CONTROL OF SUBSTRATE UTILIZATION BY O-ISLANDS AND S-LOOPS IN *ESCHERICHIA COLI* O157:H7

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ABSTRACT

Escherichia coli O157:H7 is an enteric pathogen that can cause severe gastrointestinal disease, sometimes leading to hospitalization and death. These bacteria have a variety of virulence factors that can be encoded for on pathogenicity islands (PAIs). The goal of this study was to characterize specific *E. coli* O157:H7 PAI deletion mutants using three methods: Phentotype Microarrays (PM), growth curves and survival curves were used to elucidate possible roles for the PAIs. Results from the PM study suggest that PAIs have a role in carbon substrate utilization; *i.e.*, four of the O-island (OI) deletion mutants (OI-87, 98, 102 and 172) and an S-Loop (SL-72) deletion mutant exhibited differences in substrate utilization (gains and losses in utilization) compared to parental O157:H7 strains EDL933 (OI) and Sakai (SL), respectively. All of the mutants with the exception of the OI-135 mutant exhibited differences in level of substrate utilization for substrates shown to have important roles in the bacterium. Cell growth results showed that three OI deletion mutants (OI-55, 87 and 102) and the SL (SL-72) mutant exhibited a difference in rate of growth compared to the parental strains. Cell viability results showed that seven of the OI deletion mutants (OI-51, 55, 98, 108, 135, 172 and 176) exhibited different rates of decline in cell number when transferred to sterile water compared to the parental strain. The results show that removal of PAIs from *E. coli* O157:H7 can affect carbon utilization, growth and survival demonstrating the importance of PAIs in the ecology of these bacteria.

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ABU	asymptomatic bacteriuria
A/E	attaching and effacing
AGA	N-acetyl-D-galactosamine
ATP	Adenosine-5'-triphosphate
BHI	brain heart infusion
bp	base pair
BUG	Biolog Universal growth medium
BUG+B	Biolog Universal growth medium + 5% sheep blood
Da	Dalton
DAEC	Diffuse-adhering *Escherichia coli*
DNA	deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
g	gram
Gb3	globotriaosylceramide
h	hour
HC	hemorrhagic colitis
HUS	hemolytic uremic syndrome

IF-0	inoculation fluid
IL	interleukin
kb	kilobase
kDa	kilodalton
LB	Luria-Bertani
LEE	Locus of enterocyte effacement
l	liter
m	milli
mm	millimeter
min	minute
OD	Optical Density
OD_{600}	Optical Density at 600nm
OI	O-island
ORF	Open reading frame
P	probability
PAI	pathogenicity island
PM	Phenotype microarrays
rpm	revolution per minute
SE	standard error
SL	S-Loop
Stx	Shiga toxin
STEC	Shiga toxin producing *Escherichia coli*
TNF	tumor necrosis factor

TSA	tryptic soy agar
TTSS	type III secretion system
USA	United States of America
VBNC	viable but non-culturable
VTEC	verocytotoxin-producing *Escherichia coli*
%T	percent transmission
µ	micro
ºC	degree Celsius

Chapter I

Hypothesis and Objectives

1.0 Introduction

Escherichia coli (*E. coli)* O157:H7 is an enteric human pathogen that can cause severe bouts of gastrointestinal disease, sometimes leading to hospitalization and death (Karmali 2005). The bacteria contain a variety of virulence factors that can be encoded for on pathogenicity islands (PAIs). Characterization of PAIs found in *E. coli* O157:H7 is important to help clarify the roles these islands have in regulation of gene expression in these bacteria. Investigation of these PAIs could also help elucidate possible approaches to prevention and mitigation of O157:H7 outbreaks and could help us to identify possible roles PAI have in contributing to the virulence of *E. coli* O157:H7.

The hypothesis of the thesis is that the removal of a PAI from *E. coli* O157:H7 will result in a deletion mutant that will have differences in carbon utilization, growth and survivability compared to the O157:H7 parental strain.

In this study, 13 deletion mutants of *E. coli* O157:H7 strains EDL933 and Sakai were examined and compared to their parental strains and the reference *E. coli* K12 strain DH5α. The PAIs in *E. coli* O157:H7 strain EDL933 are called O-islands (OI) and in *E. coli* O157:H7 strain Sakai they are called S-Loops (SL) (Perna *et al*. 2001; Hayashi *et al*. 2001). The main objectives of the study were to:

I) Characterize the O157:H7 deletion mutants using phenotypic profiling (the Biolog^{TM} PM plate system) to allow identification of substrate utilization patterns and, to identify differences in these patterns that could be used to

elucidate the role of the deleted PAIs in the bacteria. Specifically the study was used to:

1) Determine the PM profiles for 13 mutants and 3 controls (O157:H7 pathogenic strains EDL933 and Sakai plus the reference control *E. coli* K12 strain DH5α)
2) Determine if differences in substrate utilization confer a role to the specific OI or SL removed from the bacteria.
3) Determine if removal of a PAI results in a deletion mutant that uses subtrates in a manner more resembling a reference K-12 strain.

II) Examine the growth of OI and SL deletion mutants in order to determine if the extra-chromosomal DNA contained within the OIs and SLs of *E. coli* O157:H7 strains EDL933 and Sakai have an effect on the growth of the pathogen in a laboratory setting. Specifically:

1) Assess the differences in growth for the 13 deletion mutants and the 3 controls
2) Determine if removal of a PAI resulted in a mutant that grew more like a reference K-12 strain

III) Examine the survivability of OI and SL deletion mutants in order to determine if extra-chromosomal DNA contained within the OI and SL of *E. coli* O157:H7 strains EDL933 and Sakai contributed to its survival in water.

Chapter II

Literature Review

2.0 Background

E. coli is a gram-negative bacterium that is normally found as part of the gut microbiota of humans (Kuntz *and* Kuntz 1999). Many different strains have been characterized that generally can be sorted into three main categories: 1. commensal *E. coli* consisting of those that do not cause disease, 2. pathogenic *E. coli* that cause gastrointestinal disease and, 3. extra-intestinal *E. coli* that cause other forms of disease such as blood and urinary tract infections (Russo *et al.* 2003). Pathogenic *E. coli* can be further separated by the type of disease caused; e.g., sepsis, meningitis, and enteric or diarrheal disease (Nataro *and* Kaper 1998). The enteric group of pathogenic *E. coli* can be further divided into 6 sub-pathotypes that cause diarrhea (Table 2.1) (Nataro *and* Kaper 1998). One of these sub-pathotypes is enterohemorrhagic *E. coli* (EHEC), which is a subset of the Shiga-toxin producing *E. coli* (STEC) (Cleary 2004).

2.1 STEC and EHEC

STEC is a class of enteropathogens that produce a toxin or toxins that are closely related to the Shiga toxin produced by *Shigella dysenteriae* serotype 1. Not all STEC enteropathogens cause human disease, e.g., some STEC strains cause pig edema disease and are unlikely to cause human disease due to differences in virulence (Sonntag *et al*, 2005). EHEC is an example of a STEC that is capable of causing human disease. By contrast, EHEC forms an important class of STEC that are intestinal pathogens that have potential to cause severe infections such as hemolytic uremic syndrome (HUS),

Table 2.1: Sub-pathotypes of diarrheagenic *E. coli* and their defining characteristics*.

Pathotypes	Defining characteristics
Enterotoxigenic *E. coli* (ETEC)	Strains contain a heat stable enterotoxin or Shiga toxin and heat-labile enterotoxin groups
Enteropathogenic *E. coli* (EPEC)	Produces intestinal histopathology with characteristic attaching and effacing lesions (A/E lesions)
Enterohemorrhagic *E. coli* (EHEC)	Produce a Shiga-like toxin and A/E lesions
Enteroaggregative *E. coli* (EAEC)	Display aggregative adherence patterns
Enteroinvasive *E. coli (EIEC)*	Strains are biochemically, genetically and pathogenetically similar to *Shigella* spp.
Diffusely adherent *E. coli* (DAEC)	Display diffuse adherence patterns

* adapted from Nataro and Kaper (1998)

hemorrhagic colitis (HC) and can lead to complications such as seizures and even coma (Cleary 2004; Karch *et al.* 2005). EHEC frequently associated with human disease include the serotypes O26:H11, O103:H2, 0111:H8, O145:H28, O157:H- and O157:H7 (Yoon and Hovde 2008). EHEC serotypes have been classified into four seropathotypes based on their ability to infect humans and the frequency of disease (Table 2.2; Karmali 2005). *E. coli* O157:H7 is one of the most important serotypes in this grouping, as it typically is associated with exceptionally virulent strains (Cleary 2004) and are widely known and recognized (Karch *et al.* 2005). In Canada and the United States of America (USA), *E. coli* O157:H7 is the serotype most commonly implicated in causing HUS (Yoon and Hovde 2008). However, non-O157:H7 EHEC infections in humans do occur, but are more prevalent in continental Europe, Australia and Latin America.

2.2 Reservoirs of EHEC

One of the major reservoirs of EHEC is cattle (Gannon *et al*. 2002). Although cattle are a major reservoir, other animals such as sheep, poultry, goats and dogs can also act as reservoirs for EHEC (Karch *et al.* 2005).

Transmission to humans occurs via three possible routes; *i.e*., 1) by ingesting contaminated material such as food and drink, 2) by person to person contact and, 3) by zoonosis or animal contact. Ingestion of contaminated meat and unpasteurized milk represents one of the main ways that EHEC pathogens enter the food chain (Karch *et al.* 1999). Contamination of processed meat products such as ground beef has been responsible for various outbreaks of *E. coli* O157:H7 including an outbreak in France in

Table 2.2. Classification of serotypes into seropathotypes*.

Seropathotype	Relative incidence	Outbreaks	Severe disease (e.g., HUS)	Serotypes (examples)
A	High	Common	Yes	O157:H7, O157:NM
B	Moderate	Uncommon	Yes	O26:H11, O103:H2, O111:NM, O111:H8, O121:H19 and O145:NM
C	Low	Rare	Yes	O91:H21, O104:H21 and O113:H21
D	Low	Rare	No	Multiple

*(Adapted from Karmali 2005)

2005 (King *et al*. 2009) and in the state of Washington in the USA in 1992 and 1993 (Bell *et al*. 1994). In both cases the source was frozen hamburger patties. *E. coli* O157:H7 outbreaks have also occurred by contamination of fresh produce like lettuce, berries and bean sprouts (Sivapalasingam *et al.* 2004). In September of 2006 an outbreak of EHEC strain O157:H7 in the USA was associated to spinach; multiple states were affected (Grant *et al*. 2008). There were 205 confirmed infection cases and 3 deaths; of the cases where people were hospitalized (103 cases) 31 of those patients developed HUS (Uhlich *et al*. 2008).

