heart plaques (53). It is now accepted that infective endocarditis is the result of *A. actinomycetemcomitans* entrance into the bloodstream, where it is then targeted to the heart by an adhesin that binds to the collagen commonly found in cardiac tissues (63, 110)

There is a correlation between infective endocarditis and poor dental hygiene; people with periodontitis are 25% more likely to have heart disease (23). Periodontal patients exhibit inflamed tissue and bleeding gums, which likely increases vascular accessibility for *A. actinomycetemcomitans* and places them at risk for developing infective endocarditis. Consequently, prophylactic antibiotic treatments are commonly administered prior to dental procedures in at-risk patients. Once infective endocarditis is diagnosed, four to six weeks of intravenous antibiotic therapy is recommended, and more severe cases require surgical intervention (83). Thus, the association of this opportunistic oral pathogen with heart disease and related prophylactic and surgical procedures argues for maintaining oral health to maximize the overall health of an individual.

1.1.3 *A. actinomycetemcomitans*-associated periodontitis

Periodontitis affects the gingival tissue and teeth below the gum line in an area referred to as the gingival crevice or pocket, an environment distinct from the supragingival areas of the oral cavity such as the tooth surface or tongue (Fig 1.1). The onset of periodontitis is marked by a deepening of the gingival pocket, which leads to an influx of host immune cells and inflammatory compounds (31), and an overall increase in the Gram negative bacterial population (111). As the disease progresses, the alveolar bone and supporting ligaments that hold the tooth in place are destroyed by bacterial toxins and host factors, resulting in tooth loss (31). Unfortunately, patients often experience symptoms only periodically and the condition is diagnosed only upon dental examination when the disease is in more advanced stages (78). Once diagnosis is confirmed, mechanical debridement of the affected area is performed; however, this has been shown to be largely ineffective and antibiotics are often required to clear the infection (88). *A. actinomycetemcomitans* has the ability to live intracellularly (11, 20, 60, 91, 107), and this reservoir is thought to contribute to the return of *A. actinomycetemcomitans* to the gingival crevice after mechanical removal (91).

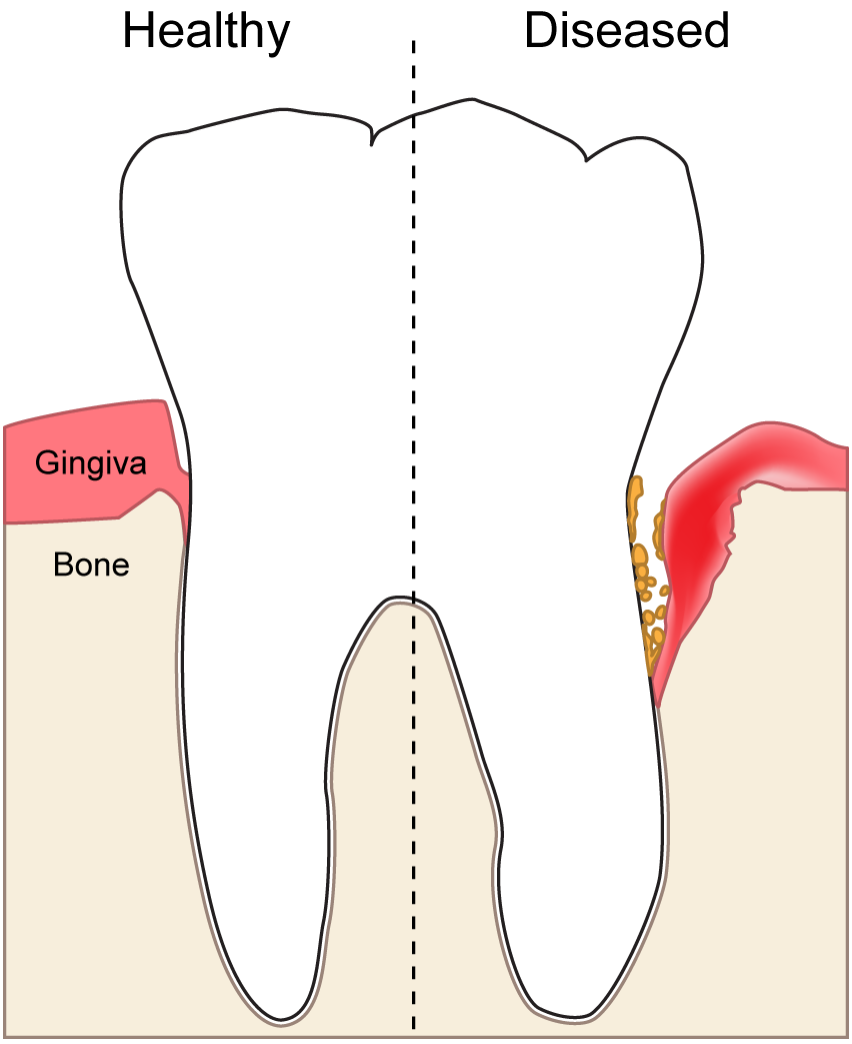

Figure 1.1. The gingival crevice. The base of the tooth is surrounded by gingival tissue, under which is alveolar bone and periodontal ligaments that hold the tooth in place. The space between the gingival and the tooth is referred to as the gingival crevice.

In the late 1800s and early 1900s, dental bacteriologist Sir Kenneth Goadby proposed that periodontitis is caused by a bacterial infection, though he could not attribute the disease process to one specific bacterium (116). Since that time it has been found that different types of periodontal disease can be caused by various members of the oral flora (22). The presence of *A. actinomycetemcomitans* in patients with periodontitis was first identified in 1976 (97). Further studies have demonstrated that *A. actinomycetemcomitans* most commonly impacts the incisors and first molars in a periodontal disease referred to as localized aggressive periodontitis (LAP), previously known as localized juvenile periodontitis (LJP) (22). *A. actinomycetemcomitans* produces a variety of virulence factors that contribute to the severity of LAP, including a leukotoxin (3, 4, 113, 114), host immune suppression factors (95, 96), and bone resorbing factors (45, 48, 72, 120). In addition, there are multiple predisposing factors that increase host susceptibility to LAP including advanced age and smoking (39, 56), diabetes (39) and African-American descent (6).

It is estimated that 90% of the population is affected by some form of periodontitis (12). Due to the episodic nature of periodontitis, the transition from mild to severe disease is poorly understood (78). Therefore, understanding the disease progression and causative agents of LAP and other periodontal diseases, and their association with heart disease, emphasize the need to investigate *A. actinomycetemcomitans*.

1.2 *A. actinomycetemcomitans* in the oral cavity

1.2.1 The gingival crevice

The gingival crevice contains a robust microbial population that includes *A. actinomycetemcomitans*. It is consistently bathed in crevicular fluid, which differentiates it from the saliva-immersed tooth surface. Crevicular fluid is a plasma exudate that passes through the gingiva and flows along the teeth (58). It contains proteins (101) and host immune components (26), in addition to a number of potential carbon sources including sugars, small organic acids, and amino acids (10, 30, 58, 121). This fluid is thought to cleanse the gingival crevice from debris and bacterial cells (26, 117) that contribute to plaque accumulation, although the presence of carbon sources in crevicular fluid suggests it also may serve as a nutrient source for these cells.

The gingival crevice is also a unique area of the oral cavity on the account of the oxygen gradient present that decreases with increasing pocket depth (57). This is significant for oral flora because the more oxygenated areas contain potentially toxic reactive oxygen species such as hydrogen peroxide and superoxide that evolve from respiration by both host cells and bacteria in aerobic environments while the deeper, anoxic portions contain numerous anaerobes that contribute to the diversity of the gingival pocket. This aspect, along with the presence of gingival crevicular fluid, the isolated nature of the crevice and reduced mechanical disturbance create an area conducive to bacterial proliferation and, consequently, an increased risk for periodontitis.

1.2.2 The polymicrobial environment in the gingival crevice

In addition to *A. actinomycetemcomitans*, the gingival crevice contains a diverse polymicrobial population that differs from the population found in supragingival portions of the oral cavity. Increased interest in the gingival crevice occurred in the 1940s and 1950s, and studies were conducted to isolate and examine members of the gingival crevicular population. Many genera were identified, including *Fusobacterium*, *Streptococcus*, *Actinomyces*, and various Spirochetes (38, 90, 103). More recent studies using 16s rDNA sequencing technology have identified numerous previously uncultured species, highlighting the microbial diversity of the gingival crevice, as well as the limits of identification by culture techniques (54, 81). Despite the high bacterial diversity in the gingival crevice, a numerically large proportion of the population is comprised of a few species (54). Gram positive streptococcal species represent the largest percentage, sometimes comprising up to 60% of the gingival crevicular population (109). During times of periodontal disease, however, there is a shift in the subgingival flora that favors Gram negative species (69, 105). Taken together, these studies suggest that the subgingival consortium is highly dynamic and necessitates examining the interactions that occur within this unique and diverse environment to understand the balance between human health and disease.

1.2.3 *A. actinomycetemcomitans* in the gingival crevice

Although the importance of *A. actinomycetemcomitans* as a common commensal organism and a potential pathogen has been appreciated for some time, virtually nothing is known about the physiology of this bacterium. However, insights can be gleaned from studying *A. actinomycetemcomitans* in the area in which it grows. Due to the dynamic subgingival population, culture techniques, and the transient nature of *A. actinomycetemcomitans* in healthy adults, the portion of the population comprised by this organism in healthy periodontal sites is difficult to assess. However, studies of patients with periodontitis indicate that *A. actinomycetemcomitans* comprises anywhere from 4 to 26% of the gingival crevice population (85, 100, 111), primarily residing in the "moderate" pockets (4 to 6 mm in depth) in the gingival crevice (25). These moderate pockets differ from deeper subgingival pockets on the basis of increased oxygen availability (57), and controlled studies in the laboratory indicate that *A. actinomycetemcomitans* exhibits enhanced growth in the presence of oxygen (77). *A. actinomycetemcomitans* is also capnophilic, demonstrating superior growth in the presence of carbon dioxide (98, 99).

1.3 Metabolism of *A. actinomycetemcomitans*

1.3.1 Metabolic pathways in *A. actinomycetemcomitans*

A. actinomycetemcomitans has a limited carbon utilization profile, capable of catabolizing only a small number of sugars including glucose, fructose, maltose, and mannose (8), as well as the small organic acids lactate and pyruvate (15). However, *A. actinomycetemcomitans* is unable to utilize sucrose, peptides or amino acids as sole carbon sources (8). The sequenced genome enables *in silico* analysis that supports these findings, as *A. actinomycetemcomitans* encodes the metabolic machinery to carry out glycolysis and the pentose phosphate pathway, but only possesses a partial tricarboxylic acid (TCA) cycle (41).

In silico analysis also suggests that sugars utilized by *A. actinomycetemcomitans* are transported by the phosphotransferase system (PTS). The PTS is a common mode for bacterial carbohydrate transport and consists of ABC transporters and accessory proteins that actively transport and phosphorylate the sugars upon entry into the cell (Fig 1.2A). *A. actinomycetemcomitans* also possesses small organic acid permeases presumed to function as antiporters or symporters to transport substrates (Fig 1.2B). Very little is known about the way in which *A. actinomycetemcomitans* uses these systems or how they impact its growth within the oral cavity.

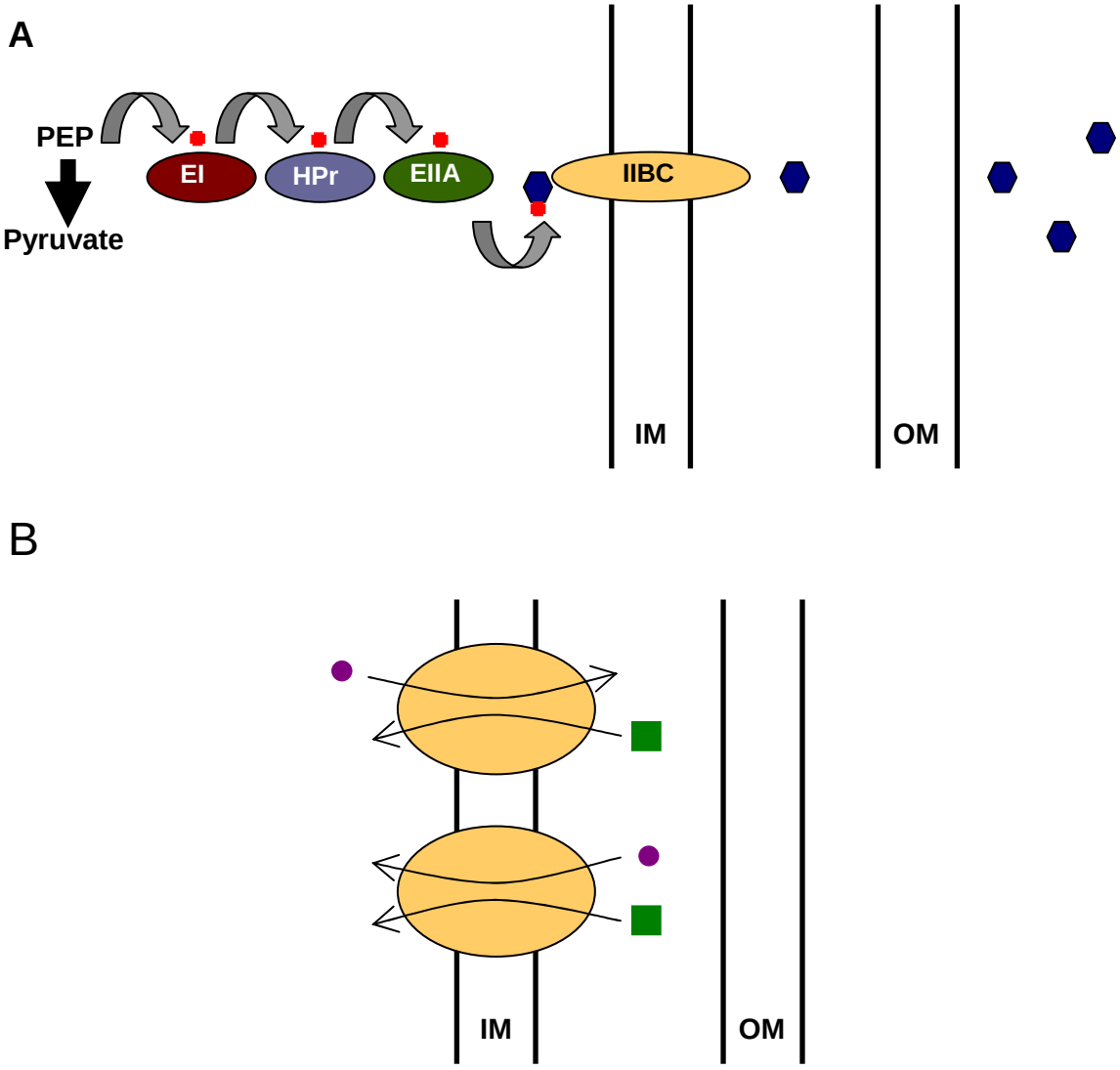

Figure 1.2. Transport systems. A) The phosphotransferase system. PTS sugars (⬢) cross the outer membrane (OM) via porins. Once in the periplasmic space, they are transported across the inner membrane (IM) by a specific transporter (IIBC). Upon entry into the cell, the sugar receives a phosphoryl group (●) that has been transferred along a series of proteins (EI, HPr, and EIIA), from an original phosphodonor, phosphoenolpyruvate (PEP). Each sugar has a specific EIIA, but all share the EI and HPr proteins. **B)** Antiporters (top) transport a substrate (■) into the cell while simultaneously transported an ion (●) out of the cell. Symporters couple the transport of an ion with transport of a substrate across the membrane.