Contamination of fresh water can lead to high levels of infection and outbreaks. This is a cause for concern because pathogenic bacteria can be spread over a large geographic area and to a wide range of people in a short period of time (Olsen *et al.* 2002). In Alpine, Wyoming in the summer of 1998 a waterborne outbreak of O157:H7 occurred (Olsen *et al*. 2002). The main cause of this outbreak was due to drinking un-chlorinated water from the town's water supply; 157 people were infected. In May of 2000 a water-borne outbreak of *E. coli* O157:H7 and *Campylobacter jejuni* was seen in Walkerton, Ontario; over 2000 people were infected and seven people died (Holme 2003). This outbreak resulted in changes to drinking water regulations in Canada (Holme 2003) and emphasized the importance of water safety in management of disease even within developed regions such as urban centers found within Canada and the United States of America (USA) (Olsen *et al.* 2002).

Transmission of bacterial pathogens from person to person is one of the main ways to transmit infections in households (Ludwig *et al.* 2002) and within other populated areas such as daycare facilities where person to person contact is common

(Karch *et al.* 2005). Snedeker *et al*. (2009) studying transmission of the bacteria found that person to person transmission occurred in settings such as nurseries and day care facilities where there is interaction between young children among themselves and their caregivers. The study focused on secondary transmission and it was found that 19 % of cases in an outbreak are secondary cases where an infected person passes the disease on to another individual either in the same household or work environment. The main age group to be affected by secondary infections is children under the age of six; this is thought to be due to their immature immune systems and poor hygiene practices. Social interactions and common use of space by contaminated and/or infected individuals likely results in spread of most types of infectious agents. Proof of infectious routes requires both good epidemiological evidence as well as molecular confirmation provided by use of microbial source tracking tools (Meyer *et al*. 2005).

Outbreaks have also occurred via zoonosis or direct transmission of bacteria from animals or their environments to humans; e.g., contaminated animals from farms and petting zoos are known to have infected humans (Crump *et al.* 2002). *E. coli* O157:H7 is known to survive for up to 10 months in some environments; therefore risk of infection can still be high even if the main source of the bacteria is removed (Karch *et al.* 2005).

2.3 Diseases caused by EHEC

When EHEC infections occur they can be asymptomatic, or have milder symptoms such as watery diarrhea (Cleary 2004). Hemorrhagic colitis (HC) occurs when large amounts of blood are seen in the stools of individuals especially when accompanied by cramping and vomiting. Progress from a milder infection to HC can take 1-2 days

(Paton *and* Paton 1998). Bloody diarrhea is more commonly associated with *E. coli* serotype O157:H7 than with any other *E. coli* serotype (Klein *et al.* 2002). HC can be life-threatening in severe cases, especially in young and old people (Coia 1998). HC is not the only complication associated with EHEC infections. Other problems include intussusceptions, where one segment of the intestine is enfolded into the other (Spalding *and* Evans 2004); colonic perforation, where a hole in the intestinal wall is formed as a result of the bacterial infection (De Bartolomeis *et al.* 2005); gangrene, resulting in decay of the body's tissues and peritonitis, an inflammation of the peritoneum (a serous membrane which lines the abdominal cavity) and, sepsis and rectal prolapse, where the rectum protrudes from the anal orifice (Huber *et al.* 1995); pancreatitis, which involves inflammation of the pancreas, leading to coma and, hemiplegia, where one side of the body is weak, subject to seizures, and uncontrolled convulsions; and HUS, a condition which is a sign of damage to the kidneys and other organs in the body (Cleary 2004).

HUS is the major complication associated with EHEC infections (Cleary 2004). Typically, it is characterized by the presence of three traits; *i.e.,* acute renal failure, hemolytic anaemia and thrombocytopaenia (Clarke 2001). HUS occurs when the walls of small blood vessels (endothelial cells) are damaged, leading to hemolysis of the red blood cells that pass through them and may result in vascular thrombosis or clotting in the blood vessels (McCarthy *et al.* 2000). Toxin-damaged endothelial cells can swell narrowing glomerular capillaries within the kidney (Moake 1994). Fibrin thrombi also can form and block capillaries as the intoxication progresses, leading to ischemic damage due to an inadequate blood supply (Proulx *et al.* 2001; Moake 1994). Blocking of renal capillaries can lead to renal failure which is seen in later stages of the disease.

Symptoms of HUS range from a subclinical illness which often is mild and escapes diagnosis, to a life threatening process. It can occur in any age group but occurs more frequently in children and the elderly (Paton *and* Paton, 1998), perhaps due to the presence of an under-developed immune system in children or a declining immune system in the elderly. In some cases, teenagers also can develop HUS where a severe EHEC infection is involved (Cleary 2004). Development of HUS is always preceded by a bout of bloody diarrhea or HC. Typically, HUS presents itself as an acute renal insufficiency, resulting in a rapid breakdown in kidney function. However, only 5-8% of children who develop HC will develop HUS and in most cases HUS is not fatal (less than 5%) if treated appropriately. A larger proportion of the affected children and/or adults who develop HUS will suffer some permanent damage from the condition (Cleary 2004).

Risk of developing HUS as a result of an *E coli* O157:H7 infection is low, but does vary from one outbreak to the next, as well as with the serotype of EHEC involved in the outbreak. Studies that looked at infections in the elderly and children have shown that the risk of developing HUS can be as high as 22% when associated with an *E. coli* O157:H7 outbreak (Cleary 2004). In some EHEC outbreaks where HUS is seen, other serotypes of EHEC also can be found (predominantly in Europe) (Karch *et al.* 2005). However, these non-O157 serotypes often are associated with a milder form of the disease (Cleary 2004).

2.4 *E. coli* O157:H7

Over 200 EHEC serotypes cause human disease but, *E. coli* O157:H7 has been associated with most outbreaks of EHEC globally (Karmali 2005). Despite an increasing

incidence of other *E. coli* serotypes being found in HUS patients, the most common serotype found is still O157:H7. In Europe *E. coli* O157:H7 is found in approximately 50 % of all HUS cases, while in North America it is found in over 95% of cases (Karch *et al.* 2005).

The bacterium, *E. coli* O157:H7 have some specific biochemical properties which are often associated with them and can be used in identification and isolation of the bacteria from clinical and environmental samples (Yoon *and* Hovde 2008). The two main characteristics are an inability to ferment sorbitol in less than 24 h and the inability to produce β-glucoronidase. Other characteristics such as an ability to use dulcitol and raffinose can be used to distinguish O157:H7 from other *E. coli* (Ratnam *et al*. 1988).

2.5 Genes found in *E coli* O157:H7 and the mechanism used for infection

The *E. coli* serotype O157:H7 is an unusually virulent bacterial strain in humans and is the most frequent EHEC serotype associated with HUS and HC (Cleary 2004). *E. coli* O157:H7 bacteria express a variety of virulence factors or proteins that cause disease that are encoded for on pathogenicity islands (PAIs) or on virulence plasmids which often can be incorporated as regions of chromosomal DNA (Kaper *and* Hacker 1999). One of the key systems that EHEC strains such as O157:H7 possess that is essential for virulence is a type III secretion system (TTSS) (Campellone *and* Leong 2003). This bacterial secretion system is needed for contact dependent translocation of bacterial proteins (effectors) into a host cell.

The TTSS is functionally conserved in most bacteria such as *E. coli* O157:H7 and acts as a "molecular syringe" (Tomoyasu *et al.* 2005). This system is encoded for within

the locus of enterocyte effacement (LEE) which is located on a PAI (Elliot *et al.* 2000). The LEE PAI is commonly found in many virulent human and plant pathogens (Deng *et al.* 2004). The TTSS secretes proteins that are involved in modulating the host's cytoskeleton, as well as other effectors such as Tir, which is also encoded for by LEE (Campellone *and* Leong 2003). Tir is the receptor for intimin, an adhesion factor needed for the bacteria to attach to the surface of cells. Association of the bacteria with the host cell surface is referred to as attachment and effacement (A/E) of the bacteria with the host (Paton *and* Paton 1998) and is required for *E. coli* O157:H7 to infect its host. Tir plays a major role in the formation of a pedestal on the host cell surface where the bacteria bind and flatten or thin the host epithelium forming an attaching and effacing lesion (as a pedestal holding a bacterium is formed) (Campellone *and* Leong 2003; Elliot *et al.* 2000).

Formation of an attaching and effacing lesion (A/E) is central to pathogenesis of *E. coli* O157:H7 on host intestinal epithelial cells (Elliot *et al.* 2000). This lesion is characterized by the bacteria's ability to attach itself to intestinal cell membranes, to destroy the microvilli (effacing) and to induce the formation of the pedestal structures, where the bacteria sit (Tomoyasu *et al.* 2005; Deng *et al.* 2004). One of the key proteins needed for attaching of the bacteria is intimin (Nataro *and* Kaper 1998). Intimin is encoded for by the *eae* gene which is located on LEE, the same island that codes for TTSS. The O157:H7 serotype produces extensive A/E lesions which demonstrate the bacteria's ability to intimately adhere to intestinal epithelial cells. In O157:H7 strains where the *eae* gene is knocked out, the bacteria are unable to colonize intestinal epithelial cells, demonstrating the importance of intimin to the progression of the disease (Nataro *and* Kaper 1998). However, not all EHEC serotypes have the *eae* gene indicating that

other adherence factors also can be involved in bacterial virulence; *i.e*., infections with these strains can still cause HUS and HC. During the course of the disease A/E lesions disappear, but are always present at onset of the disease (Nataro *and* Kaper 1998); e.g., in rabbits and piglets development of intestinal symptoms mimicking HC is due to the presence of A/E lesions. In EHEC strains, including O157:H7 the A/E lesions are only present in the large intestine while in other *E. coli* classes, A/E lesions can also be found in the small intestine.

A/E lesions are not the only contributors to the development of HUS and HC. It has been speculated that while A/E lesions are needed to produce diarrheal or intestinal disease symptoms, Shiga toxin (Stx) is required to produce bloody diarrhea and HC. Stx is the major factor and defining virulence characteristic of EHEC (Nataro *and* Kaper 1998). *E. coli* O157:H7 produces at least one Stx that is similar to the Stx produced by *S. dysenteriae*. In fact, Stx1 from *E. coli* O157:H7 differs from the Stx of *S. dysenteriae* by only one amino acid (Yoon *and* Hovde 2008). Before the toxin produced by pathogenic forms of *E. coli* was characterized and found to be similar to the toxin produced by *S. dysenteriae*, Stx was referred to as a verotoxin because of the toxigenic effect it had on cultured Vero cells. Bacteria producing this toxin were called verotoxigenic *E. coli* (VTEC) resulting in the VT nomenclature seen in older literature (Nataro *and* Kaper 1998**;** Paton *and* Paton 1998). Since then, *E. coli* producing these toxins have been called STEC and, now the two terms STEC and VTEC are used interchangeably. EHEC is a subset of STEC/VTEC that causes human illness (Karch *et al.* 2005). Exposure to Stx can potentially lead to complications or death in patients who have contracted HUS.