1.3.2 Metabolism and virulence

For hundreds of years, the human body has been studied for its ability to serve as a host for bacterial pathogens. Successful pathogenesis relies on the ability to acquire nutrients necessary for growth and survival, yet relatively little is understood about the *in vivo* physiology and metabolism of most human pathogens. It is now being appreciated that nutritional availability can impact virulence in human pathogens including *Pseudomonas aeruginosa*, *Mycobacterium tuburculosis*, and uropathogenic *E. coli* (79, 80, 89). Interestingly, there has been a link between virulence and metabolism in *A. actinomycetemcomitans.* One study found that leukotoxin synthesis, important for *A. actinomycetemcomitans* pathogenesis, is influenced by levels of fructose present (46). Another study reported that leukotoxin production is dependent upon expression of a gene required to transport fructose and other PTS sugars (47). These findings underscore the importance of carbon source utilization for microbial disease progression in the oral cavity.

While investigations into the metabolism of individual members of the gingival crevicular flora are important, it is also beneficial to understand the metabolic interplay between the microbiota in this environment. The gingival pocket of most individuals is dominated by *Streptococcus* species, which divide rapidly and consume sugars (109). Therefore, it is likely that *A. actinomycetemcomitans* and other species must interact and compete with *Streptococcus* and each other for the carbon sources they are able to utilize. Indeed, these carbon source-related interactions are known to exist for some members of the oral cavity. For instance, the Gram negative anaerobe *Veillonella*

alcalescens is unable to ferment glucose, and therefore, associates with *Streptococcus mutans* to feed off of the metabolic byproduct lactate produced by *Streptococcus* (61). Thus, the cross-feeding relationship that occurs between *Streptococcus* and *Veillonella* species suggests that members of the subgingival population have adapted to both the host environment and the presence of each other, setting precedence for understanding the metabolic processes and microbial interactions of the potential pathogen *A. actinomycetemcomitans* in the gingival crevice.

1.4 Dissertation objectives

A. actinomycetemcomitans is found exclusively in the mammalian oral cavity, yet neither the physiology nor metabolism of this organism in its natural environment, or the metabolic networks required for growth and persistence in the gingival crevice have been well studied. *A. actinomycetemcomitans* is consistently exposed to a serum exudate containing defined carbon sources and host immune factors, in addition to nearby microorganisms competing for a stronghold in this oral community. Therefore, the focus of my dissertation was to achieve an understanding of how *A. actinomycetemcomitans* competes for and utilizes carbon sources in the complex, polymicrobial environment in which it is found.

This dissertation is composed of four chapters. Chapter one is an introduction to *A. actinomycetemcomitans*, the diseases with which it is associated, and the current state of knowledge regarding its metabolic capacity. In chapter two I use a chemically

defined medium to examine carbon source utilization and preference. Through transcriptome analyses and radiolabeled substrate studies, I discovered a novel carbon resource partitioning mechanism in which lactate, an inferior carbon source in terms of energy and growth yield, is used preferentially over the PTS sugar glucose. I named this process PTS substrate exclusion, and I have proposed a model for this phenomenon. Chapter three describes the purification and characterization of the L-lactate dehydrogenase of *A. actinomycetemcomitans*, an enzyme required for its PTS substrate exclusion. Kinetic studies and inhibition assays with this enzyme provide evidence that supports our model. Finally, in chapter four I discuss the conclusions and future directions established by my work.

Chapter 2: A Novel Exclusion Mechanism for Carbon Resource Partitioning in *A. actinomycetemcomitans*

2.1 Introduction

The mammalian oral cavity is a diverse environment that contains an estimated 500 bacterial species (52). Although the microbiology of the oral cavity has been the subject of study for many years, surprisingly little is known about the basic nutritional preference of oral bacteria. Compared to many common bacteria within the oral cavity, such as the streptococci, *A. actinomycetemcomitans* grows slowly and possesses the enzymatic capabilities to utilize only a small number of carbon sources including glucose, fructose, maltose, mannose, and lactate (7). Streptococci on the other hand, are fast-growing bacteria capable of quickly transporting and consuming a diversity of carbohydrates to produce small organic acids, primarily lactate. Importantly, cultural studies and investigations using specific DNA methods (54, 104) provide evidence for multiple species of streptococci in the gingival crevice. Depending upon the subject and method of sampling, the concentration of streptococci capable of producing lactate in the gingival crevice can vary from approximately 5% (69) to over 60% (109) of the recoverable flora.

The first step in carbon catabolism is solute transport into the cell by active or passive means. In many bacteria, transport is accomplished by carbon-specific systems including the phosphoenolpyruvate (PEP)-dependent phosphotransferase systems

(PTS) (84). PTS utilize phosphoryl transfer proteins to ultimately transfer phosphate from PEP to the transported carbohydrate. Aside from solute transport, PTS also function to exclude other non-PTS carbohydrates in a process termed inducer exclusion (92). Inducer exclusion involves inhibition of proteins involved in transport/metabolism of non-PTS carbon sources by PTS system proteins; thus providing the bacterium with a molecular preference mechanism for PTS carbon sources.

The slow growth rate and limited carbon catabolic capabilities of *A. actinomycetemcomitans* present theoretical problems for survival of this bacterium in the oral cavity. For example, *A. actinomycetemcomitans* possesses overlapping carbon substrate utilization profiles with many faster-growing bacteria in the oral cavity (such as streptococci). This is important in the oral cavity as metabolizable carbon likely limits growth and composition of the oral microbiota (32, 58, 62, 108). One potential survival mechanism for *A. actinomycetemcomitans* is to evolve carbon usage preferences that reduce competition with faster growing members of the community. In many environments, competition for nutrients often leads to the evolution of resource (niche) partitioning, a phenomenon by which resources (such as catabolizable carbon sources) are subdivided such that competition is minimized. This study provides evidence that when provided the physiologically relevant carbon sources glucose, fructose, and lactate *A. actinomycetemcomitans* prefers lactate. This occurs despite the fact that *A. actinomycetemcomitans* grows faster and obtains higher cell yields during growth with carbohydrates. This preference for lactate is mediated by a novel exclusion mechanism in which metabolism of lactate to pyruvate inhibits uptake of PTS carbohydrates.

2.2 Materials and Methods

2.2.1 Bacterial Strains and Growth Media.

A. actinomycetemcomitans strains VT1169 (65) and Y4 (3) along with *S. gordonii* strain Challis DL1.1 (ATCC 49818) were used in these studies. Liquid cultures were grown in the chemically defined Socransky's medium (102) lacking DL-mevalonic acid and hemin (referred to as Chemically Defined Medium, CDM). Cultures were grown with shaking at 165 RPM in a 37˚C incubator with a 10% CO_2 atmosphere. For growth curves, overnight cultures grown in CDM supplemented with 20 mM glucose, fructose, or L-lactate were diluted to an optical density at 600 nm (OD_{600nm}) of 0.015 in CDM supplemented with 20 mM carbon source.

2.2.2 DNA manipulations and construction of *A. actinomycetemcomitans* mutants.

Standard methods were used to manipulate plasmids and DNA fragments (2). Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs. Chromosomal DNA from *A. actinomycetemcomitans* was isolated using DNeasy Tissue kits (Qiagen) and plasmid isolations were performed using QIAprep spin miniprep kits (Qiagen). DNA fragments were purified using QIAquick mini-elute PCR purification kits (Qiagen), and PCR was performed using the Expand Long Template PCR System (Roche).

For creation of the *A. actinomycetemcomitans lctD* mutant, a 550 bp internal fragment of *lctD* was amplified by PCR using the primers ldh-for (GAAGATCTCTGACGGTGGTTCTTATGCAG) and ldh-rev (GGGGTACCCATACCTGAGTGCATATCCCG) and cloned into the T/A cloning vector pGemT-easy (Promega) to create pSB100. pSB100 was digested with EcoRI to remove the *lctD* internal fragment and ligated into the EcoRI digested suicide plasmid pVT1461 (64) to create pSB101. pSB101 was transformed into *E. coli* SM10 λ pir and introduced into *A. actinomycetemcomitans* by conjugation (65). *A. actinomycetemcomitans lctD* mutants containing an integrated copy of pSB101 (by Campbell-type recombination) were selected on tryptic soy agar plates containing 0.5% yeast extract and 50 μg/ml spectinomycin. Inactivation of *lctD* was confirmed using PCR.

2.2.3 Microarray studies.

Logarithmic *A. actinomycetemcomitans* (OD_{600nm} = 0.1) were collected and mixed 1:1 with the RNA stabilizing reagent RNALater (Ambion). RNA was isolated using the Gentra Versagene RNA isolation kit. DNA contamination was assessed with PCR amplification of the *clpX* gene, and RNA integrity was monitored with agarose gel electrophoresis of glyoxylated samples (Ambion). RNA was prepared for hybridization to a custom *A. actinomycetemcomitans* Affymetrix GeneChip microarray as previously described (87, 94). Washing, staining, and scanning of the GeneChips was performed at the University of Iowa DNA core facility using an Affymetrix fluidics station. GeneChips were performed in duplicate or triplicate for each condition. Data analysis was performed using GeneChip Operating Software version 1.4 (GCOS).

2.2.4 Carbohydrate and small molecule measurements.

A. actinomycetemcomitans grown in CDM supplemented with 20 mM glucose, fructose, or L-lactate were washed with warm CDM containing no carbon source and resuspended to an OD_{600nm} of 0.1 in 50 ml warm CDM supplemented with 2 mM each fructose, glucose, and L-lactate. One ml samples were collected at various time points, centrifuged in a tabletop centrifuge at 8000 rpm for 2 minutes, and the supernatants were filter sterilized through a 0.2 µm filter. Supernatants were stored at -20˚C until analyzed for the presence of fructose and glucose using the fructose assay kit and glucose assay kit (Sigma-Aldrich, Catalog # SA-20 and GAHK-20 respectively). Lactate concentrations were determined using a lactate assay kit (SUNY at Buffalo, Catalog # A-108). For lactate spike studies, overnight cultures of *A. actinomycetemcomitans* grown in CDM supplemented with 20 mM glucose or fructose were washed with warm CDM containing no carbon source and resuspended to an OD_{600nm} of 0.1 in warm CDM containing 2 mM glucose or fructose. After 1 hour incubation, 2 mM L-lactate was added and consumption of carbohydrates and L-lactate was monitored over time as described above. For intracellular pyruvate levels, cell extracts were prepared as outlined (112) using logarithmic carbohydrate and L-lactate-grown bacteria, and pyruvate levels measured as previously described (1) using sodium pyruvate (Sigma) to generate a standard curve.

2.2.5 Radiolabeled substrate studies.

For glucose uptake studies, an overnight culture of *A. actinomycetemcomitans* grown in CDM supplemented with 20 mM glucose was diluted 1:1 with warm CDM supplemented with 20 mM glucose and incubated for 1 hour shaking at 37˚C. The cells were then washed with warm CDM containing no carbon source and resuspended to an OD_{600nm} of 0.1 in CDM supplemented with 200 µM or 2 mM L-lactate, 2 mM fructose, or water. The cultures were incubated in a 37℃ heating block. After 5 minutes, 100 µM uniformly ^{14}C labeled glucose [U-^{14}C-glucose] (Amersham) was added to each sample. At various time points, 100 µl samples were removed and quenched in 2.9 ml ice-cold CDM containing 20 mM unlabeled glucose. Each sample was filtered through a 0.2 µm filter, followed by 3 ml ice-cold CDM, followed by 10 ml air. Filters were placed in scintillation vials containing 4 ml Ecolite scintillation fluid and counted in an LS6500 scintillation counter (Beckman-Coulter). As a control for non-specific (background) radioactivity, glucose-grown heat-killed *A. actinomycetemcomitans* (95℃ for 10 min.) were utilized.

2.2.6 *A. actinomycetemcomitans* consumption of *S. gordonii*-produced lactate.

Overnight cultures of *S. gordonii* were washed and suspended in CDM supplemented with 2 mM sucrose. Growth was monitored and samples collected throughout the growth curve and frozen at -20℃ until analyzed for sucrose with a sucrose assay kit (Sigma, product code #SCA-20) and lactate as described above. Once

in stationary phase, cells were removed by centrifugation, the pH of the supernatant was adjusted to 7.0 and the supernatant was filter sterilized through a 0.2 μm filter. The supernatant was then inoculated with *A. actinomycetemcomitans* to and OD_{600nm} of 0.025. Samples were removed and analyzed for lactate disappearance.

2.3 Results

2.3.1 Growth kinetics of *A. actinomycetemcomitans* in defined medium with in vivo relevant carbon sources.

The composition of crevicular fluid is similar to that of plasma and as such, contains several potential energy sources for *A. actinomycetemcomitans* including glucose, fructose, mannose, and lactate. Two of these energy sources (glucose and lactate) are commonly found at high levels (approximately 1 to 5 mM) (73) while the others are found at low micromolar levels in crevicular fluid/plasma (73, 106). We chose to focus on *A. actinomycetemcomitans* growth using glucose and lactate since they are the most prevalent in vivo, and fructose since it is a well-studied PTS-transported carbohydrate present at low levels in vivo (46, 67, 74-76). To examine growth using these in vivo relevant substrates, we utilized a chemically defined medium (CDM) supplemented with these substrates as sole energy sources. Growth kinetics indicate that *A. actinomycetemcomitans* grows well in CDM supplemented with glucose, fructose, and L-lactate as sole energy sources (Fig. 2.1). Doubling times were faster in glucose

(139 min) and fructose (143 min) than in lactate (204 min), and final cell yields were approximately two fold higher after growth with carbohydrates as compared to lactate (Fig. 2.1).