Sequencing of the *E. coli* O157:H7 genome (Perna *et al.* 2001) has revealed other possible virulence factors, many of which are found on the 9 large OIs of the bacteria (Table 2.3) (Perna *et al.* 2001). Discovery of these islands (Shen *et al.* 2004) has led to speculation that different O-islands may contribute to differences in virulence of *E. coli* O157:H7 and other EHEC serotypes. Shen *et al.* (2004) showed that the EHEC serotype O113:H21 has acquired parts of two OIs from the especially virulent serotype O157:H7 (OI #48 and OI#122). This acquisition of OIs that have a role in virulence from O157:H7 by non-O157:H7 strains could elucidate or explain the increasing virulence of non-O157:H7 strains and why some STEC outbreaks with cases of HUS are not due to the O157:H7 serotype but due to non-O157:H7 serotypes.

2.6 Shiga toxins in *E. coli* O157:H7

Stx is a compound toxin composed of five glycolipid binding β-subunits (7.7 kDa monomers) and one catalytically active α−subunit (32 kDa) (Karch *et al.* 2005; Paton *and* Paton 1998). There are two main types of Stx (Stx1 and Stx2) with at least three variants existing for Stx2 (Table 2.4) (Paton *and* Paton 1998). One or both of these Stx (Stx1 or Stx2, or variants) can be present in EHEC serotypes; *i.e.*, *E. coli* O157:H7 isolates produce one or both of these toxins (Kuntz *and* Kuntz 1999). It appears that Stx2 and its variants are more important to the cytotoxicity associated with HUS than Stx1 (Nataro *and* Kaper 1998); *i.e.*, humans infected with O157:H7 strains just producing Stx2 appear more prone to HUS than strains having Stx1 alone or both Stx1 and Stx2.

In an EHEC infection, once the bacteria adhere to a cell Stx needs to be translocated across the intestinal epithelium into the blood stream of the host in order to

Table 2.3. O-islands encoding putative virulence factors in *E. coli* O157:H7 strain EDL933*.

The O-island	The putative virulence factor
OI #7	Macrophage toxin and ClpB-like chaperon
OI #28	RTX- toxin like exoprotein and transport system
OI #43/48	Two urease gene clusters
OI #47	An adhesion polypeptide and/or fatty acid biosynthesis system
OI # 115	TTSS and secreted proteins similar to *Salmonella-Shigella inv-spa* host cell invasion system
OI #122	Two toxins and PagC like virulence factor
OI #138	Fatty acid biosynthesis system
OI #148	LEE

* Adapted from Perna *et al.* (2001)

Table 2.4. Main types of Stx and variant forms*.

Previous nomenclature	Proposed new nomenclature	
	Gene	Protein
Shiga toxin (Stx)	stx	Stx
Shiga-like toxin I (SLT-I) or verotoxin 1 (VT1)	stx_1	Stx1
SLT-II or VT2	stx_2	Stx2
SLT-IIc or VT2c	stx_{2c}	Stx2c
SLT-IIe or VT2e	stx_{2e}	Stx2e

*Adapted from Paton *and* Paton (1998).

reach the target cells (Paton *and* Paton 1998). The target cells for the toxin are cells that have high levels of the globotriaosylceramide (Gb3) receptor; *i.e*., the glycolipid receptor that can bind to Stx. In humans many Gb3 receptors are found in the kidneys. The Stx works by binding to the glycolipid receptors of target cells and then enters the cells by receptor mediated endocytosis (Paton *and* Paton 1998). Once in the cell, one of two events can occur: 1) The Stx is either degraded by fusion with lysosomes or 2) the Stx is transported to the Golgi apparatus, where it subsequently gets directed back into the endoplasmic reticulum, and then to the cytosol. During the course of this transfer process, the α–subunit of the stx is nicked by a protease (furin) which activates the toxin. The activated toxin interrupts protein production by inhibiting peptide elongation; this can eventually lead to cell death.

Another complication seen associated with HUS is vascular damage due to the toxin (Matussek *et al.* 2003). The toxin induces an inflammatory response in host tissues by activating tumor necrosis factor alpha (TNF-α) as well as interleukins (IL-8 and IL-6) (Nataro *and* Kaper 1998). These factors (TNF-α, IL-8 and IL-6) enhance the effect of the toxin on human endothelial cells because they induce expression of Gb3 and therefore increase binding of toxin to these cells. This immune response to the toxin could result in the vascular damage seen associated with HUS (Matussek *et al.* 2003).

2.7 Use of Phenotype Microarrays to assess bacteria

Phenotype microarrays (PMs) can be used to find functions for genes (Zhou *et al.* 2003) and to see how genes network together by testing various cellular phenotypes simultaneously (Potera 2006a). PMs provide a physiological assessment of a

cell which can complement genomic and proteomic studies studying bacteria (Bochner 2003). PMs can be used to test a wide range of basic cellular activities ranging from nutritional pathways, to osmotic response, pH and chemical sensitivity. These arrays also allow researchers to test for more than one cell phenotype at a time (broad testing); e.g., some mutations/loss of function can affect other cell phenotypes that may not be expected. These changes can be seen with PMs since they test a wide variety of phenotypes at the same time (Bochner 2003; Zhou *et al.* 2003).

PMs measure cell respiration calorimetrically using a tetrazolium dye (Bochner *et al*., 2001). When the dye is reduced, it turns purple (reduction is irreversible) and accumulates in the test mixture. This accumulation can be measured using a spectrophotometer and translated as an absorption measurement, reflecting cell respiration over time. This change in color is easy to monitor, quantify and reproduce. Each PM plate has 96 wells with 95 different substrates and a negative control (water). A cell mixture including tetrazolium dye is then used to inoculate the wells, after which the plates are incubated for 24 to 48 hr. The plates are read using a plate reader; *i.e*., a positive reaction will turn the inoculums purple because of the tetrazolium dye, meaning that the substrate was metabolized by the bacteria. This color change is quantified using the plate reader and is used to create a PM profile.

PMs can be used in comparative genomic studies to compare mutant bacteria to wild type cells. They provide a straightforward way to determine the effects of a mutation on a genome-wide scale (Zhou *et al*. 2003). Zhou *et al*. (2003) created mutations to study two-component regulatory systems in *E .coli* K12 by making 37 different two-component mutants and studying the effects of these mutations using PM plates. They found that 22

out of the 37 two-component mutants they made exhibited phenotypic differences. In one of the two-component systems studied, the role of the CusS/CusR system in copper ion efflux from the cell was examined. In this system *cusRS* mutants showed hypersensitivity to 1, 10-phenanthroline, a chelator with high affinity for copper ions. Their results supported the hypothesis that the CusS/CusR system plays a role in copper efflux from the cell. These arrays can be used to characterize phenotypic or observed traits found in cells by helping to identify the phenotypic range of traits affected by mutations and in some cases can help focus in on a domain within a genome responsible for a phenotypic trait (Bochner 2003).

PMs have also been used to compare pathogenic and non-pathogenic isolates of *E. coli* (Bochner 2003). When Murthy (2006) looked at differences between non-pathogenic *E. coli* K12 and uropathogenic *E. coli*, they identified 37 differences in substrate utilization between the two bacterial strains, suggesting that some of the metabolic pathways used by the bacteria are different. They suggested there were examples of environmental adaptation by bacteria. PMs have also been used to help clarify the roles of pathogenicity islands (PAIs) and the phenotypes they confer to a cell (Bochner 2003). Chouikha *et al*. (2006) used PMs to elucidate the role of a new genomic island (AGI-3) inserted into the *sel*C locus (a "hot spot" for insertion of PAIs) of the extra-intestinal avian pathogenic *E. coli* BEN2908 strain. The role of the AGI-3 genomic island was studied by removing parts of the island and by performing various tests to characterize the island, including PMs. It was found that parts of AGI-3 are also present in other pathogenic *E. coli*, as well as other pathogenic species and that it had a role in carbon assimilation and virulence. These differences in carbon source utilizations can

help clarify the roles of PAIs and identify possible PAIs by determining differences with non-pathogenic strains and/or similarities between pathogenic strains of bacteria.

The PM method has been used successfully in comparative genomics by comparing selected mutant cells to wild type controls as seen with the above mentioned experiment by Zhou *et al*. (2003) looking at two component regulatory systems. Ito *et al.* (2005) also used PMs to study knock out mutants from the KO library (Mori *et al*, 2000) and compare it to the wildtype parental strain BW25113 [lacIq rrnBT14 DlacZWJ16 hsdR514 DaraBADAH33 DrhaBADLD78] which was used to create all the null mutants. They found that these mutants affected various systems in the wildtype strains and often the substrates affected were for mannose, acetate and α-ketoglutarate. The regulatory network for utilization of these substrates may be important.

As well, phenotypic profiling has been used to compare different strains of the same bacterial species such as environmental and clinical isolates of *E. coli* and *Salmonella* (Bochner, 2003). The profiles of some bacterial species can be found in the Biolog™ core database which is being developed commercially for the purpose of bacterial identification (Bochner 2003). One example where PMs were used to compare different strains from the same species was done by Durso *et al*. (2004), who looked at commensal and pathogenic *E. coli* strains. They found that differences in substrate utilization did occur between *E. coli* K-12 strains and *E. coli* O157:H7 strains. For example the substrates D-saccharic acid and D-serine are used by ~80% of commensal strains while only ~15 % of the O157:H7 strains use these two substrates. Their results indicate that for these substrates as well as others, there are differences between strains of the same species.

2.8 Growth Curves

Growth curves are an important tool to study bacteria and are often used to monitor bacterial growth under defined conditions (Fujikawa *et al*. 2004). Bacterial growth curves, including those for *E. coli* typically are sigmoidal in shape (Coleman *et al.* 2003). They are characterized as having a lag phase, an exponential growth phase, a stationary phase and a death phage where the bacteria die. Duplication of identical growth conditions is extremely important since different conditions affect growth of bacteria and need to be taken into account when using growth curves to study bacteria. Roszak *and* Colwell (1987) mentioned that the normal state of bacteria is quite different than the laboratory state of bacteria and that, laboratory conditions are radically different from the natural growth conditions of the natural habitat where the studied bacteria are found; these conditions need to be taken into account when comparing bacterial growth to their natural habitat because bacteria grow differently under different conditions.

E. coli O157:H7 growth has been monitored under different conditions, such as variation in pH and agitation of the culture (*i.e*., with agitation and without agitation) and with different strains initially isolated under different conditions (Coleman *et al*. 2003). For example at 10 °C, the rate of growth changed depending on whether a low or high density of bacteria was used to inoculate the growth medium; differences across 3 bacterial strains were seen in each case. Valero *et al.* (2010) looked at growth of different *E. coli* serotypes under the same conditions; *i.e*., pH, temperature and inoculum levels. It was found that under all conditions, *E. coli* serotype O158:H23 had a faster growth than all the other serotypes tested, including O157:H7. They also found that O157:H7 could not grow at a temperature of 8 °C in a pH of 5.5 with a low inoculum

level, while serotypes O55:H6 and O158:H23 grew under those same conditions. This indicates that growth curves generated under identical growth conditions can be used to elucidate differences between species of the same bacteria.