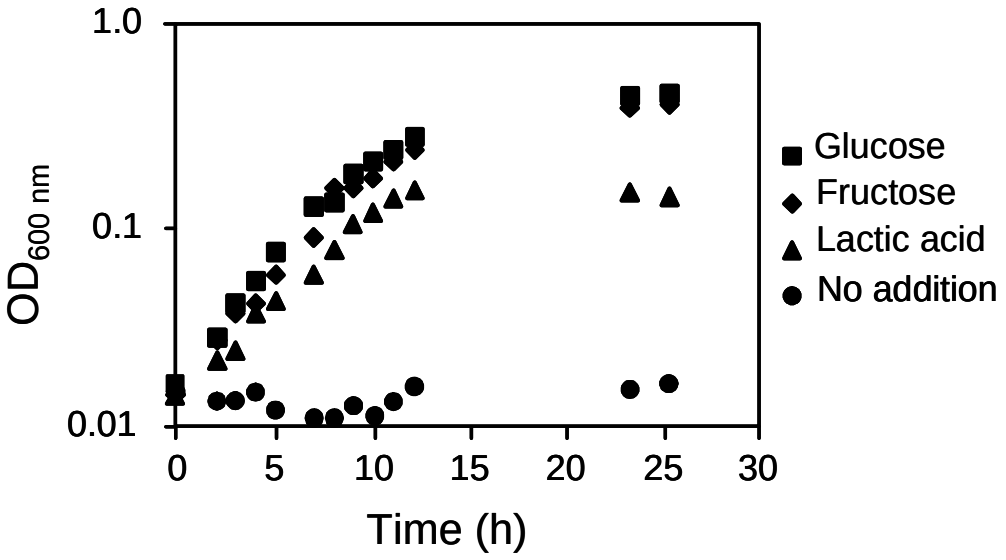

Figure 2.1. Growth of *A. actinomycetemcomitans* in chemically defined medium. CDM containing glucose (■), fructose (♦), L-lactate (▲), and no energy source (●). Bacteria were grown shaking (165 rpm) at 37℃ with a 10% CO_2 atmosphere with 20 mM carbon source. Growth curves were performed at least three times and representative data is provided.

2.3.2 *A. actinomycetemcomitans* prefers lactate to glucose and fructose.

Microorganisms often exhibit catabolic preference for specific carbon sources. Although *A. actinomycetemcomitans* is capable of growth using glucose, fructose, and lactate, nothing is currently known about carbon preference in this bacterium. To examine preference, exponential *A. actinomycetemcomitans* were washed, suspended in CDM containing these three substrates, and the cultures examined for carbon substrate disappearance. Within 2 hours, *A. actinomycetemcomitans* consumed all detectable lactate while most of the glucose (~90%) and fructose (>90%) remained (Fig. 2.2). Upon disappearance of lactate, glucose and fructose were consumed concomitantly with glucose disappearance occurring at a slightly elevated rate compared to fructose. These results indicate that although *A. actinomycetemcomitans* doubles approximately one hour slower with lactate compared to glucose/fructose, this bacterium preferentially catabolizes lactate. The growth substrate of the bacterial inoculum did not impact this preference, as identical carbon consumption profiles were observed when bacteria were grown in glucose, lactate, and fructose before washing and resuspension (data not shown).

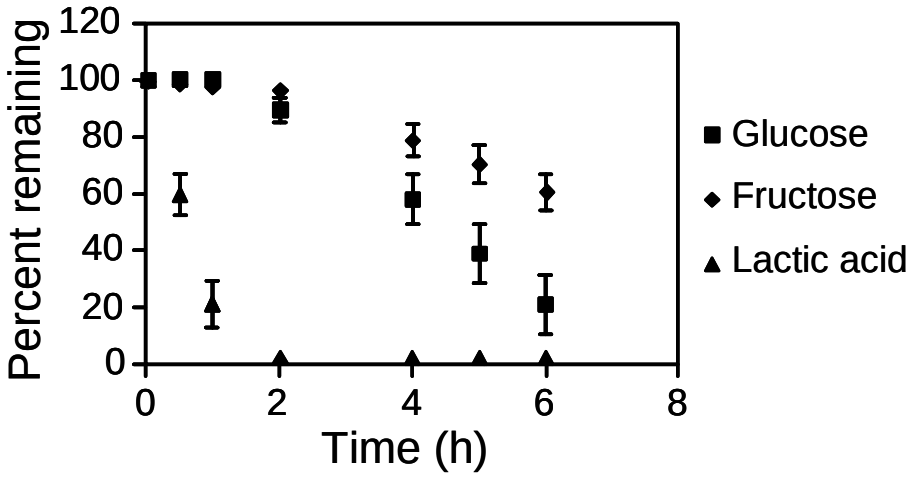

Figure 2.2. Glucose, fructose, and L-lactate consumption by *A. actinomycetemcomitans*. *A. actinomycetemcomitans* was grown in the presence of equimolar (2 mM) glucose, fructose, L-lactate and disappearance of these carbon substrates evaluated over time. Experiments were performed in duplicate. Error bars represent standard deviation and in some cases are too small to be seen.

2.3.3 Lactate rapidly inhibits glucose consumption.

Our carbon consumption experiments indicated that when provided equimolar amounts of glucose, fructose, and lactate, *A. actinomycetemcomitans* will selectively consume all detectable lactate before glucose and fructose. From these experiments, it is not clear if addition of lactate immediately inhibits glucose consumption, or if the effect is delayed. The kinetics of lactate-mediated inhibition of carbohydrate catabolism are critical, as it will provide clues to the mechanism of this process. To examine the kinetics of this process, *A. actinomycetemcomitans* was suspended in CDM containing glucose. After one hour, *A. actinomycetemcomitans* consumed approximately 20% of the glucose (Fig. 2.3A). Lactate was then added to the culture and glucose and lactate levels were measured for the next two hours. Lactate completely inhibited glucose consumption by *A. actinomycetemcomitans*, and this inhibition was not alleviated until all detectable lactate was consumed (Fig. 2.3A). Similar experiments revealed that lactate also inhibited fructose consumption, consistent with its effect on glucose (Fig. 2.3B).

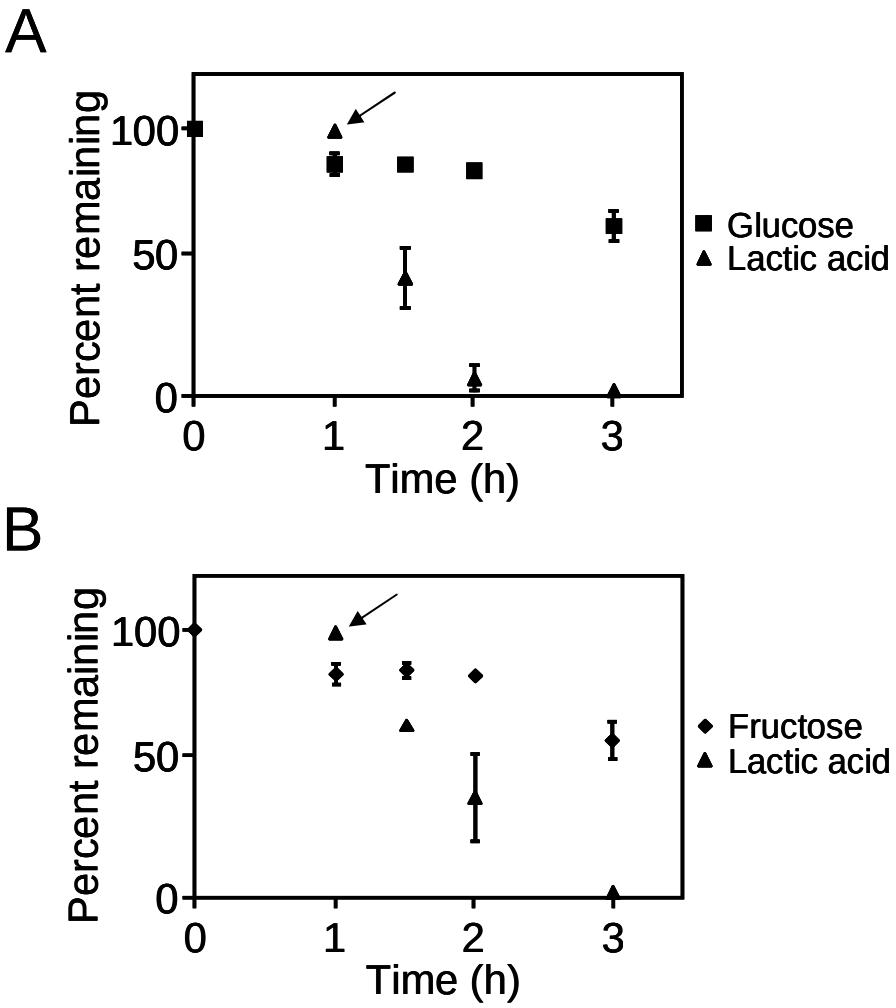

Figure 2.3. Addition of lactate inhibits glucose and fructose consumption by *A. actinomycetemcomitans*. *A. actinomycetemcomitans* was inoculated into CDM containing glucose (A) or fructose (B) at time zero. After growth for one hour, L-lactate was added (designated by the arrow) and consumption of each carbon source monitored for three hours. Experiments were performed in duplicate. Error bars represent standard deviation and in some cases are too small to be seen.

2.3.4 Lactate inhibits glucose transport in *A. actinomycetemcomitans*.

Although it is clear that *A. actinomycetemcomitans* preferentially metabolizes lactate, the mechanism of lactate-mediated catabolite repression is unknown. In many bacteria, carbon preference is often controlled at the transcriptional level. Since lactate is the preferred carbon source in *A. actinomycetemcomitans*, one potential mechanism is that lactate represses transcription of genes required for glucose and fructose catabolism. To test this mechanism, a transcriptome analysis of *A. actinomycetemcomitans* was performed for glucose, fructose, and lactate-grown cells using a custom Affymetrix GeneChip microarray. Results from these experiments revealed that genes involved in transport and catabolism of glucose and lactate are not significantly regulated (five-fold change or greater) at the transcriptional level by the different carbon-sources (Table 2.1). RT-PCR was used to verify expression of AA0332, AA0335, AA1601, and AA1876 (data not shown).

Table 2.1. mRNA levels for carbon-specific transport/catabolic genes.

	Signal Intensity (x1000)*		
Gene[†]	Fructose (SD)	Glucose (SD)	Lactate (SD)
Fructose catabolism			
AA0332, fructose-specific IIA	**16 (0.8)**	**7.9 (0.3)**	**10 (0.3)**
AA0335, fructose-specific IIBC	**12 (0.9)**	**6.8 (0.8)**	**8.2 (0.6)**
Glucose catabolism			
AA1601, glucose specific IIA	**25 (0.2)**	**32 (1.3)**	**28 (1.5)**
AA1876, glucose-specific IIBC	**4.3 (0.1)**	**5.1 (0.6)**	**7.7 (0.6)**
Lactate catabolism			
AA2749, L-lactate dehydrogenase	**28 (0.6)**	**36 (1.6)**	**31 (2.6)**
AA2751, Lactate permease	**13 (0.6)**	**13 (0.4)**	**11 (1.9)**
Shared PTS components			
AA1600, PTS system, enzyme I	**14 (0.5)**	**21 (2.2)**	**16 (1.1)**
AA1599, Phosphocarrier protein HPr	**23 (0.8)**	**29 (2.0)**	**25 (0.1)**

* Obtained from Affymetrix GeneChips using GCOS software. Four comparisons each were performed for logarithmic bacteria growing with fructose, glucose, or lactate. SD is standard deviation. RT-PCR was used to verify expression of AA0332, AA0335, AA1601, and AA1876 (data not shown).

[†] ORF numbers are from the *A. actinomycetemcomitans* genome sequencing project www.genome.ou.edu/act.html.

In silico examination of the *A. actinomycetemcomitans* genome reveals that transport of four of the five known growth substrates (glucose, fructose, mannose, and maltose) likely proceed via PTS (although alternative transport mechanisms may exist for maltose); while transport of lactate likely proceeds via a proton driven transporter (encoded by *lctP*) (www.genome.ou.edu/act.html). Thus, a potentially effective mechanism for lactate preference is for lactate to inhibit uptake of PTS carbohydrates into the cell. This mechanism is supported by our observation that addition of lactate rapidly and completely abrogates consumption of the PTS-transported carbohydrates glucose/fructose (Fig. 2.3). To examine the impact of lactate on carbohydrate transport, exponential glucose-grown *A. actinomycetemcomitans* were washed and resuspended in CDM containing no catabolizable carbon source. Lactate, fructose, or water (as a control) was added to the cell suspensions followed by ^{14}C-labeled glucose, and cell-associated radioactivity examined. Linear up-take of glucose was observed in bacteria treated with water and fructose (Fig. 2.4A); however no radioactivity (compared to the background) was found associated with bacteria exposed to lactate. These results indicate that lactate addition inhibits uptake of glucose by *A. actinomycetemcomitans*. The inhibition of glucose transport was not specific to the concentration of lactate utilized as experiments with 10-fold less lactate (200 μM) exhibited similar results (data not shown). This inhibition of glucose transport is also not strain specific as lactate inhibited glucose transport in another common laboratory strain of *A. actinomycetemcomitans* (Fig. 2.4B).