2.9 Survivability Curves

E. coli O157:H7 can survive for long periods of time in water making it an important pathogen when involved in waterborne outbreaks (Flint 1987). Studies looking at survival of *E. coli* O157:H7 in different water environments have been undertaken. Cook *et al.* (2007) compared the survival of *E. coli* O157:H7 and *Campylobacter jejuni* in ground water and found that *E. coli* O157:H7 could survive just as well in nutrient free water as in water containing carbon or nitrogen while *C. jejuni* died off 2.5 to 13 times quicker than *E. coli* in groundwater. Smith *et al.* (1994) conducted experiments with *E. coli* in polar marine environments for 54 days; at the conclusion of the experiment it was found that the cells were no longer able to grow on selective medium but direct viable counts indicated that cells were still present but in a viable but not culturable (VBNC) state. *E. coli* can enter a VBNC state when exposed to stresses such as changes in pH, available nutrients and/or temperature or in starvation conditions (Liu *et al*. 2008).

One method used to study survivability of *E. coli* in water is the BacLight Live/Dead Bacterial Viability kit by Invitrogen. Liu *et al.* (2008) used the kit to count the number of *E. coli* O157:H7 cells present in water in a starvation experiment designed to force *E. coli* O157:H7 into a VBNC state for subsequent tests. After 13 weeks, cells were no longer culturable but were still viable using the Live/Dead staining; these cells were still viable after 16 weeks. Lisle *et al.* (1998) used the Live/Dead kit to assess the effect

of starvation on *E. coli* O157:H7 prior to testing resistance to chlorine to examine drinking water safety. They used the Live/Dead kit to assess membrane integrity while inducing starvation and found no difference in the BacLight assay in comparison to other assays for number of bacteria in the culture during starvation. Petit *et al.* (2000), used the BacLight kit to measure membrane integrity of *E. coli* cells in 2 different microcosms with river water; they found that neither light or starvation affected bacterial counts and that the counts remained stable during the course of the experiment.

The BacLight Live/Dead kit uses a mixture of a SYTO 9 green-fluorescent nucleic acid stain and a propidium iodide red-fluorescent nucleic acid stain. The SYTO-9 penetrates the membranes of all bacteria, living or dead, intact or damaged and the propidium iodide only penetrates damaged or dead cells. This results in a mixture of cells that are either, alive and stained green, or dead and stained red. This allows the researcher to perform direct bacterial counts using fluorescence microscopy. The counts can then be used to create survivability curves to compare different strains of bacteria such as *E. coli* to each other.

Chapter III

Effect of Pathogenicity Islands on Substrate Utilization in *E. coli* O157:H7

3.1 Introduction

The *E. coli* serotype O157:H7 is an unusually virulent bacterial strain that is the most frequent EHEC serotype isolated that is known to be involved in HUS and HC (Cleary 2004). *E. coli* O157:H7 express a variety of factors that can be encoded for on pathogenicity islands (PAIs) or on plasmids, such as pO157 (Kaper *and* Hacker 1999). Pathogenicity islands (PAIs) are segments/regions of DNA found within the chromosomal DNA, where virulence and fitness genes often are grouped together (Kaper *and* Hacker 1999). These genomic blocks of DNA differ between pathogenic and non-pathogenic strains of bacteria from the same or closely-related species (Maurelli *et al*. 1998; Gal-Mor *and* Finlay 2006). They usually span a large continuous genomic region, ranging in size from ~10 to 200 kb (Wang *et al*. 2010). Generally, genes associated with adhesions, invasins, iron uptake systems, toxins and type II, III and IV secretion systems are found on PAIs (Kaper *and* Hacker 1999).

Characterization of PAIs is important not only to development of an understanding of pathogenesis and the development of O157:H7 disease, but also to allow for development of vaccines or new methods to identify the bacterium (Wang *et al*. 2010). One method available to characterize these PAIs is through use of gene deletions. A gene deletion involves removal of a gene or a group of genes from the organism, effectively knocking out gene function (Kabir *et al.* 2005). This method has been used in our laboratory to create a series of O157:H7 deletion mutants, missing specific PAIs, also known as OIs and SLs (Perna *et al*. 2001; Hayashi *et al*. 2001). These OIs and SLs are

DNA segments unique to the strains they are found in; *i.e.*, OIs (OI) were first identified in *E. coli* O157:H7 EDL933 while, SLs (SL) were first identified in *E. coli* O157:H7 Sakai (Perna *et al*. 2001; Hayashi *et al*. 2001). The O157:H7 deletion mutants each are missing one OI or one SL. In this study we examine 13 deletion mutants in total, 12 of which are O157:H7 EDL933 OI mutants and one of which is a Sakai SL mutant. All islands appear to be O157:H7 specific; *i.e.*, they appear to be found only in O157:H7 bacterial isolates.

One approach to characterization of bacterial mutants is through use of phenotypic microarrays (Bochner 2003). Phenotype microarrays (PMs) can be used to find functions for genes (Zhou *et al.* 2003) and to see how genes network together by testing various cellular phenotypes simultaneously (Potera 2006a). Deletion of as yet uncharacterized pieces of DNA such as OI's may lead to a change in function in more than one phenotype; this change in function can be assessed using PMs since they test a wide variety of phenotypes, and not just one (Bochner 2003; Zhou *et al*. 2003).

PMs can also be used in comparative genomics to compare mutant phenotypes to that of wild type cells. They provide a straightforward way to determine the effects of a mutation on a genome-wide scale (Zhou *et al*. 2003). They can also be used to help isolate and characterize mutations that have occurred in a cell by narrowing the phenotypic range and in some cases can be used to narrow the domain of the mutation (Bochner 2003). Zhou *et al*. (2003) created mutations to study *E .coli* K12's two-component regulatory systems by making 37 different two-component mutants and studying the effects of the mutations using PM plates. They found that 22 of the two-component mutants exhibited phenotypic differences while 15 did not. One example of a

two-component system that was studied is the CusS/CusR membrane transport system which is thought to play a role in a copper ion efflux from the cell. The *cusRS* mutant showed hypersensitivity to 1, 10-phenanthroline, which is a chelator with high affinity for copper. These results supported the hypothesis that the CusS/CusR system plays a role in a copper efflux cell transport.

PMs have also been used to compare pathogenic and non-pathogenic *E. coli* (Bochner 2003). Murthy (2006) looked at differences between non-pathogenic *E. coli* K12 and uropathogenic *E. coli* strain OPE013 using Biolog's PM plates. They found 37 differences in substrate utilization between the two, suggesting that the metabolic pathways are different for these two strains. Durso *et al*. (2004) compared *E. coli* O157:H7 strains to commensal *E. coli* strains and found that there was a difference in carbon source utilization pattern between the two groups. There were 19 carbon sources that were used by *E. coli* O157:H7 in comparison to commensal *E. coli* strains. Chouikha *et al*. (2006) used PMs to help elucidate the role of the genomic island, AGI-3 which was inserted into the *sel*C locus (a "hot spot" for insertion of PAIs) of an extra-intestinal avian pathogenic *E. coli* strain BEN2908. The role of the AGI-3 genomic island was studied by removing parts of the island and by performing various tests to characterize the island, including PMs. It was found that parts of this island are also present in other pathogenic *E. coli*, as well as other pathogenic species and that it had a role in carbon assimilation and virulence. These differences in carbon source utilization can help clarify the roles of pathogenicity islands (PAIs) and identify possible PAIs by identifying differences with non-pathogenic strains and/or similarities between pathogenic strains of bacteria.

In this study the PM profiles for 13 PAI deletion mutants and three controls (O157:H7 strains EDL933 and Sakai plus the *E. coli* K-12 reference strain DH5α) were determined. The mutants were compared to their respective O157:H7 control as well as the *E. coli* K-12 reference strain DH5α. Specific objectives of this study were:

1) To create PM profiles for each of the mutants and the three controls.

2) To compare O157:H7 strains EDL933 and Sakai to the *E. coli* K-12 reference strain DH5α.

3) To compare O157:H7 strains EDL933 and Sakai to each other.

4) To compare the OI deletion mutants and the SL deletion mutant to the *E. coli* K-12 reference strain DH5α and their respective parental strains EDL933 and Sakai.

3.2 Materials and Methods

3.2.1 Bacteria and Cultures

Bacteria used in this study included the *E. coli* K-12 reference strain DH5α, and the pathogenic *E. coli* O157:H7 strains EDL933 and Sakai (both isolated from humans), as well as 13 OI and one SL deletion mutant; *i.e.*, OI-39, OI-51, OI-55, OI-59, OI-87, OI-89, OI-98, OI-102, OI-108, OI-135, OI-172, OI-176 and SL 72 (OI deletion mutants were created by removing an OI from *E. coli* O157:H7 strain EDL933 and the SL deletion mutant was created by removing an SL from *E. coli* O157:H7 strain Sakai). Single colonies of bacteria were streaked on to Tryptic Soy Agar (TSA) and grown overnight for 12-16 h at 37 °C; this was repeated twice in order to ensure purity of the cultures and to ensure the bacteria are in the same physiological state. After the final overnight incubation, 2 colonies from each TSA plate containing pure cultures was streaked on to Biolog Universal Growth Medium™ (BUG) plus 5 % sheep blood (BUG+B). The BUG+B plates were incubated overnight for 16-18 h at 37 °C.

3.2.2 The Biolog™ System

The following protocols for the Biolog™ system were adapted from an M. Sc. thesis study, "Enterococci as Source-Tracking Agents" by Lang (2005), and from "PM Procedures for *E. coli* and other GN Bacteria" (Biolog Inc. 2005).

3.2.3 Preparation of PM inoculating fluids.

The inoculating fluid (IF-0) was prepared by adding 25 ml of sterile water into the bottle containing 125 ml of 1.2 x IF-0. From this bottle 16 ml of 1x IF-0 was removed

and stored into a sterile tube, this tube was then put aside for future use. To the bottle containing the IF-0 solution, 1.8 ml of dye mix (dye mix A) and 23.2 ml of water were added to make an IF-0 + dye mixture. Then 25 ml of this IF-0 + dye mixture was dispensed into a sterile reservoir and stored until future use.

3.2.4 Preparation of cell suspension cultures for inoculation of Biolog™ plates

PM1 plates (Biolog™, Catalogue # 12111) were removed from refrigeration and stored at room temperature until needed. A turbidity meter (Biolog™) was calibrated prior to making the cultures for inoculation of the phenotypic plates (Biolog™). An uninoculated tube containing 16 ml of IF-0 was prepared while making the inoculating fluid, cleaned with a paper tissue (Kimwipe™), inserted into the turbidity meter and the instrument set to 100 % transmittance (%T). A bacterial turbidity standard (Biolog™) pre-calibrated for 85 % ± 3 % transmittance was mixed and then used to calibrate the turbidity meter. After calibration a clean IF-0 tube was inserted into the turbidity meter and the machine set to 100 % T. This was done for each new tube of IF-0 used to dilute a bacterial culture in to as the tubes were not optically uniform. Then a long sterile swab (~30 mm in length) was moistened with IF-0 medium and then gently scraped over the BUG+B agar with an overnight culture so as to pick up some bacterial cells but, not any nutrients from the agar. The swab was then twirled inside the IF-0 tube just below the fluid level to mix in the bacteria. After depositing the cells, the tube was mixed with the swab in an up and down motion so as to not introduce any bubbles in the tube. The above was continued until the IF-0 tube had a %T of 42 % ± 3 %. This mixture was used to create the final inoculum for the plates. In the reservoir containing 25 ml of IF-0 + dye

mixture, 5 ml of the IF-0 with bacteria with a %T of 42 % ± 3 % was added and mixed gently so as to not introduce any bubbles in the reservoir, creating a uniform solution. The final cell density of this mixture was 85 % T ± 3 %.

3.2.5 Inoculation of the PM plates (PM1)

The prepared culture with a %T between 82 – 88 % was used to inoculate the PM1 plates. A multichannel repeating pipettor (8 channels) was set to dispense 100 µl of culture. The pipettor was primed by taking up the cell suspension and dispensing 100 µl of culture back into the reservoir. After priming the 8-channel pipettor 100 µl of cell suspension was dispensed into the wells of the PM plate until all 96 wells were filled. The PM1 wells all contained carbon sources except for well A1 which was a blank / control which contained distilled water. The tetrazolium in the inoculum was used to measure substrate utilization and turned from clear to purple when the substrate was utilized. Inoculated plates were incubated overnight for 72 h at 37 ºC with readings taken every 24 h.

3.2.6 Reading the PM plates (PM1)

Following overnight incubation, the PM plates were read using a Biolog MicroStation™. The MicroStation read each well of the plate at 590 nm and then again at 750 nm to measure the color change. The value from the A1 well (blank) was subtracted from the reading obtained from the substrate-containing wells in order to account for any carry over from the BUG+B agar that might have contaminated over the wells. The final output values were calculated by subtracting the value at 750 nm from

the value at 590 nm. Results were then entered into an Excel spreadsheet for analysis. This protocol was followed for each strain and was repeated 2 times for a total of 3 measurements for each *E. coli* strain tested.

3.2.7 Analysis of PM data

All data was analyzed using Excel and statistical functions from this program. This allowed the data from each of the strains to be compared for similarities and differences. A positive result was considered one that had an absorbance value of 0.100 or higher; a negative result was considered one that had an absorbance value lower than 0.100. The data was also analyzed for significant differences in levels of substrate utilization, when comparing the mutants and the O157:H7 parental controls EDL933 and Sakai. A significant difference in either substrate utilization or in level of substrate utilization was determined by a two-tailed T-test assuming equal variance with a P value of 0.05.

After evaluation of collected data only data collected after 48 h of incubation was used because some of the substrates required 48 h for full color development and by 72 h, background color had developed which interfered with the plate readings. The O157:H7 control EDL933 was used to assess the OI-mutants, while the O157:H7 control Sakai was used to assess the SL mutant. The reference K-12 strain DH5α was used to assess differences with both the O157:H7 parental strains and their deletion mutants.

3.3 Results

3.3.1 Phenotypic profiling

In order to characterize the metabolic capabilities of different strains of *E. coli* O157:H7, cells were grown on phenotypic microarray (PM1) plates (Biolog^{TM}) for 48 h and the results assessed to identify similarities and differences among the bacterial isolates examined.

3.3.2 Confirmation of known properties of *E. coli* O157:H7

E. coli O157:H7 is known to have specific biochemical properties (Figure. 3.1) that can be used for identification and isolation of the bacteria; e.g., most *E. coli* O157:H7 are able to ferment the sugar, dulcitol, but cannot ferment the sugar sorbitol (Ratnam *et al*. 1988). PM1 plates (Biolog^{TM}) contain the substrates, dulcitol and sorbitol. When *E. coli* O157:H7 control strains EDL933 and Sakai, as well as the thirteen OI and SL deletion mutants used in this study were grown on PM1 plates, all cells were positive for dulcitol utilization (Figure. 3.2), but negative for sorbitol utilization (Figure. 3.3).

Absorbance values for dulcitol utilization were not the same in the control strains. On average values varied from 0.3 in Sakai to 0.5 in EDL933 (Figure. 3.2) while values in the OI deletion mutants of EDL933 ranged from 0.1 to 0.6, a six fold range in activity and, averaged 0.1 in the SL 72 deletion mutant produced from the Sakai strain. These results suggest that dulcitol utilization in *E. coli* O157:H7 can be influenced by the presence and/or absence of O islands and SLs in the bacteria.

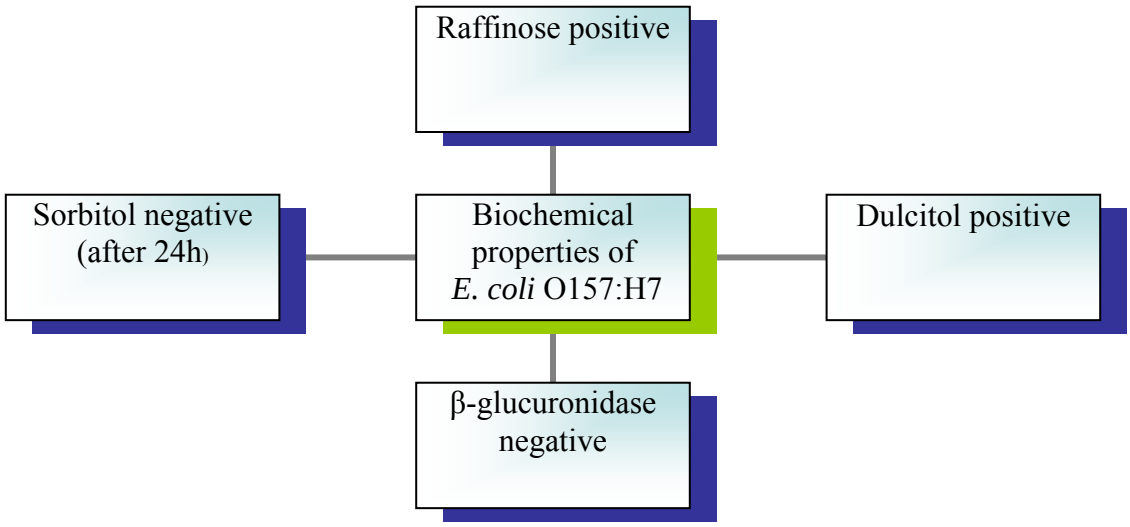


Figure. 3.1: Biochemical properties associated with most *E. coli* O157:H7.

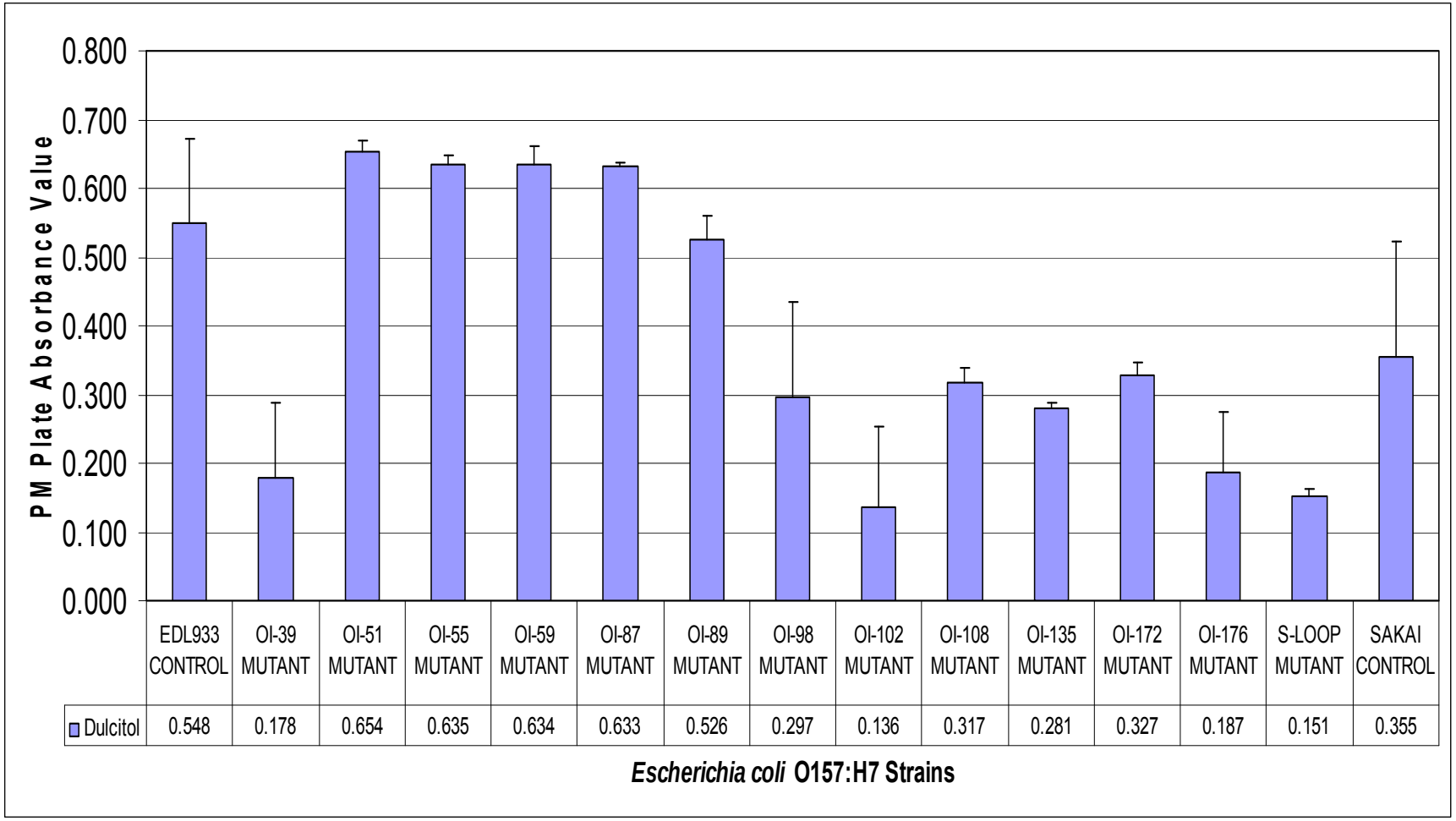


Figure. 3.2: Dulcitol utilization in *E. coli* O157:H7 strains EDL933 and Sakai plus thirteen OI and SL deletion mutants. All strains were tested 3 times using Biolog™ PM1 plates and the average absorbance at 48 h plotted. Values > 0.1 were considered positive.