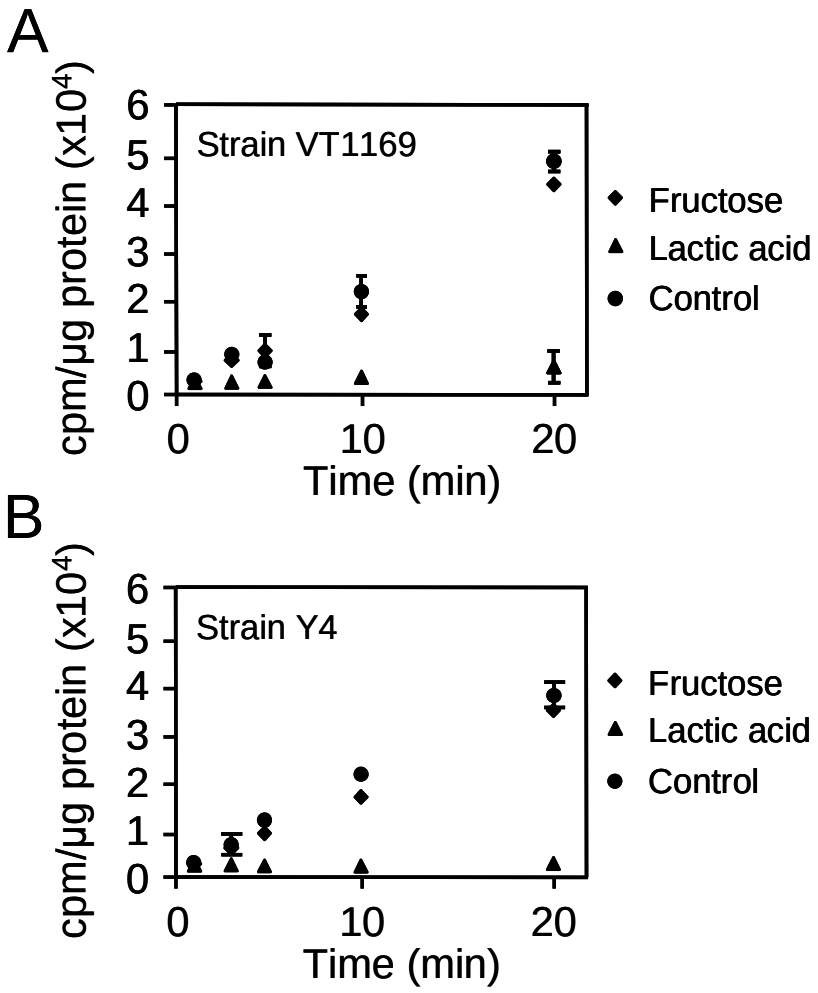

Figure 2.4. Lactate inhibits up-take of glucose in *A. actinomycetemcomitans* strains VT1169 (A) and Y4 (B). *A. actinomycetemcomitans* was suspended in CDM containing fructose (♦), L-lactate (▲), or no energy source (●). Radiolabelled glucose (U-^{14}C-glucose) was then added and uptake examined as outlined in Materials and Methods. Uptake of radiolabelled glucose is expressed as counts per minute (cpm)/µg of *A. actinomycetemcomitans* protein. Experiments were performed in triplicate. Error bars represent standard deviation and in some cases are too small to be seen. Background radioactivity using heat-killed cells was 187±27 cpm/µg protein.

2.3.5 Pyruvate also inhibits glucose transport in *A. actinomycetemcomitans.*

Our results indicate that addition of lactate inhibits transport of glucose; however it is not clear if this effect is mediated by lactate itself or a downstream product of lactate metabolism. The first step in lactate catabolism is the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase. There are several potential fates of pyruvate but the primary products formed during lactate fermentation are mixtures of propionate, acetate, formate, and succinate (44, 119). Thus, it is plausible that one of these products, and not lactate itself, is responsible for the inhibition of glucose transport and consumption. To test this, the impact of pyruvate, propionate, acetate, formate, and succinate on glucose transport was assessed. Results from these experiments reveal that pyruvate, but not the other organic acids, inhibit glucose uptake by *A. actinomycetemcomitans* (Fig. 2.5A&B).

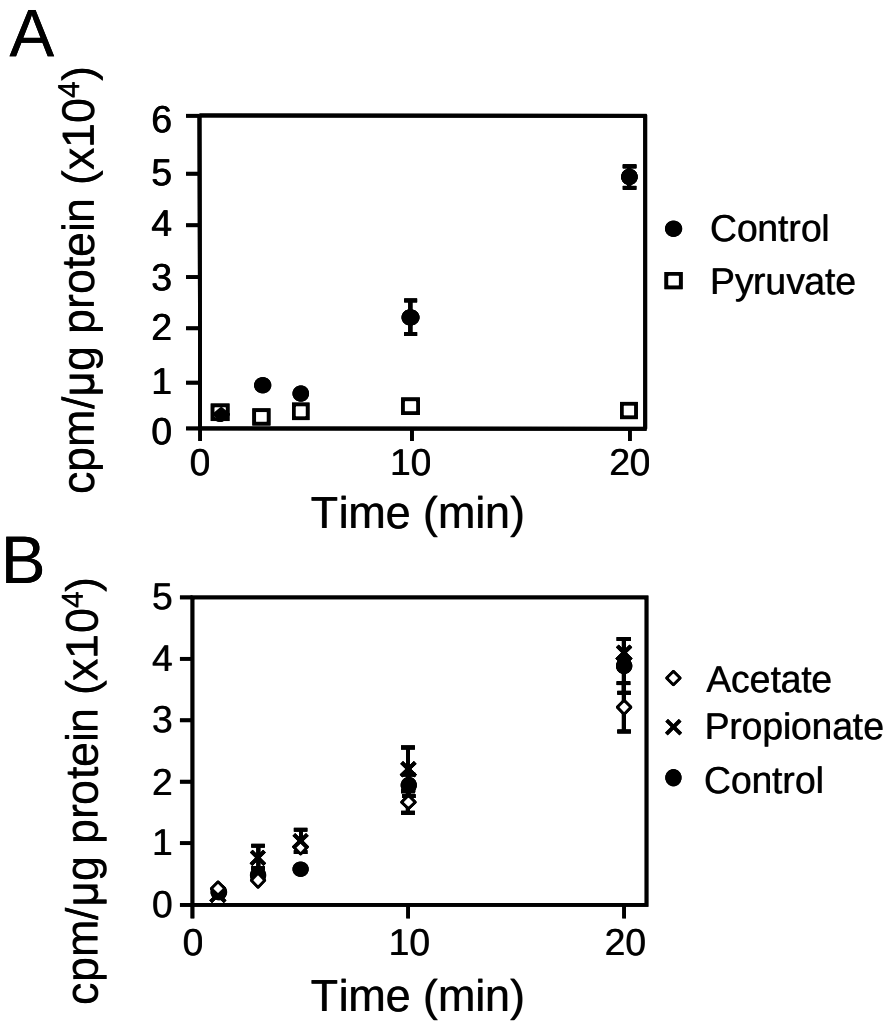

Figure 2.5. Pyruvate inhibits uptake of glucose in *A. actinomycetemcomitans*. *A. actinomycetemcomitans* was suspended in CDM and incubated with no energy source (●), pyruvate (□) (A) or no energy source (●), acetate (◊), propionate (×) (B). Radiolabelled glucose (U-^{14}C-glucose) was then added and uptake examined as outlined in Materials and Methods. Uptake of radiolabelled glucose is expressed as counts per minute (cpm)/µg of *A. actinomycetemcomitans* protein. Formate and succinate also had no effect on glucose transport (data not shown). Experiments were performed in duplicate. Error bars represent standard deviation and in some cases are too small to be seen.

2.3.6 Lactate catabolism is required for inhibition of glucose transport.

Two possibilities exist to explain the observations that both lactate and pyruvate inhibit *A. actinomycetemcomitans* glucose transport: 1) both pyruvate and lactate effectively inhibit glucose transport; 2) pyruvate alone is responsible for this transport inhibition. To discriminate between these possibilities, we genetically inactivated the L-lactate dehydrogenase gene (*lctD*) in *A. actinomycetemcomitans*. LctD is required for conversion of L-lactate to pyruvate, thus the *lctD* mutant is unable to grow using L-lactate as a sole energy source but grows normally on glucose (data not shown). We then examined the impact of lactate on glucose transport in the *lctD* mutant, with the rationale that the inability of this strain to convert lactate to pyruvate will provide clues to the direct effect of lactate on glucose transport. Results from these experiments reveal that lactate does not inhibit glucose transport in the *lctD* mutant (Fig. 2.6) indicating that the ability to catabolize lactate is required for lactate-mediated inhibition of glucose transport. It should be noted that as in the wt, pyruvate inhibits glucose transport in the *lctD* mutant (data not shown). These data strongly support that pyruvate, and not lactate, is critical for inhibition of glucose transport during growth with lactate.

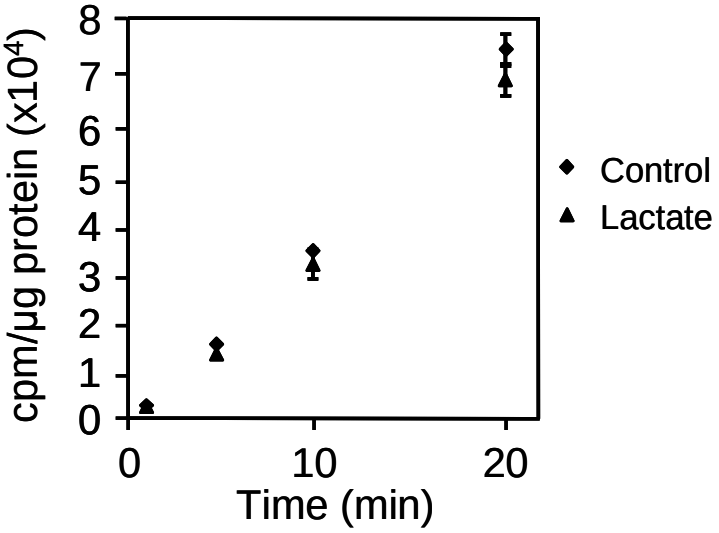

Figure 2.6. Lactate dehydrogenase is required for inhibition of glucose uptake. The *A. actinomycetemcomitans* L-lactate dehydrogenase (*lctD*) mutant was suspended in CDM and incubated with no energy source (♦) or L-lactate (▲). Radiolabelled glucose was then added and uptake examined as outlined in Materials and Methods. Uptake of radiolabelled glucose is expressed as counts per minute (cpm)/µg of *A. actinomycetemcomitans* protein. Experiments were performed in duplicate. Error bars represent standard deviation and in some cases are too small to be seen.

2.3.7 Growth with lactate increases intracellular levels of pyruvate.

Although it is clear that pyruvate inhibits glucose transport in *A. actinomycetemcomitans*, the molecular mechanism is not known. Obviously pyruvate will be generated during growth on all substrates; however it is possible that the levels of intracellular pyruvate generated are substrate specific. Based on previous studies showing that high levels of pyruvate significantly inhibit PTS carbohydrate uptake (42, 92, 93), we hypothesized that intracellular levels of pyruvate increase during *A. actinomycetemcomitans* growth with lactate. To test this hypothesis, intracellular pyruvate levels were measured during growth with lactate and the PTS transported sugars glucose and fructose. Results from these experiments indicate that the intracellular levels of pyruvate increase significantly (>10-fold) during growth using lactate as opposed to PTS carbohydrates (Fig. 2.7).

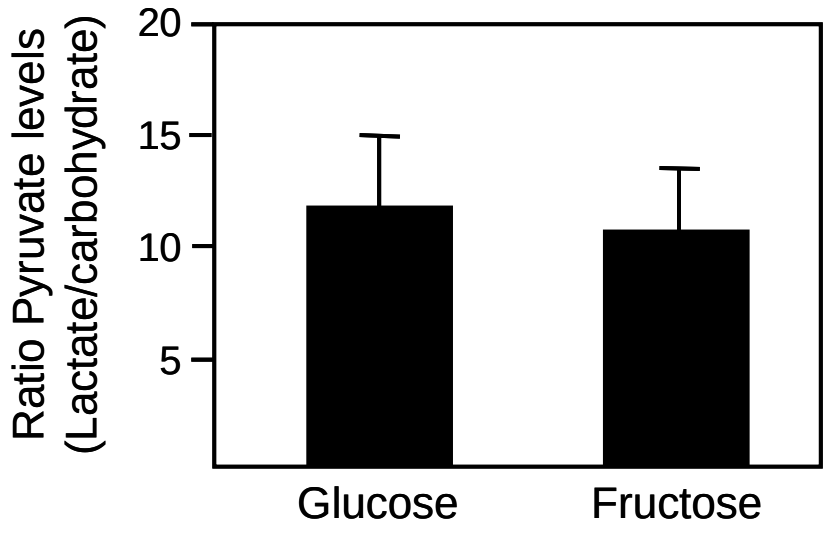

Figure 2.7. Lactate-grown *A. actinomycetemcomitans* contain higher levels of pyruvate. Intracellular levels of pyruvate were measured for logarithmic glucose, fructose, and L-lactate-grown *A. actinomycetemcomitans* as previously described (1) and are presented as fold increases during growth with L-lactate. Error bars represent standard deviation, and experiments were performed in triplicate. Intracellular volume was calculated based on normal size measurements of *A. actinomycetemcomitans* (1.6 μm x 0.5 μm x 0.5 μm).

2.3.8 The biological significance of lactate preference.

A. actinomycetemcomitans resides in the human oral cavity in a multi-species consortium including numerous species of the genus *Streptococcus*. *Streptococcus spp.* generally possess fast doubling times (<1 hour) and comprise a large percentage of the total bacteria within the oral cavity (24, 33). Most streptococci produce significant amounts of lactate which cause localized decreases in pH. Based on *A. actinomycetemcomitans* preference for lactate, we hypothesized that *A. actinomycetemcomitans* would consume lactate produced by streptococci. To test this hypothesis, *Streptococcus gordonii*, a prevalent streptococci within the oral cavity, was grown in CDM containing sucrose. As expected, *S. gordonii* consumed the sucrose with a concomitant production of lactate (Fig. 2.8). Upon addition of *A. actinomycetemcomitans*, lactate was consumed (Fig. 2.8). These results indicate that although *A. actinomycetemcomitans* does not grow using sucrose, this bacterium is capable of growing on lactate produced by sucrose-grown *Streptococcus gordonii*.

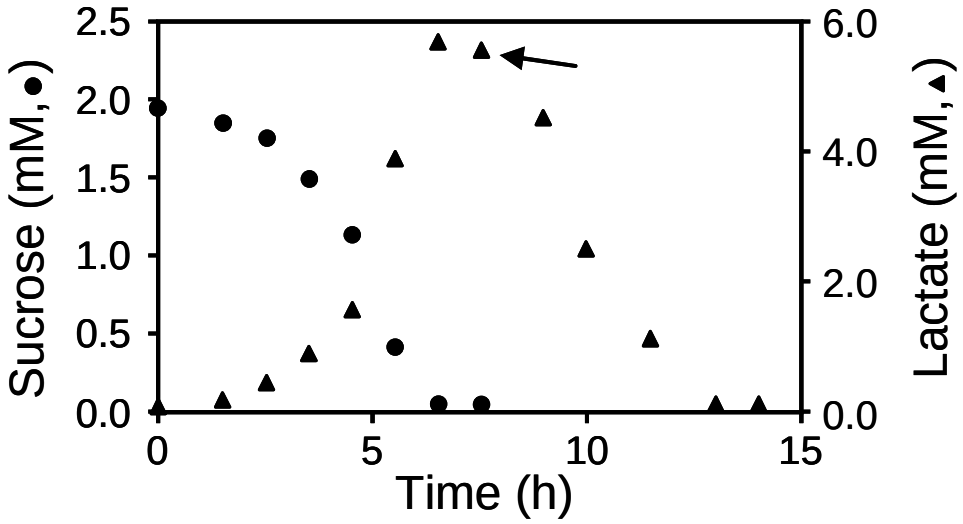

Figure 2.8. *A. actinomycetemcomitans* utilizes lactate produced by S. gordonii. *S. gordonii* was grown in CDM containing sucrose, and sucrose/lactate levels measured throughout growth. After 8 hours, *A. actinomycetemcomitans* was added (designated by the arrow) and lactate levels measured. Experiments were performed in duplicate. Standard deviations are <5% of the mean for sucrose and <9% of the mean for lactate and were not included for clarity.

2.4 Discussion

The human oral cavity is a nutritionally complex environment that supports growth and colonization of over 500 bacterial species (52). Although multiple carbon substrates exist in this environment, previous studies suggest that competition for carbon and energy is intense in this environment (32, 58, 62, 108); thus it is likely that to minimize competition, bacteria in the oral cavity have evolved carbon source preferences that do not overlap (resource partitioning). In the presence of multiple carbon substrates, bacteria often utilize only one carbon substrate at a time. Several mechanisms have been identified to allow preferential consumption of energy sources including transcriptional control of substrate-specific catabolic genes and inducer exclusion (16, 92, 115). Our data illustrate that *A. actinomycetemcomitans* exhibits little/no differential transcription of catabolic genes based on the carbon substrate (Table 2.1), but instead mediates preference at the level of carbohydrate transport. Lactate preference in *A. actinomycetemcomitans* is somewhat analogous to inducer exclusion, a regulatory process whereby one carbon source inhibits transport/catabolism of other carbon sources. However the paradigm for inducer exclusion is PTS sugars inhibiting transport/catabolism of other non-PTS sugars; thereby preventing these sugars from entering cells and transcriptionally activating specific carbon catabolism genes (92). Our results demonstrate the converse scenario, in which a non-PTS carbon substrate (lactate) inhibits transport of PTS sugars, and that transcriptional induction of specific catabolic genes is not required. Thus *A. actinomycetemcomitans* has evolved a novel mechanism for carbon source preference which we refer to as PTS substrate exclusion.

Although the molecular mechanism of PTS substrate exclusion is unknown, our results provide strong evidence that pyruvate and not lactate mediates this process (Fig. 2.5 and 2.6). The observation that intracellular levels of pyruvate increase >10-fold during growth with lactate compared to PTS transported sugars (Fig. 2.7) provide clues to the mechanism. PTS-mediated carbohydrate transport involves transfer of phosphate from phosphoenolpyruvate (PEP) through a series of proteins and ultimately to the carbohydrate (84). The first step in PTS sugar transport involves an autophosphorylation reaction in which protein E1 catalyzes the transfer of phosphate from PEP to itself yielding phophorylated E1 and pyruvate (Fig. 2.9). Previous studies of E1 from *E. coli* (which is 84% similar to the putative E1 from *A. actinomycetemcomitans*) indicate that high levels of pyruvate (≥ 20 mM) inhibit autophosphorylation, thereby preventing flow of phosphate to the carbohydrate (93). Interestingly, our measurements indicate pyruvate levels of greater than 50 mM during *A. actinomycetemcomitans* growth with lactate, suggesting that these levels are sufficient to significantly inhibit carbohydrate transport via PTS. From these data, we propose a model in which the high levels of intracellular pyruvate produced during growth with lactate inhibit E1 autophosphorylation (Fig. 2.9). Since E1 phosphorylation is required for transport of all PTS sugars, this mechanism provides *A. actinomycetemcomitans* with a single mechanism for exclusion of PTS sugars. Alternative mechanisms exist for lactate-mediated carbohydrate exclusion including the possibility that instead of inhibiting E1 autophosphorylation, high levels of intracellular pyruvate initiate the conversion of phosphorylated E1 and pyruvate to E1 and PEP (reverse reaction of that shown in Fig. 2.9). Although this reaction has been demonstrated in vitro (118), we do not favor this model since this reaction is

thermodynamically unfavorable compared to the forward reaction and therefore not likely to occur in vivo.

It is interesting that although *A. actinomycetemcomitans* grows fastest and obtains higher cell yields during growth with carbohydrates (Fig. 2.1), this bacterium has evolved to prefer lactate. The paradigm for carbon source preference is that bacteria prefer carbon sources that provide faster growth rates and/or higher growth yields. This is clearly not the case with *A. actinomycetemcomitans*, suggesting that growth in the oral cavity has selected for preference of energy sources not ideal for monoculture growth in vitro. Other bacteria in the oral cavity, including *Veillonella atypica* (27, 61), utilize lactate as a carbon/energy source; however these bacteria do not possess the capabilities to grow using carbohydrates such as glucose. *A. actinomycetemcomitans* is unique in that it possesses carbohydrate utilization pathways, but has evolved preference for lactate.

The ecological importance of lactate preference in vivo is unknown. An obvious potential benefit is that the ability to catabolize lactate provides *A. actinomycetemcomitans* with an alternative carbon substrate that is not utilized by most fast-growing community members such as streptococci. A more intriguing question is how inhibition of glucose transport benefits *A. actinomycetemcomitans* in vivo. One benefit may lie in the interactions of *A. actinomycetemcomitans* with other oral community members. During fermentation of carbohydrates, streptococci produce lactate from pyruvate to regenerate the NAD necessary for growth and biosynthesis. Recent evidence indicates that high lactate levels inhibit this reaction, and pyruvate is instead converted to acetyl phosphate and H_2O_2 by pyruvate oxidase (5). Under these conditions, H_2O_2 can accumulate to levels sufficient for killing many bacterial community

members. Thus it is conceivable that preferential consumption of lactate by *A. actinomycetemcomitans* within the gingival crevice may reduce H_2O_2 production by streptococci, thereby protecting *A. actinomycetemcomitans* from H_2O_2-mediated stress. These models are being currently tested and should further our understanding of the ecological significance of PTS substrate exclusion in multi-species communities.

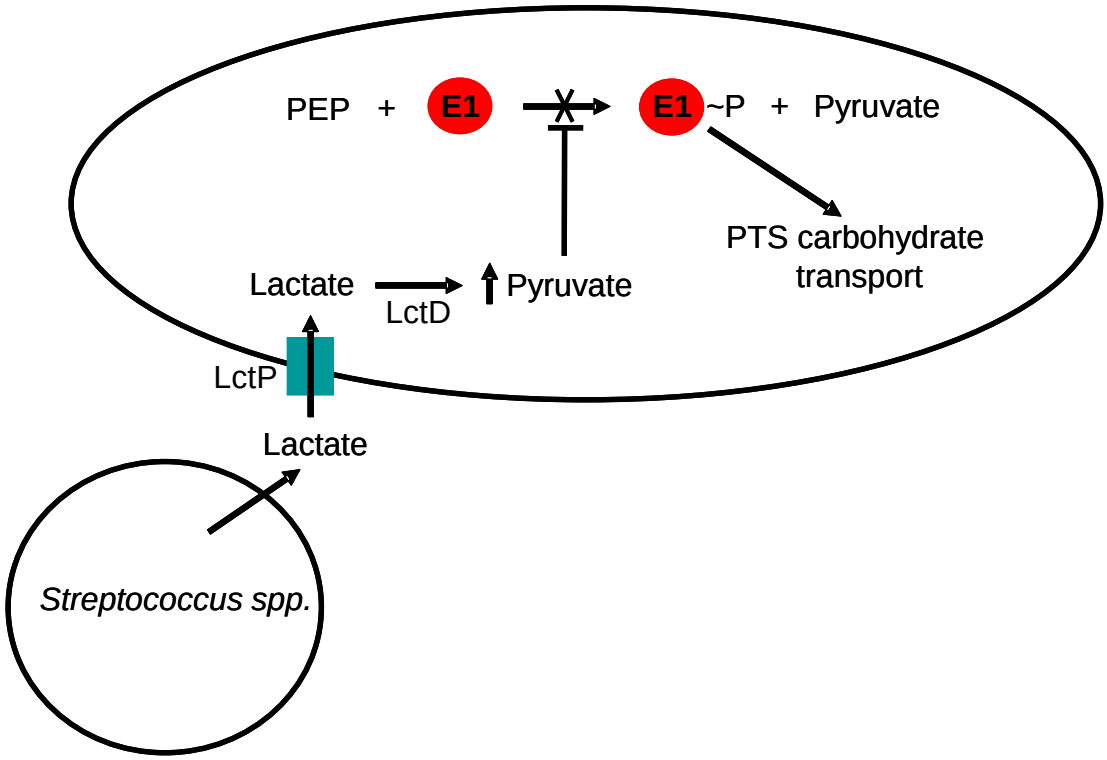

Figure 2.9. Model for lactate-mediated inhibition of glucose transport in *A. actinomycetemcomitans* during growth with streptococci. Lactate produced by *Streptococcus* spp is transported into *A. actinomycetemcomitans* by lactate permease (LctP). Once inside the cell, intracellular pyruvate levels increase due to the conversion of lactate to pyruvate by lactate dehydrogenase (LctD). Protein E1 normally autophosphorylates using phosphoenolpyruvate (PEP) as the phospho-donor; however elevated intracellular pyruvate levels inhibit this autophosphorylation. Since E1 is the first step in transport of PTS sugars, inhibition of E1 autophosphorylation inhibits uptake of all PTS carbohydrates.

Chapter 3: Characterization of the L-lactate dehydrogenase from *A. actinomycetemcomitans*

3.1 Introduction

We recently showed that although *A. actinomycetemcomitans* divides faster and achieves higher cell yields when catabolizing glucose, L-lactate is preferentially utilized (15). Interestingly, L-lactate addition to a chemically defined medium inhibited *A. actinomycetemcomitans* uptake of glucose, a process referred to as PTS substrate exclusion (15). Glucose transport in *A. actinomycetemcomitans* utilizes the phosphotransferase system (PTS). The PTS involves transport of glucose across the cytoplasmic membrane through a sugar-specific channel and concomitant phosphorylation upon entry into the cell to produce glucose-6-phosphate. The phosphoryl group originates from the phosphodonor, phosphoenolpyruvate (PEP), and is subsequently passed through a series of PTS proteins and ultimately to glucose (Fig. 1.2). The first step in PTS transport involves protein EI, which undergoes autophosphorylation in the presence of PEP to yield pyruvate and EI~P. The phosphoryl group is then transferred to HPr, followed by a sugar-specific EII protein, which then phosphorylates the incoming sugar (84). The intracellular ratio of PEP:pyruvate plays a crucial role in PTS transport. Indeed, as the PEP:pyruvate ratio declines, the model Gram-negative organism *Escherichia coli* displays reduced uptake of several PTS carbohydrates (42). Interestingly, L-lactate-grown *A. actinomycetemcomitans* produces extremely elevated intracellular levels of pyruvate (15), supporting a model in which

elevated intracellular levels of pyruvate during catabolism of L-lactate inhibit glucose transport via reduction of the PEP:pyruvate ratio (Fig. 2.9).

One of the intriguing questions regarding this model is how the extremely high levels of intracellular pyruvate (approximately 50 mM) are produced during growth with L-lactate. In this study, we hypothesized that potential clues might be gained by examining the *A. actinomycetemcomitans* enzyme required for the first step in L-lactate catabolism, namely L-lactate oxidation to pyruvate. We show that the gene AA02749 (*lctD*) encodes for an NAD-independent L-lactate dehydrogenase that is critical for growth of *A. actinomycetemcomitans* with L-lactate. Interestingly, inhibitor studies reveal that unlike homologous enzymes, *A. actinomycetemcomitans* LctD maintains significant enzymatic activity, even at extremely high pyruvate levels (50 mM).

3.2 Materials and Methods

3.2.1 Bacterial strains and culture conditions

A. actinomycetemcomitans strain VT1169 (65) and the *A. actinomycetemcomitans lctD* mutant (15) was grown in chemically defined Socransky's medium (102) lacking DL-mevalonic acid and hemin (referred to as Chemically Defined Medium, CDM) or tryptic soy broth with 0.5% yeast extract (TSBYE). Cultures were grown with shaking at 165 RPM at 37˚C with a 10% CO_2 atmosphere. For

growth analysis, overnight TSBYE-grown cultures were washed three times with warm (37ºC) CDM containing no catabolizable carbon source and diluted to an optical density at 600 nm (OD_{600nm}) of 0.015 in CDM supplemented with 20 mM L-lactate or glucose. *E. coli* DH5α and BL21(DE3) were grown in Luria broth (LB), shaking at 250 RPM at 37℃, with 75 µg/ml ampicillin for plasmid maintenance or 100 µg/ml for selection.

3.2.2 Construction of N- and C-terminally tagged LctD

The *lctD* gene from *A. actinomycetemcomitans* strain 1169 was amplified by PCR using primers lctDN-for (5'-GGAATTCCATATGATTATTTCGTCCGCTAACG-3') and lctDN-rev (5'- CCGCTCGAGGCGTATATAAAATACGCCGTTTG-3') or lctDN-for and lctDC-rev (5'- CTCGAGCTTACTTAAATCTACTAATGC-3') to create the N-terminally or C-terminally his_6-tagged constructs respectively. Forward primers contained an NdeI site and reverse primers contained an XhoI site. Products were digested with NdeI and XhoI and ligated into the NdeI/XhoI-digested pET15b expression vector (Novagen) for the his_6 N-terminal tag or the NdeI/XhoI-digested pET21a(+) expression vector (Novagen) for the his_6 C-terminal tag. The resultant plasmids were transformed into *E. coli* DH5α and sequenced using T7 promoter and SP6 terminator primers. Resulting plasmids were named pSB201 (his_6 N-terminal tag) and pSB203 (his_6 C-terminal tag). Plasmids were transformed into the expression strain *E. coli* BL21(DE3) for expression and purification.