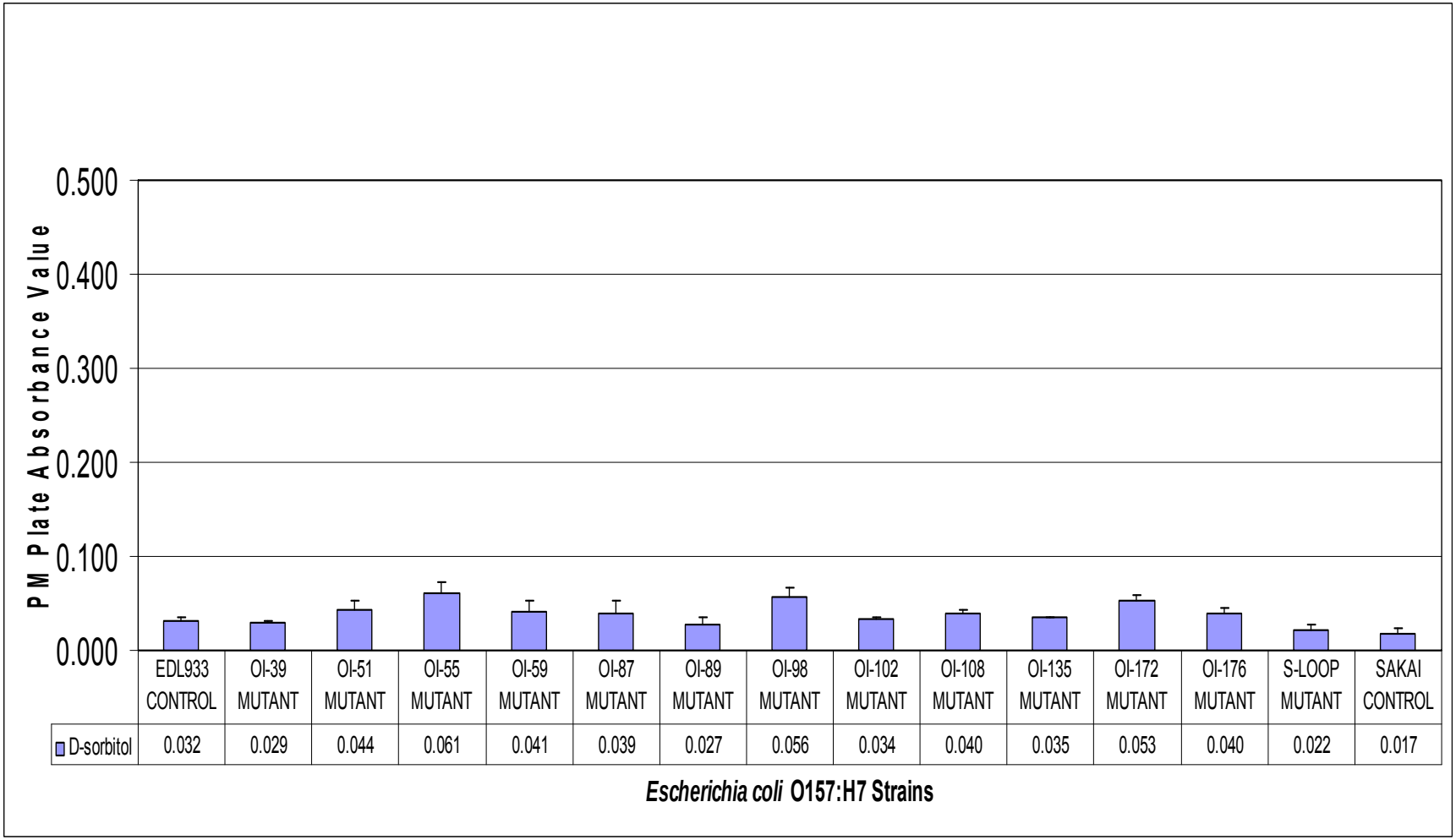


Figure. 3.3: Sorbitol utilization in *E. coli* O157:H7 strains EDL933 and Sakai plus thirteen OI and SL deletion mutants. All strains were tested 3 times using PM1 plates (Biolog^{TM}) and the average absorbance at 48 h plotted. Values $\geq$ 0.1 were considered positive.

3.3.3 Confirmation of known properties of *E. coli* strains using the metabolic pathway for glycolysis.

Glycolysis involves conversion of glucose to pyruvate to produce ATP in the absence of oxygen (Figure. 3.4). Bacteria, including *E. coli* which undergo these processes should be positive for substrate intermediates associated with this process. PM1 plates contain three of the substrates found in the glycolytic pathway; *i.e.*, glucose, glucose-6-phosphate, fructose-6-phosphate and the product of glycolysis pyruvic acid. All of the *E. coli* strains tested were positive for utilization of these substrates (Figure. 3.5).

3.3.4 Comparison of substrate utilization patterns in *E. coli* DH5α and *E. coli* O157:H7 strain EDL933

Substrates used by both *E. coli* DH5α and *E. coli* O157:H7 strain EDL933 were similar.. However, significant differences in substrate utilization were observed for 15 substrates ($P < 0.05$). *E. coli* O157:H7 strain EDL933 was positive for use of the substrates (above threshold value of ≥ 0.100) dulcitol, α-keto-butyric acid, α-D-lactose, lactulose, sucrose, α-hydroxy butyric acid and propionic acid (Figure.3.6), while *E. coli* DH5α was negative for these substrates (below threshold value of < 0.100). The opposite was seen for the substrates D-saccharic acid, D-serine, D-sorbitol, L-glutamic acid, D-galactonic acid-γ-lactone, M-tartaric acid, glycolic acid and L-lyxose (Figure.3.7); *i.e.*, *E. coli* DH5α was positive for these substrates while *E. coli* O157:H7 strain EDL933 was negative ($P < 0.05$).

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate also were seen (Table 3.1). *E. coli* O157:H7 strain