3.2.3 Purification

E. coli BL21(DE3) carrying pSB201 or pSB203 were grown in TB (EMD) containing 75 µg/ml ampicillin to an OD_{600nm} = 0.6, and induced overnight at 16℃ with 100 µM IPTG in the presence of 100 µM riboflavin (Sigma). Induced cells were harvested by centrifugation for 15 minutes at 6100 x g in a Beckman Coulter Avanti J-E centrifuge at 4℃. The pellet was resuspended in 3 ml b uffer A (25 mM phosphate buffer, 0.5 M NaCl, 20 mM imidazole, 10 µM flavin adenine dinucleotide (FAD), pH 7.07) containing one-half tablet complete Mini protease inhibitor cocktail (Roche), 25 U Benzonase Nuclease (Novagen) and 10 µM FAD (Alfa Aesar). The cells were passed three times through a French press (American Instruments Company) at 20,000 pounds/square inch. The resulting lysate was centrifuged at 60,000 x g in a Beckman-Coulter OptimaL 100K Ultracentrifuge for one hour at 4℃ to remove cellular debris and insoluble protein. The lysate was then applied to a HisTrap HP column (GE Healthcare). The column was washed with 3 ml cold buffer B (25 mM phosphate buffer, 0.5 M NaCl, 10 µM FAD, pH 7.07) containing 0.15 M imidazole, followed by elution with cold buffer B containing 0.5 M imidazole. The eluted protein was concentrated, and a buffer exchange was performed with 25 mM phosphate buffer containing 10 µM FAD. Samples were separated on a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue (Pierce). Bradford analysis to quantify protein was performed with the Bio-Rad Protein Assay as outlined by the manufacturer. Western blot analysis was performed as outlined (2) using a commercially available anti-his_6 antibody (Sigma) and a stabilized goat anti-mouse

HRP-conjugated secondary antibody (Pierce). Chemiluminescent detection was performed using the SuperSignal West Dura Extended Duration Substrate as outlined by the manufacturer (Thermo). Blots were imaged with a G-box gel documentation system (Syngene). As a control, a purification procedure was carried out as described above with BL21(DE3) cells containing the parent plasmid pET21a(+).

3.2.4 Characterization of LctD-his$_6$ enzymatic activity

Activity assays were performed with phenazine methosulfate (PMS) (Acros Organics) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Calbiochem) as previously described (34, 35, 51). To initially examine enzymatic activity of LctD-his$_6$, reactions (1.5 ml) were carried out in 0.08 M Tris buffer containing 120 µg/ml PMS, 60 µg/ml MTT, 2 mM substrate (L-lactate, D-lactate, or pyruvate), and 100 nM LctD-his$_6$. Reactions containing pyruvate as the substrate also contained 1 mM NADH. Half of the reaction volume was removed at time zero, heated at 65˚ C for 5 minutes, chilled on ice, and stored at -80˚ C overnight. The remaining half of the reaction was processed in the same manner at 15 minutes, and all samples were filtered through a 0.2 µm Nanosep centrifugal device (Pall). Product analysis was carried out on a Varian HPLC using a Varian Metacarb 87H 300 x 6.5 mm column at 35˚C. Samples were eluted with 0.025 N H_2SO_4 isocratic elution buffer with a flow rate of 0.5 ml/minute. A Varian refractive index (RI) detector at 35˚C was used for product detection with commercially available L-lactate, D-lactate, and pyruvate as standards.

For kinetic analysis, assays were performed monitoring a PMS-coupled reduction of MTT as previously described (34, 35, 51). The assay was performed in 0.08M Tris-HCl (pH 8.6) containing 60 µg/ml MTT, 240 µg/ml PMS, and a range of purified enzyme. Absorbance changes in MTT were measured at 570 nm over 10 minutes. Absorbance values obtained from enzyme containing reactions were adjusted by subtracting background absorbance values from a reaction containing no enzyme. Calculations were performed using an extinction coefficient of 17 $mM^{-1}cm^{-1}$ (49). For inhibition studies, the activity assays described above were performed in the presence of increasing concentrations (0.05 to 50 mM) of pyruvate (Alfa Aesar) or oxalate (Fisher).

3.3 RESULTS

3.3.1 AA02769 is required for growth with L-lactate

A. actinomycetemcomitans resides within the gingival crevice where it likely encounters levels of L-lactate ranging from 1-5 mM (106), and previous work in our lab demonstrated that this bacterium preferentially catabolizes L-lactate (15). The first step in L-lactate catabolism is the conversion of L-lactate to pyruvate via the enzyme L-lactate dehydrogenase. Examination of the *A. actinomycetemcomitans* HK1651 genome sequence revealed two genes with high homology to lactate dehydrogenases: AA02769 (gene designations from www.oralgen.lanl.gov) putatively encodes a protein with 75% identity (E value $<10^{-169}$ using BLASTp) to the catabolic L-lactate dehydrogenase (LctD)

from *E. coli* K12 and AA02749 putatively encodes a protein with 73% identity (E value $<10^{-141}$ using BLASTp) to the fermentative D-lactate dehydrogenase (LdhA) from *E. coli* K12. Based on the fact that LdhA homologs are primarily utilized for lactate biosynthesis in fermentative reactions (9, 17), and that *A. actinomycetemcomitans* does not grow with D-lactate as the sole carbon source (data not shown), we hypothesized that AA02769 likely encodes the enzyme required for oxidation of L-lactate to pyruvate. To test this hypothesis, the ability of an *A. actinomycetemcomitans* strain containing an insertion in AA02769 to grow with L-lactate as the sole catabolizable carbon source was assessed. Inactivation of *lctD* eliminated the ability of *A. actinomycetemcomitans* to grow with L-lactate, but not glucose, as the sole catabolizable carbon source (Fig. 3.1). Based on these results, along with those described below, we will refer to AA02769 as *lctD*.

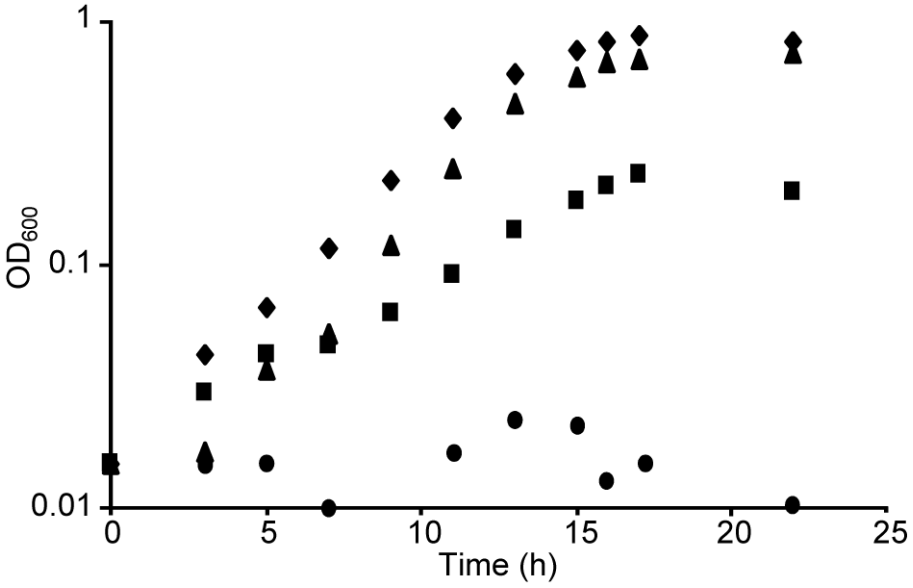

Figure 3.1. LctD is required for growth using L-lactate. Growth curves of wild-type *A. actinomycetemcomitans* and the *A. actinomycetemcomitans lctD* insertion mutant in chemically defined medium (CDM) containing 20 mM glucose or 20 mM L-lactate as the sole source of energy. Glucose-grown *A. actinomycetemcomitans* (♦), L-lactate-grown *A. actinomycetemcomitans* (■), glucose-grown *A. actinomycetemcomitans lctD*$^-$ (▲), L-lactate-grown *A. actinomycetemcomitans lctD*$^-$ (●).

3.3.2 Over-expression and purification of LctD

A. actinomycetemcomitans lctD putatively encodes a 42 kDa cytoplasmic protein that is a proposed member of a family of NAD-independent L-lactate dehydrogenases that convert L-lactate to pyruvate in a unidirectional manner (37). Due to its high homology to the catabolic L-lactate dehydrogenase from *E. coli* and the observation that inactivation of *lctD* eliminated L-lactate-dependent growth of *A. actinomycetemcomitans* (Fig. 3.1), we hypothesized that *A. actinomycetemcomitans* LctD catalyzes the oxidation of L-lactate to pyruvate. To test this hypothesis, *A. actinomycetemcomitans lctD* was cloned into the pET21a(+) expression vector (pSB103) to create C-terminally his_6-tagged LctD. Affinity purification using a nickel column resulted in nearly pure LctD-his_6 as demonstrated by a prominent band at approximately 42-kDa on a Coomassie stained gel (Fig. 3.2A) and a single band in a Western blot using an anti-his_6 antibody (Fig. 3.2B). As a control, a purification procedure was performed using cells containing pET21a(+), which resulted in no proteins readily apparent by SDS-PAGE analysis after purification and no L-lactate dehydrogenase enzyme activity (data not shown).

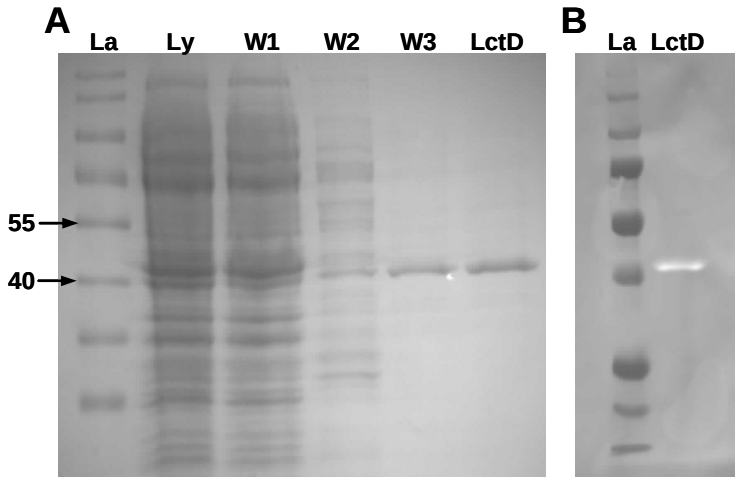

Figure 3.2. Purification of LctD-his$_6$. (A) SDS-PAGE analysis of LctD-his$_6$. LctD-his$_6$ was purified using a HisTrap nickel column and examined by SDS-PAGE and Coomassie staining. Lane designations above the gel are: (La), molecular weight ladder; (Ly), cell lysate; (W1), buffer A flow-through; (W2), buffer B with 0.15 M imidazole flow-through; (W3), buffer B with 0.5 M imidazole flow-through; (LctD), phosphate buffer-exchanged LctD-his$_6$. Numbers to the left of the SDS-PAGE gel represent size standards in kilodaltons. Phosphate buffer-exchanged LctD-his$_6$ was used for enzymatic activity studies. (B) Western blot analysis of purified LctD-his$_6$. Purified LctD-his$_6$ was separated on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane, and detected using an anti-his$_6$ antibody and chemiluminescence. Image represents an overlay of a white light image and a chemiluminescent image.

In addition to C-terminally his_6-tagged LctD, N-terminally his_6-tagged LctD was also constructed and purified. While both purified enzymes displayed enzymatic activity, they were extremely unstable and exhibited significant loss of enzymatic activity in a variety of buffers after overnight storage at 4, -20, or -80℃; therefore all assays were carried out using freshly purified protein. LctD-his_6 was used for enzymatic characterization as its yields were somewhat higher than those of his_6-LctD.

3.3.3 Kinetic characterization of *A. actinomycetemcomitans* LctD

Once purified protein was obtained, the enzymatic activity of LctD-his_6 was assessed. The final electron acceptor of many NAD-independent enzymes is unknown, as these enzymes are likely coupled to electron transport (37). Therefore, LctD-his_6 activity was determined in the presence of the electron carriers 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and phenazine methosulfate (PMS) as previously described (34, 35, 51). LctD-his_6 catalyzed the oxidation of L-lactate to pyruvate (Fig. 3.3A and B); however, no activity was observed with the D-lactate isomer (Fig. 3.3C). Additionally, LctD-his_6 was unable to catalyze the reverse reaction (pyruvate to lactate) even in the presence of the reduced substrate NADH (data not shown). These data indicate that, as expected, LctD-his_6 is an NAD-independent L-lactate dehydrogenase that catalyzes the oxidation of L-lactate to pyruvate in a unidirectional manner.

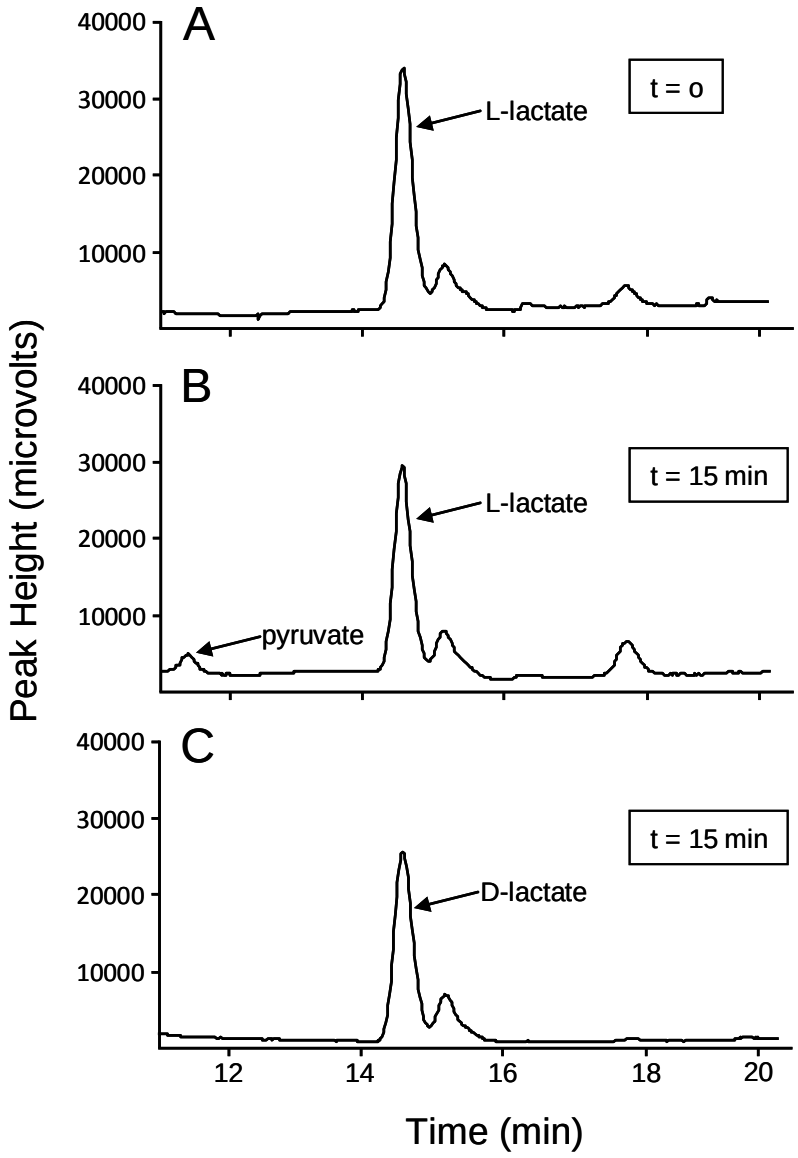

Figure 3.3. LctD-his$_6$ catalyzes oxidation of L-lactate, but not D-lactate, to pyruvate. HPLC chromatogram of LctD-his$_6$ in vitro enzyme reactions with (A) L-lactate as the substrate at time 0, (B) L-lactate as the substrate after 15 min., and (C) D-lactate as the substrate after 15 min. Pyruvate and D- and L-lactate were detected using a refractive index detector, and data are displayed as peak height (microvolts). Using commercially available standards, it was determined that pyruvate is detected at approximately 11.6 to 12.1 min and lactate (D and L) is detected at approximately 14.6 to 15.2 min. Representative data are shown for experiments that were performed in duplicate or triplicate.

After determining that the product of LctD-his$_6$ L-lactate oxidation was pyruvate, kinetic analysis was performed. For kinetic analysis, reduction of MTT (measured as the change in absorbance at 570 nm) was utilized to monitor LctD-his$_6$ activity as previously described (34, 35, 51). Activity assays were carried out in the presence of saturating substrate concentrations, and it was determined that with 8 nM enzyme, 4 mM lactate, and 60 µg/ml MTT, 240 µg/ml PMS achieved linear results for a 10 minute duration (Fig. 3.4A). To acquire a K_m value, activity assays were performed in the presence of increasing lactate concentrations, and a K_m value of approximately 150 µM was calculated using SigmaPlot 10.0.1 software (Fig. 3.4B).

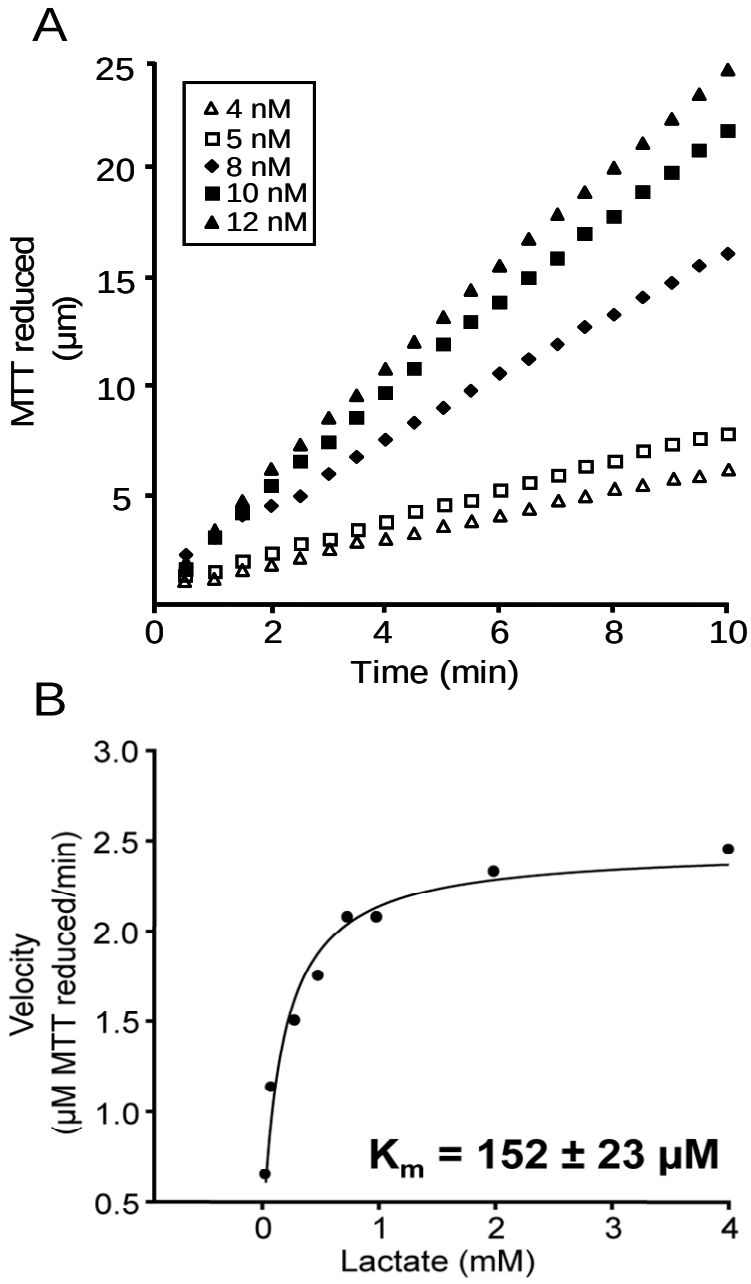

Figure 3.4. Kinetic analysis of LctD-his$_6$. LctD-his$_6$ was incubated with L-lactate and enzymatic activity assessed by monitoring reduction of MTT as described (34, 35, 51). (A) Enzymatic activity was assessed over time for multiple LctD-his$_6$ concentrations (4, 5, 8, 10, and 12 nM) in the presence of saturating substrate concentrations. For K_m calculations, 8 nM LctD-his$_6$ was used. Representative data are shown. (B) The LctD-his$_6$ K_m for lactate was calculated as 152 ± 23 µM (average ± standard deviation) by averaging values from quadruplicate experiments. Representative data are shown.

3.3.4 Pyruvate is a poor inhibitor of LctD

Our model for PTS substrate exclusion involves the production of high levels of intracellular pyruvate during growth with L-lactate (Fig. 2.9). Based on this model, we hypothesized that *A. actinomycetemcomitans* LctD-his_6 would not be sensitive to feedback inhibition by product (pyruvate) accumulation; thus allowing intracellular accumulation of pyruvate to the high levels (50 mM) previously observed (15). Interestingly, LctD homologs from other bacteria are often inhibited by relatively low levels (5 mM) of pyruvate (68). To assess the impact of pyruvate on *A. actinomycetemcomitans* LctD-his_6 activity, inhibition assays were performed in the presence of increasing concentrations of pyruvate as well as the common lactate dehydrogenase inhibitor oxalate (43, 59). Pyruvate displayed poor inhibition of LctD-his_6 activity, with approximately 90% activity remaining in the presence of 5 mM pyruvate and 50% activity remaining in the presence of 50 mM pyruvate (Fig. 3.5). As expected, oxalate was a potent inhibitor of LctD activity, with 50% inhibition observed at approximately 2 mM oxalate (Fig. 3.5).

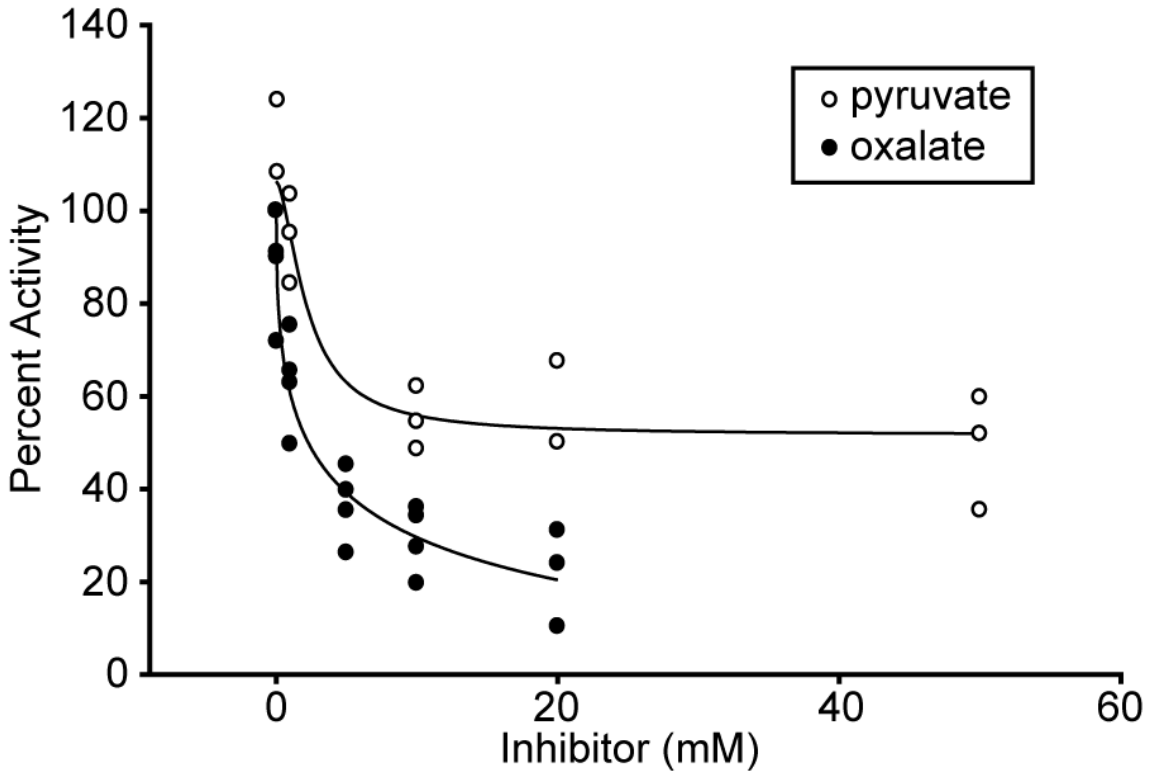

Figure 3.5. Pyruvate is a poor inhibitor of LctD-his$_6$. The ability of pyruvate and oxalate to inhibit LctD-his$_6$ enzymatic activity was assessed as described in Materials and Methods. 50% inhibition of LctD-his$_6$ activity was observed with 10-50 mM pyruvate and 2.1 mM oxalate.

3.4 Discussion

A. actinomycetemcomitans is found exclusively in the mammalian oral cavity, a diverse environment where microbes likely compete for limited carbon sources including glucose, L-lactate, and fructose (73, 106). While a large number of oral bacteria preferentially catabolize carbohydrates, previous results from our laboratory revealed that *A. actinomycetemcomitans* has evolved a preference for L-lactate despite its apparent inferiority as a carbon and energy source (15). This unique mechanism for preferential L-lactate consumption, referred to as PTS substrate exclusion (15), may have benefits for *A. actinomycetemcomitans* as it may mitigate L-lactate-mediated acidification of the gingival crevice as well as reduce production of the antimicrobial H_2O_2 by oral streptococci (5).

We recently proposed a model for PTS substrate exclusion in which intracellular levels of pyruvate inhibit glucose uptake in L-lactate-grown *A. actinomycetemcomitans* (Fig. 2.9). As this model is predicated on the accumulation of pyruvate to intracellular levels approximately 10 times higher than normally found in glucose growing bacteria (15), we hypothesized that the *A. actinomycetemcomitans* L-lactate dehydrogenase (LctD) is not feedback inhibited by high levels of pyruvate. To test this hypothesis, *A. actinomycetemcomitans* LctD was purified as a C-terminal his_6 fusion protein and determined to be an NAD-independent L-lactate dehydrogenase that catalyzes irreversible oxidation of L-lactate to pyruvate (Fig. 3.3B). LctD-his_6 displayed a K_m for L-lactate of approximately 150 μM, a value within the range of other characterized NAD-

independent lactate dehydrogenases (37, 50, 68), including the highly homologous *E. coli* LctD enzyme (75% identity) that has a K_m value of 21–70 µM (50).

A. actinomycetemcomitans LctD-his_6 was highly resistant to feedback inhibition by pyruvate, displaying 50% activity at 50 mM pyruvate (Fig. 3.5). This property is unique in that homologous enzymes in other bacteria display feedback inhibition by substantially lower levels of pyruvate. Indeed, the NAD-independent lactate dehydrogenase of *Propionibacterium pentosaceum* is inhibited by levels of pyruvate (1-5 mM) commonly observed intracellularly (68). Our results reveal that LctD allows *A. actinomycetemcomitans* to specifically convert L-lactate to pyruvate in a unidirectional manner. Since this enzyme displays reduced inhibition by pyruvate, intracellular levels of pyruvate rise to levels known to inhibit PTS transport in other bacteria (42, 118), resulting in PTS substrate exclusion. While the PTS enzyme(s) affected by high levels of pyruvate is not known, we hypothesize that inhibition occurs at the first step of PTS transport, phosphorylation of protein EI by PEP (Fig. 2.9), and studies in our laboratory are currently addressing this hypothesis.