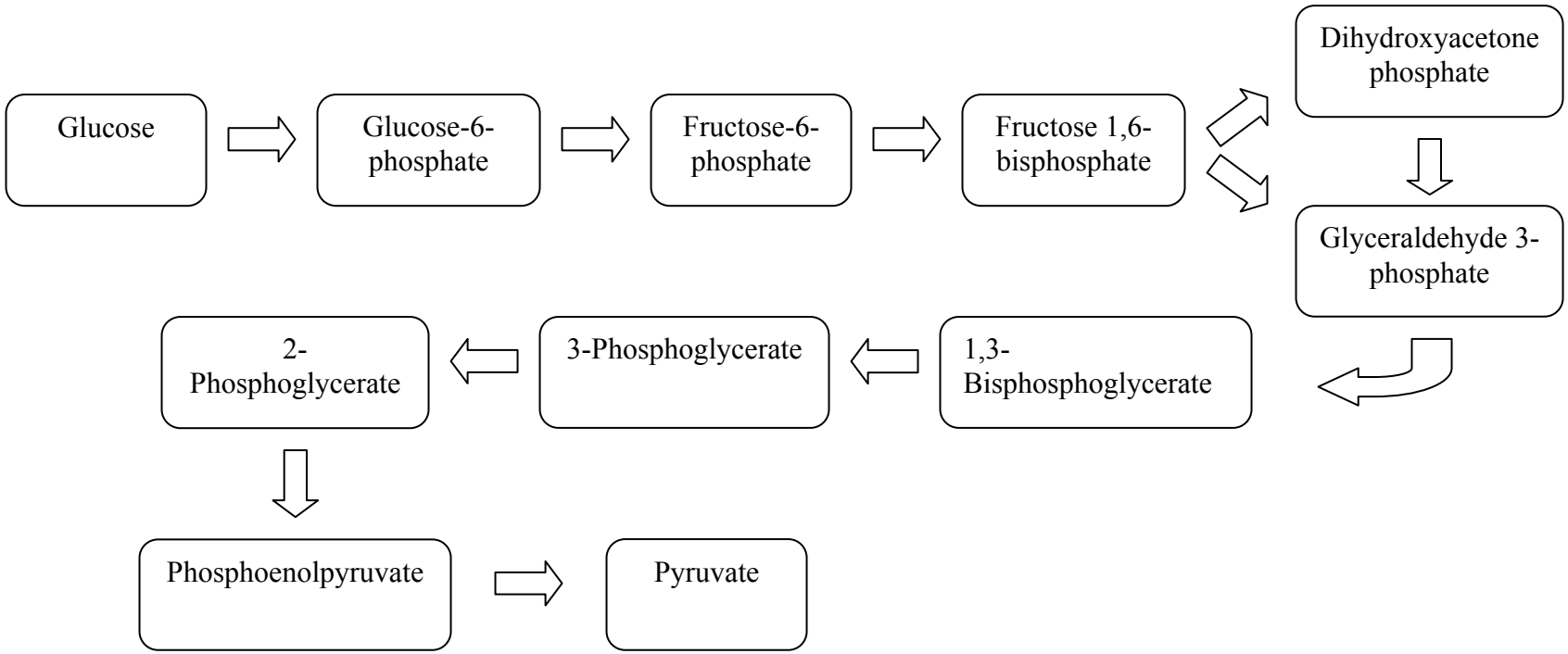
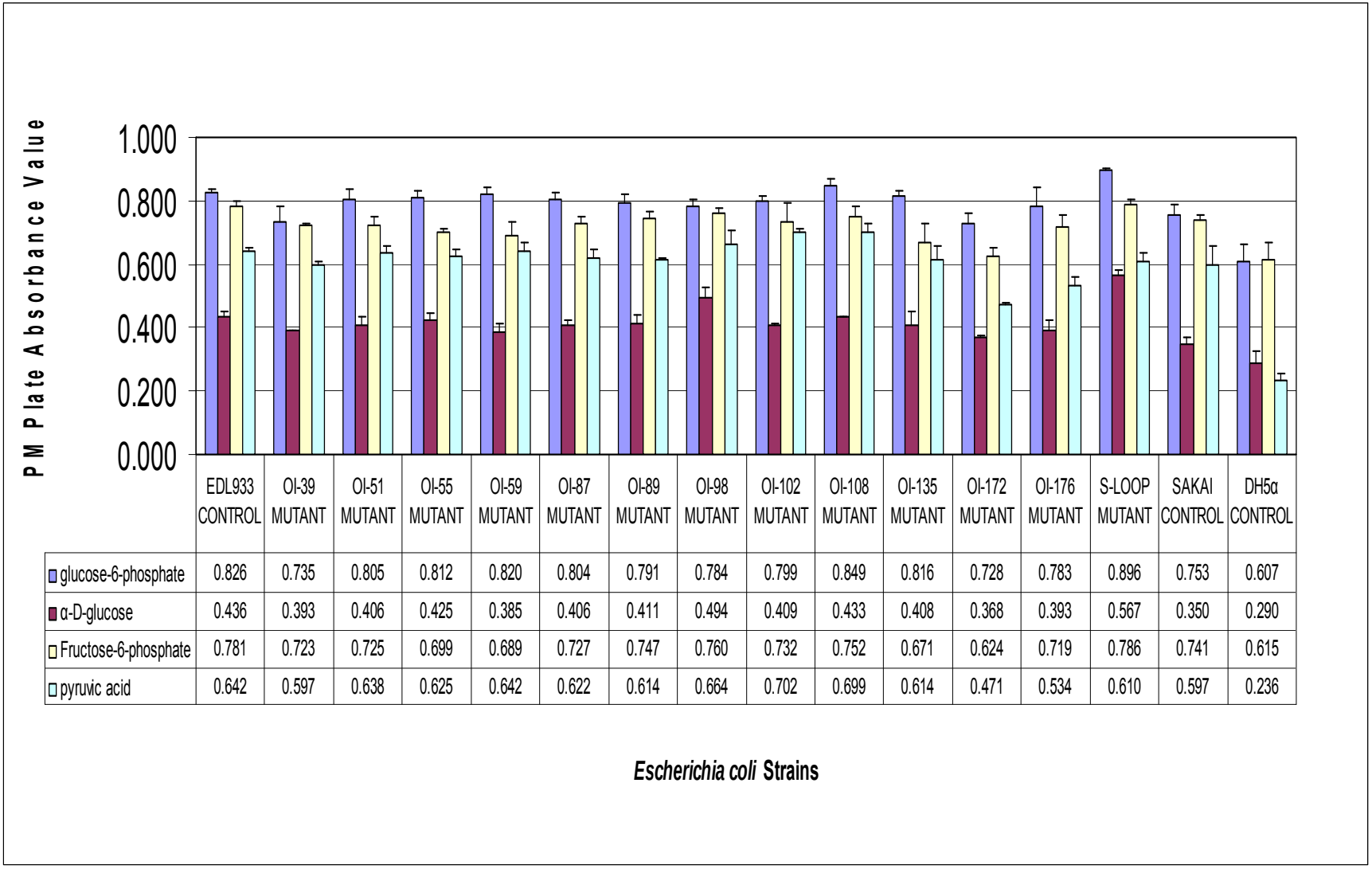
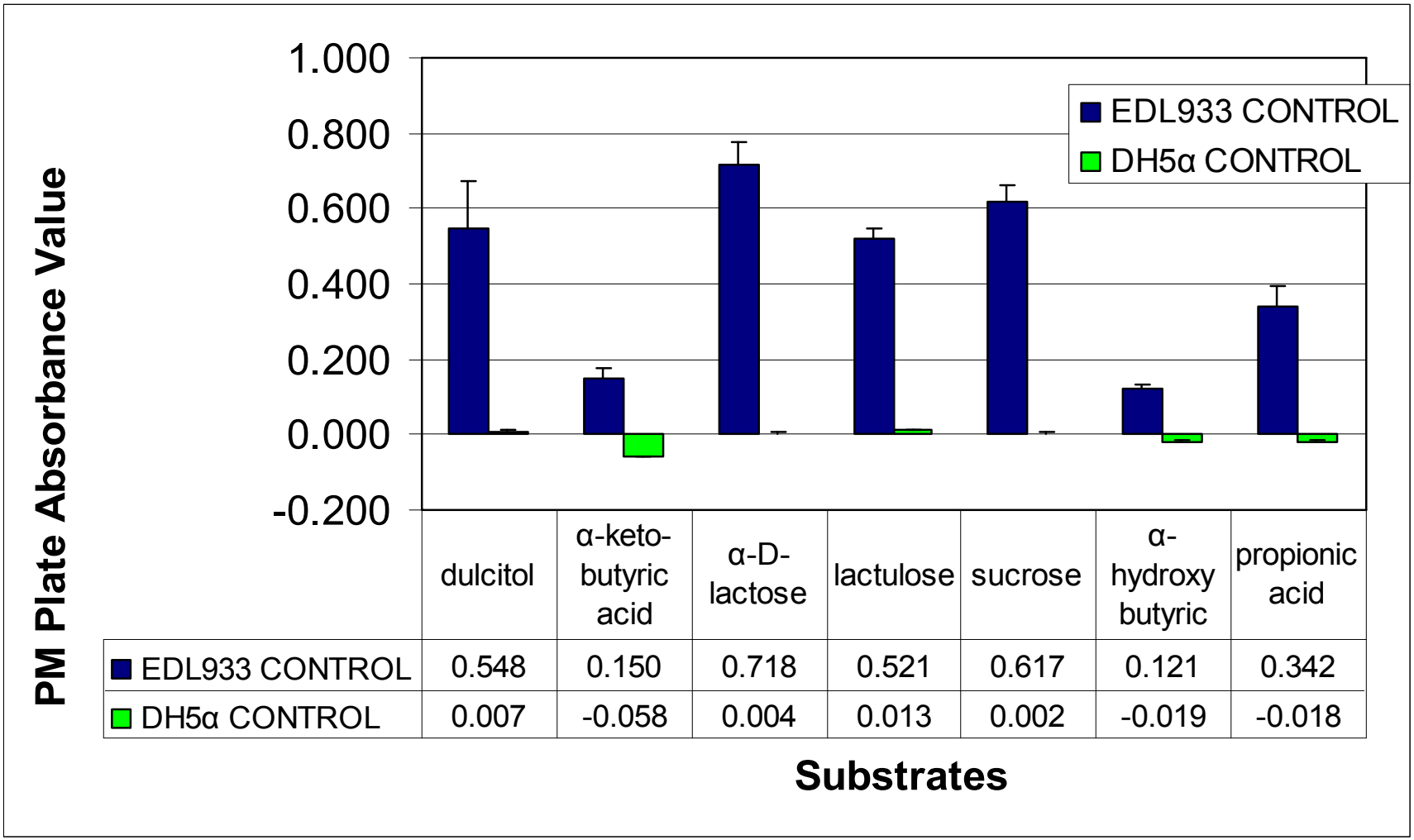


Figure. 3.4. The glycolysis pathway.



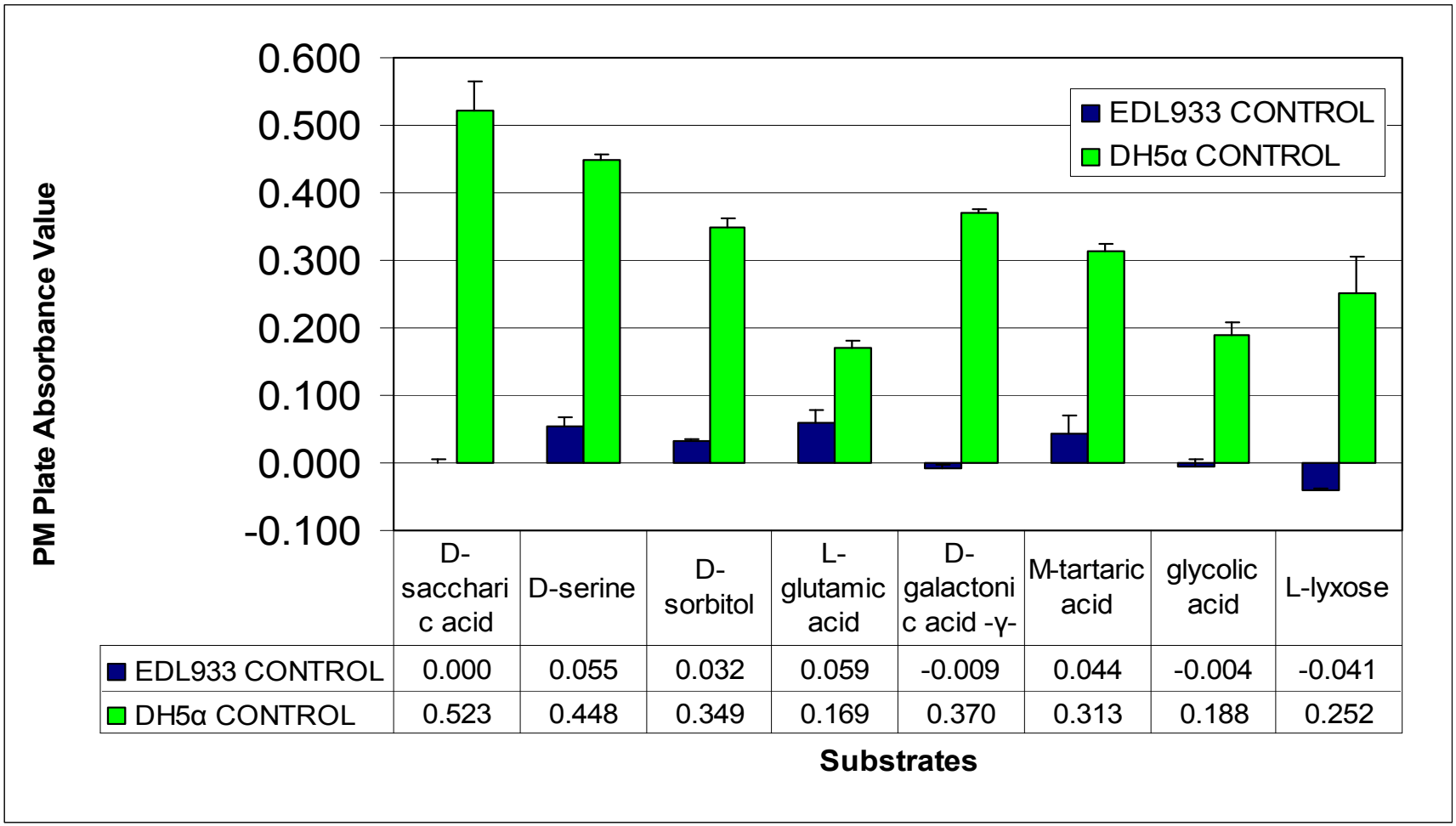
	EDL933 CONTROL	OI-39 MUTANT	OI-51 MUTANT	OI-55 MUTANT	OI-59 MUTANT	OI-87 MUTANT	OI-89 MUTANT	OI-98 MUTANT	OI-102 MUTANT	OI-108 MUTANT	OI-135 MUTANT	OI-172 MUTANT	OI-176 MUTANT	S-LOOP MUTANT	SAKAI CONTROL	DH5α CONTROL
glucose-6-phosphate	0.826	0.735	0.805	0.812	0.820	0.804	0.791	0.784	0.799	0.849	0.816	0.728	0.783	0.896	0.753	0.607
α-D-glucose	0.436	0.393	0.406	0.425	0.385	0.406	0.411	0.494	0.409	0.433	0.408	0.368	0.393	0.567	0.350	0.290
Fructose-6-phosphate	0.781	0.723	0.725	0.699	0.689	0.727	0.747	0.760	0.732	0.752	0.671	0.624	0.719	0.786	0.741	0.615
pyruvic acid	0.642	0.597	0.638	0.625	0.642	0.622	0.614	0.664	0.702	0.699	0.614	0.471	0.534	0.610	0.597	0.236

Figure. 3.5. PM results for *E. coli* O157:H7 strains and *E. coli* DH5α for substrates involved in glycolysis.



	dulcitol	α-keto-butyric acid	α-D-lactose	lactulose	sucrose	α-hydroxy butyric	propionic acid
EDL933 CONTROL	0.548	0.150	0.718	0.521	0.617	0.121	0.342
DH5α CONTROL	0.007	-0.058	0.004	0.013	0.002	-0.019	-0.018

Figure.3.6 Comparison of *E. coli* O157:H7 strain EDL933 and the *E. coli* K-12 reference strain DH5α for the substrates dulcitol, α-keto-butyric acid, α-D-lactose, lactulose, sucrose, α-hydroxy butyric acid and propionic acid. The threshold value for substrate utilization was <0.100. The EDL933 strain is above the threshold value in each case and the DH5α strain is below the threshold value.