Chapter 4: Conclusions and Future Directions

4.1 OVERVIEW

4.1.1 A Novel Carbon Resource Partitioning Mechanism

The physiology of *A. actinomycetemcomitans* is poorly understood, in part due to the fact that growth of this organism has not been studied in a suitable chemically defined medium. In chapter two I describe the use of a modified Socransky's chemically defined medium to begin to characterize carbon utilization by *A. actinomycetemcomitans.* Because *A. actinomycetemcomitans* is found solely in the mammalian oral cavity, focus was given to carbon sources available in this environment. Using physiologically relevant concentrations of carbon sources present in gingival crevicular fluid, I determined that *A. actinomycetemcomitans* preferentially utilizes lactate despite its inferiority as a growth substrate (Fig. 2.2). Additionally, I found that the presence of lactate or pyruvate in the growth medium inhibits the transport and metabolism of PTS sugars (Figs. 2.4 and 2.5), and transcriptome analyses determined that regulation of this event does not occur at the level of transcription (Table 1) (15). Therefore, I hypothesized that growth on lactate produces elevated intracellular levels of pyruvate that inhibit the PTS system. Enzymatic assays were then used to examine intracellular pyruvate concentrations within *A. actinomycetemcomitans* growing on lactate or PTS sugars, and the results revealed an accumulation of 10-fold more

pyruvate in lactate-grown cells (Fig. 2.7) (15). This phenomenon had never before been demonstrated, and we termed this process PTS substrate exclusion.

Based on the above results, I developed a model to explain PTS substrate exclusion (Fig 2.9) (15). The model predicts that L-lactate found in the gingival crevicular fluid and produced by neighboring *Streptococcus* species is preferentially taken into the cell and oxidized by L-lactate dehydrogenase (LctD), creating elevated intracellular pools of pyruvate. The increased concentrations of pyruvate interfere with the PEP:pyruvate ratio, inhibiting the PTS. PTS substrate exclusion impacts transport and metabolism of the PTS sugars glucose and fructose; therefore, I hypothesize that inhibition occurs at EI, the first protein in the PTS that is shared between all PTS sugars (Fig. 1.2A).

The results of these experiments identified L-lactate as the preferred carbon source of *A. actinomycetemcomitans* and led to the description of a novel carbon resource partitioning mechanism likely used by this organism in the oral cavity. Additionally, this work provides strong evidence suggesting that the most energetically favorable carbon source may not be the preferred substrate in the environment in which an organism is found.

4.1.2 Purification and Characterization of the L-lactate dehydrogenase

The first step in the model described in chapter two is the oxidation of lactate to pyruvate by LctD (Fig. 2.9). Based on this model, I hypothesized that the LctD from *A. actinomycetemcomitans* is unlike LctD from other organisms and does not exhibit

feedback inhibition in the presence of elevated pyruvate levels. *In silico* analysis revealed that LctD belongs to a group of enzymes that catalyze the production of pyruvate from lactate without the use of NAD as an electron acceptor, which differentiates LctD and other NAD-independent enzymes from the NAD-dependent ones. In fact, the final electron acceptors for many of the NAD-independent enzymes are unknown and thought to be coupled to the electron transport chain. These enzymes are less studied than their NAD-dependent counterparts and are often difficult to purify and exhibit rapid loss of activity (37).

In chapter three I His-tagged the LctD from *A. actinomycetemcomitans* and purified it for analysis. Kinetic studies indicated that the K_m for L-lactate is approximately 152 µM, similar to that of the *E. coli* NAD-independent lactate dehydrogenase (Fig 3.4). Further kinetic studies using increasing concentrations of pyruvate and oxalate (a known lactate dehydrogenase inhibitor) (43, 59) revealed that LctD remains active in the presence of 50 mM pyruvate (Fig. 3.5) (14), whereas another characterized NAD-independent lactate dehydrogenase from *Propionibacterium pentosaceum* exhibits inhibition at 5 mM pyruvate (68). Importantly, 50 mM pyruvate is found in lactate-grown *A. actinomycetemcomitans* (15). The results of these studies provide strong support for the model in which we hypothesize that LctD does not exhibit feedback inhibition in response to elevated pyruvate levels, thus allowing intracellular concentrations of pyruvate to increase sufficiently inhibit the PTS and subsequent sugar transport/metabolism.

4.2 Future Directions

4.2.1 Metabolic End Products

In silico analysis of the *A. actinomycetemcomitans* genome reveals that it is capable of carrying out glycolysis and the pentose phosphate cycle, but only possesses a partial TCA cycle (41); however, it is unknown how these pathways are utilized during growth in the oral cavity. In chapter two I have described the preferential use of lactate as a carbon source, but it is not currently known what end products are being formed from lactate catabolism. Additionally, as *A. actinomycetemcomitans* is classified as a facultative anaerobe, one must also consider the decreasing oxygen gradient in the gingival pocket and how oxygen availability, especially a lack of oxygen in the deep gingival pockets, influences the carbon utilization profile of *A. actinomycetemcomitans*. Current studies analyzing anaerobic and aerobic metabolic end products are underway in our laboratory that will shed light on these and other facets of *A. actinomycetemcomitans* metabolism.

4.2.2 Verification of EI Phosphorylation Inhibition

Because we observed PTS substrate exclusion with both fructose and glucose, we hypothesize that phosphorylation of the non-specific EI protein is blocked by elevated intracellular pyruvate levels (Fig. 2.9). Thus, further studies should be conducted to determine the phosphorylation state of EI in the presence of increasing pyruvate levels.

Experiments of this variety are challenging for several reasons. For instance, EI undergoes autophosphorylation during growth on PTS sugars at a conserved histidine residue, but phosphorylated histidines are often unstable and heat-labile. Additionally, EI phosphorylation is transient, as the phosphoryl group is rapidly transferred to HPr before modification of the incoming sugar (84). Therefore, it may be necessary to purify EI and HPr to reconstitute a partial PTS system *in vitro* to conduct phosphorylation assays using radiolabeled phosphorus. Progress has been made on this project, as I have his-tagged and purified the EI enzyme. These studies will provide further insight into the mechanism of PTS substrate exclusion.

4.2.3 Carbon Source and Virulence in *A. actinomycetemcomitans*

The preferential utilization of lactate as a carbon source is unique and interesting, but is there any evidence that this influences survival and/or virulence of *A. actinomycetemcomitans in vivo*? To survive in the oral cavity, *A. actinomycetemcomitans* must compete for nutrients with neighboring bacteria, namely *Streptococcus* species. *Streptococcus* species constitute a large percentage of the gingival pocket community (109) and are known to grow rapidly on sugars, producing lactate as a metabolic byproduct. In chapter two I provided evidence that *A. actinomycetemcomitans* can catabolize L-lactate produced by *Streptococcus gordonii* (Fig. 2.8). I hypothesized that although lactate is an energetically inferior carbon source, it is utilized preferentially as a means to avoid competition with other nearby bacteria for sugars. Therefore, I would predict that, when co-cultured, the *A. actinomycetemcomitans lctD*$^{-}$ mutant would be unable to compete for sugars with the more rapidly growing *S.*

gordonii, and the culture would ultimately be overtaken by *S. gordonii*. Studies are currently being conducted in our laboratory to address competition and survival of the *lctD*$^-$ mutant and wild-type (wt) *A. actinomycetemcomitans* when grown in the presence of *S. gordonii*.

In addition to survival, carbon source utilization may influence pathogenesis. There have been previous studies addressing carbon source availability and production of the virulence factor leukotoxin in *A. actinomycetemcomitans* (46, 47), but none studying lactate utilization and virulence. I performed microarray analyses on *A. actinomycetemcomitans* grown on lactate, glucose, or fructose, and these did not reveal any differential expression of known *A. actinomycetemcomitans* virulence factors. However, differential expression of virulence factors is not necessarily a requirement for disease, as it has been acknowledged that a pathogen cannot cause disease if it is incapable of establishing residence in the host (13, 36). This has previously been shown in the pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae,* where mutants unable to catabolize lactate were defective for colonization and survival in murine models (28, 29). This may hold true for *A. actinomycetemcomitans* and additional studies can answer this question. There is a murine abscess model that is presently being used to address colonization of and abscess formation by the *A. actinomycetemcomitans lctD*$^-$ mutant compared to wt.

Additionally, there is interest in determining the impact of co-culture on virulence. Work in our laboratory has shown that interactions between *A. actinomycetemcomitans* and *S. gordonii* result in upregulation of an *A. actinomycetemcomitans* outer membrane protein that aids in host immune evasion (86). It is possible that other interactions

between these two organisms could impact the periodontal disease process. Experiments are in progress that will determine the effect of co-culture on abscess formation in the murine model.

4.3 Model for Preferential Lactate Utilization

The question remains, why would *A. actinomycetemcomitans* evolve to utilize an inferior carbon source? The paradigm in microbiology is that organisms will use the carbon source that provides the most energy and growth yield; however, my work has shown that *A. actinomycetemcomitans* preferentially catabolizes an energetically inferior carbon source. There must be an advantage for *A. actinomycetemcomitans* to use lactate over glucose or fructose, and clues to possible benefits of *A. actinomycetemcomitans* lactate utilization have been revealed via studies of the co-habiting species *S. gordonii*. It was known that H_2O_2 produced by *Streptococcus* species inhibits growth of other neighboring bacterial species, and these studies focused on H_2O_2 production by *S. gordonii* in the presence of sugars or lactate. The results demonstrated that while the presence of sugars resulted in approximately equimolar production of H_2O_2, elevated concentrations of lactate caused an increase in H_2O_2 production (5). If the converse is also true, then this suggests that the preferential use of lactate by *A. actinomycetemcomitans* not only acts as a carbon resource partitioning mechanism, but may also indirectly lead to a decrease in the amount of toxic H_2O_2 to which it is exposed. Additionally, host salivary peroxidase can react with H_2O_2 to form hypothiocyanate, a product shown to be bacteriostatic to oral streptococci (18, 19).

This may also suggest that other interspecies interactions are at play, as the removal of H_2O_2 from the environment is likely beneficial to other species in the gingival crevice. The study of the interactions that occur between the numerous species within the gingival crevice will ultimately lead to new insight and understanding of the complex disease process of periodontitis and its association with heart disease.

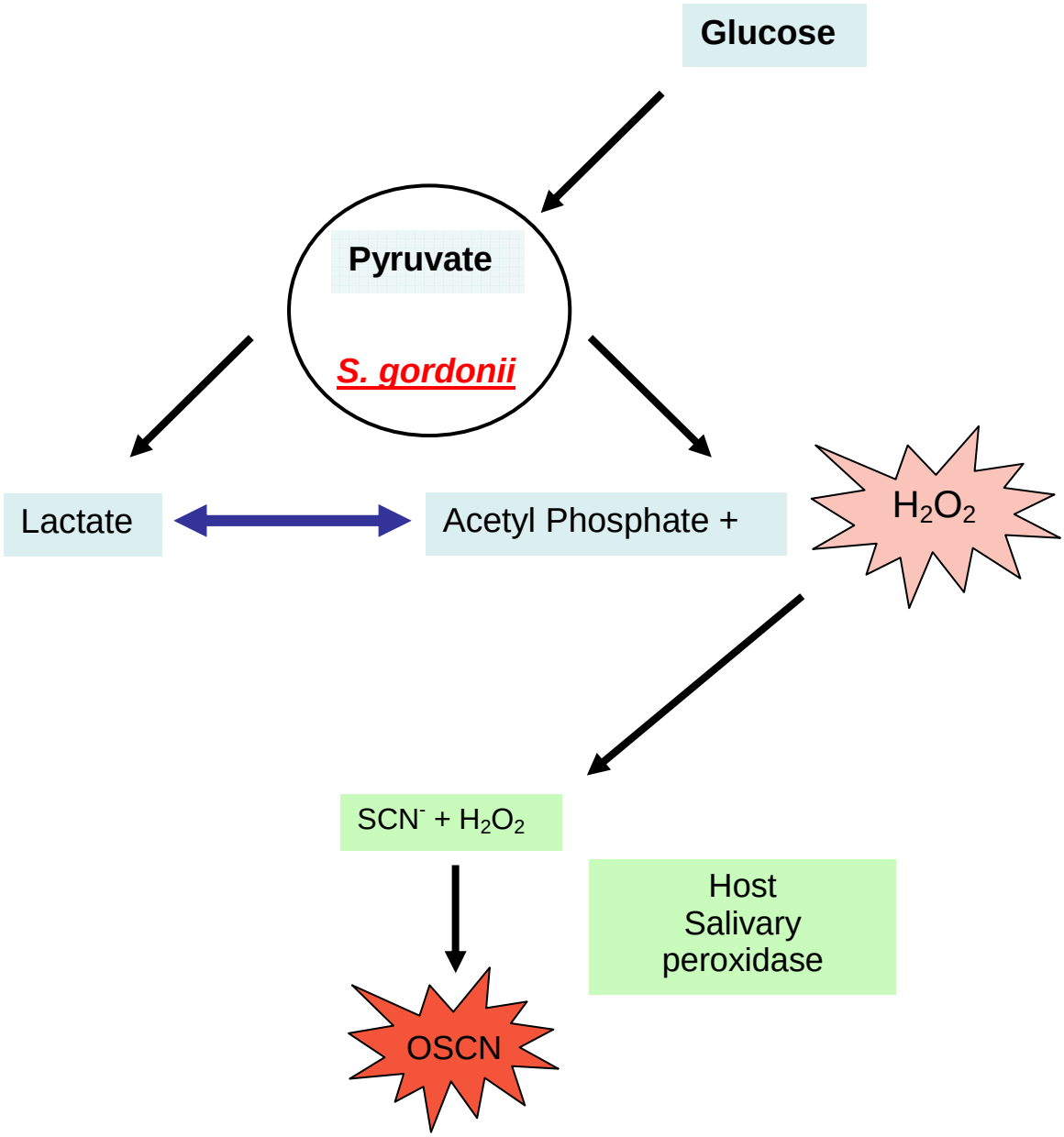

Figure 4.1. Hypothiocyanate production in the oral cavity. S. *gordonii* consumes sugars, producing lactate and H_2O_2 as metabolic byproducts. H_2O_2 in the oral cavity reacts with host salivary peroxidase and thiocyanate to form hypothiocyanate, a bactericidal compound.

4.4 Final Discussion

A. actinomycetemcomitans has been known as a causative agent of periodontitis and infective endocartitis for quite some time; however, the metabolic processes employed during this disease process are not well studied. In this work I have described a novel carbon resource partitioning mechanism and provided data that supports our hypothesis describing the molecular mechanisms governing this phenomenon. Additionally, this work has provided a foundation for continued characterization of this mechanism and elucidation of the impact of carbon source utilization on colonization, survival and virulence of *A. actinomycetemcomitans in vivo*.

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