	D-saccharic acid	D-serine	D-sorbitol	L-glutamic acid	D-galactonic acid -γ-	M-tartaric acid	glycolic acid	L-lyxose
EDL933 CONTROL	0.000	0.055	0.032	0.059	-0.009	0.044	-0.004	-0.041
DH5α CONTROL	0.523	0.448	0.349	0.169	0.370	0.313	0.188	0.252

Figure. 3.7 Comparison of *E. coli* O157:H7 strain EDL933 and the *E. coli* K-12 reference strain DH5α for the substrates D-saccharic acid, D-serine, D-sorbitol, L-glutamic acid, D-galactonic acid-γ-lactone, M-tartaric acid, glycolic acid and L-lyxose. The threshold value for substrate utilization was <0.100. The DH5α strain is above the threshold value in each case and the EDL933 strain is below the threshold value.

Table 3.1. Percentage difference* in levels of substrate utilization for the pathogenic *E. coli* O157:H7 EDL933 strain and the reference *E. coli* K-12 strain DH5α where all substrates were used by both bacteria.

PM # of Substrate	SOURCE	EDL933 Control	DH5α Control	Percentage Difference (%)
2	L-arabinose	0.461	0.289	37.4
3	N-acetyl-D-glucosamine	0.529	0.272	48.5
5	succinic acid	0.614	0.422	31.2
6	D-galactose	0.517	0.297	42.6
7	L-aspartic acid	0.655	0.412	37.2
8	L-proline	0.629	0.265	57.9
9	D-alanine	0.597	0.386	35.3
10	D-trehalose	0.721	0.302	58.1
11	D-mannose	0.528	0.350	33.7
15	glycerol	0.564	0.351	37.8
16	L-fucose	0.615	0.325	47.1
19	D,L-α-glycerol-phosphate	0.436	0.261	40.1
20	D-xylose	0.660	0.303	54.1
21	L-lactic acid	0.495	0.318	35.8
25	glucose-6-phosphate	0.826	0.607	26.5
27	D,L-malic acid	0.714	0.382	46.4
28	D-ribose	0.566	0.290	48.8
33	α-D-glucose	0.436	0.290	33.4
34	maltose	0.540	0.377	30.3
35	D-melibiose	0.678	0.405	40.3
36	thymidine	0.722	0.464	35.8
37	L-asparagine	0.647	0.400	38.2
44	α-methyl-D-galactoside	0.534	0.348	34.8
48	uridine	0.698	0.527	24.5
49**	**L-glutamine**	**0.234**	**0.388**	**39.6**
51	glucose-1-phosphate	0.805	0.545	32.3
52	Fructose-6-phosphate	0.781	0.615	21.2
56	β-methyl-D-glucoside	0.433	0.300	30.7
58	maltotriose	0.610	0.414	32.1
59	2-deoxy adenosine	0.904	0.560	38.0
60	adenosine	0.755	0.456	39.5
61	glycyl-L-aspartic acid	0.607	0.415	31.6
65	fumaric acid	0.574	0.280	51.2

66	bromo succinic acid	0.636	0.228	64.2
72	inosine	0.766	0.426	44.3
75	L-serine	0.532	0.373	29.9
77	L-alanine	0.501	0.304	39.4
80	N-acetyl-β-D-mannosamine	0.354	0.242	31.6
82	methyl pyruvate	0.588	0.326	44.6
84	L-malic acid	0.641	0.329	48.6
92	pyruvic acid	0.642	0.236	63.3
93	L-galactonic acid-γ-lactone	0.607	0.221	63.6
94	D-galacturonic acid	0.793	0.582	26.6

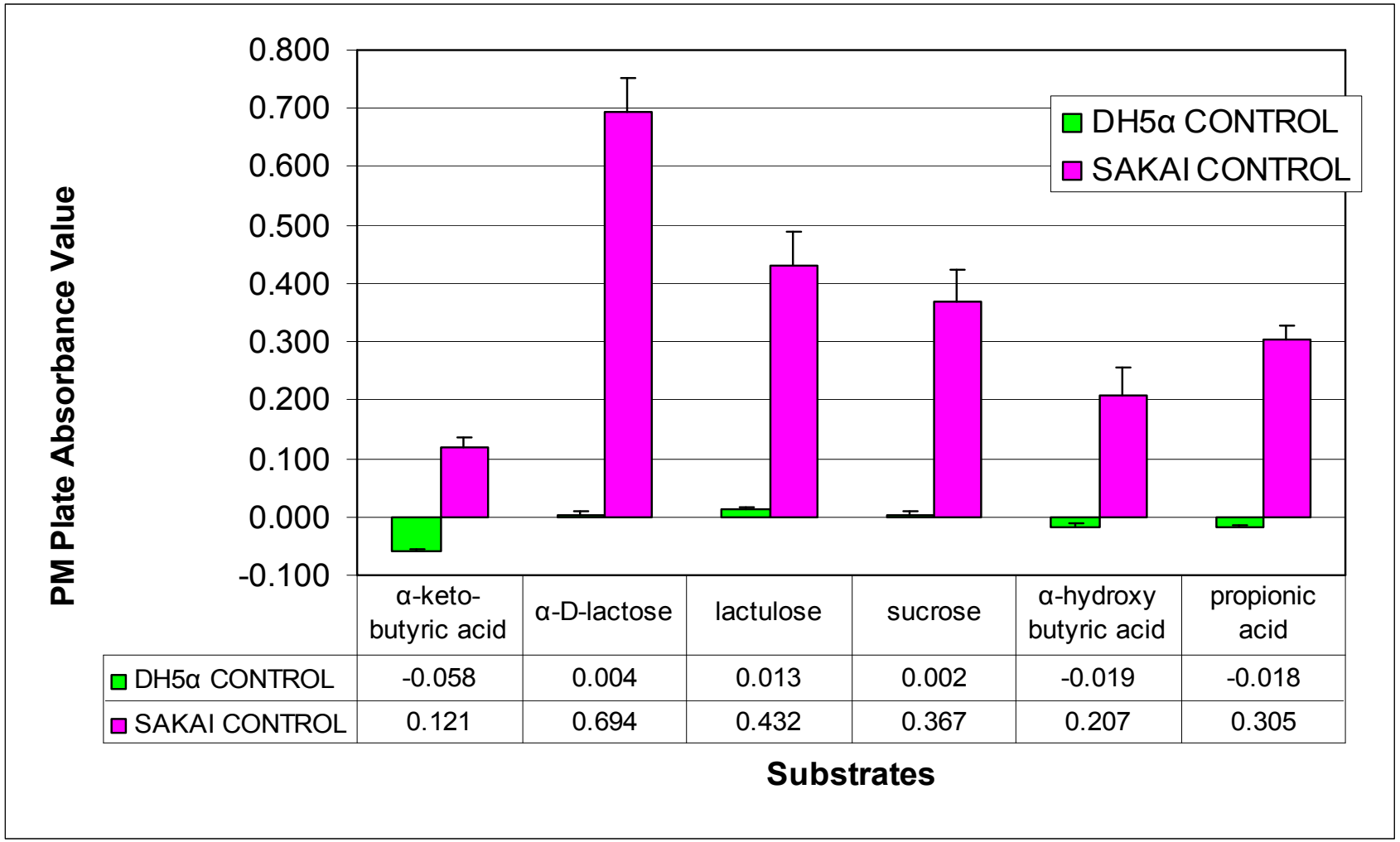
*All values were significantly different ($P < 0.05$).

** Bolded value is the case where DH5α has a higher level of substrate utilization.

EDL933 utilized significantly higher levels of 42 of the 43 positive-testing substrates. These substrates were L-arabinose, N-acetyl-D-glucosamine, succinic acid, D-galactose, L-aspartic acid, L-proline, D-alanine, D-trehalose, D-mannose, glycerol, L-fucose, D,L-α-glycerol phosphate, D-xylose, L-lactic acid, glucose-6-phosphate, D,L,-malic acid, D-ribose, α-D-glucose, maltose, D-melibiose, thymidine, L-asparagine, α-methyl-D-galactoside, uridine, glucose-1-phosphate, fructose-6-phosphate, β-methyl-D-glucoside, maltotriose, 2-deoxy adenosine, adenosine, glycyl-L-aspartic acid, fumaric acid, bromo succinic acid, inosine, L-serine, L-alanine, N-acetyl-β-D-mannosamine, methyl pyruvate, L-malic acid, pyruvic acid, L-galactonic acid-γ-lactone and D-galacturonic acid. In contrast, strain DH5α used significantly more L-glutamine than *E. coli* EDL933. Percentage differences in utilization for each of these substrates were 37.4%, 48.5%, 31.2%, 42.6%, 37.2%, 57.9%, 35.3%, 58.1%, 33.7%, 37.8%, 47.1%, 40.1%, 54.1%, 35.8%, 26.5%, 46.4%, 48.8%, 33.4%, 30.3%, 40.3%, 35.8%, 38.2%, 34.8%, 24.5%, 32.3%, 21.2%, 30.7%, 32.1%, 38.0%, 39.5%, 31.6%, 51.2%, 64.2%, 44.3%, 29.9%, 39.4%, 31.6%, 44.6%, 48.6%, 63.3%, 63.6%, 26.6% and 39.6% respectively (Table. 3.1).

3.3.5 Comparison of substrate utilization patterns in *E. coli* DH5α and *E. coli* O157:H7 strain Sakai

Substrate utilization for the *E. coli* DH5α and *E. coli* O157:H7 strain Sakai also was similar. However, significant differences in substrate utilization ($P < 0.05$) were seen for 18 substrates. *E. coli* O157:H7 strain Sakai was positive (above threshold value of 0.100) for α-keto-butyric acid, α-D-lactose, lactulose, sucrose, α-hydroxy butyric acid and propionic acid (Figure. 3.8). By contrast, *E. coli* DH5α was negative (below the



	α-keto-butyric acid	α-D-lactose	lactulose	sucrose	α-hydroxy butyric acid	propionic acid
DH5α CONTROL	-0.058	0.004	0.013	0.002	-0.019	-0.018
SAKAI CONTROL	0.121	0.694	0.432	0.367	0.207	0.305

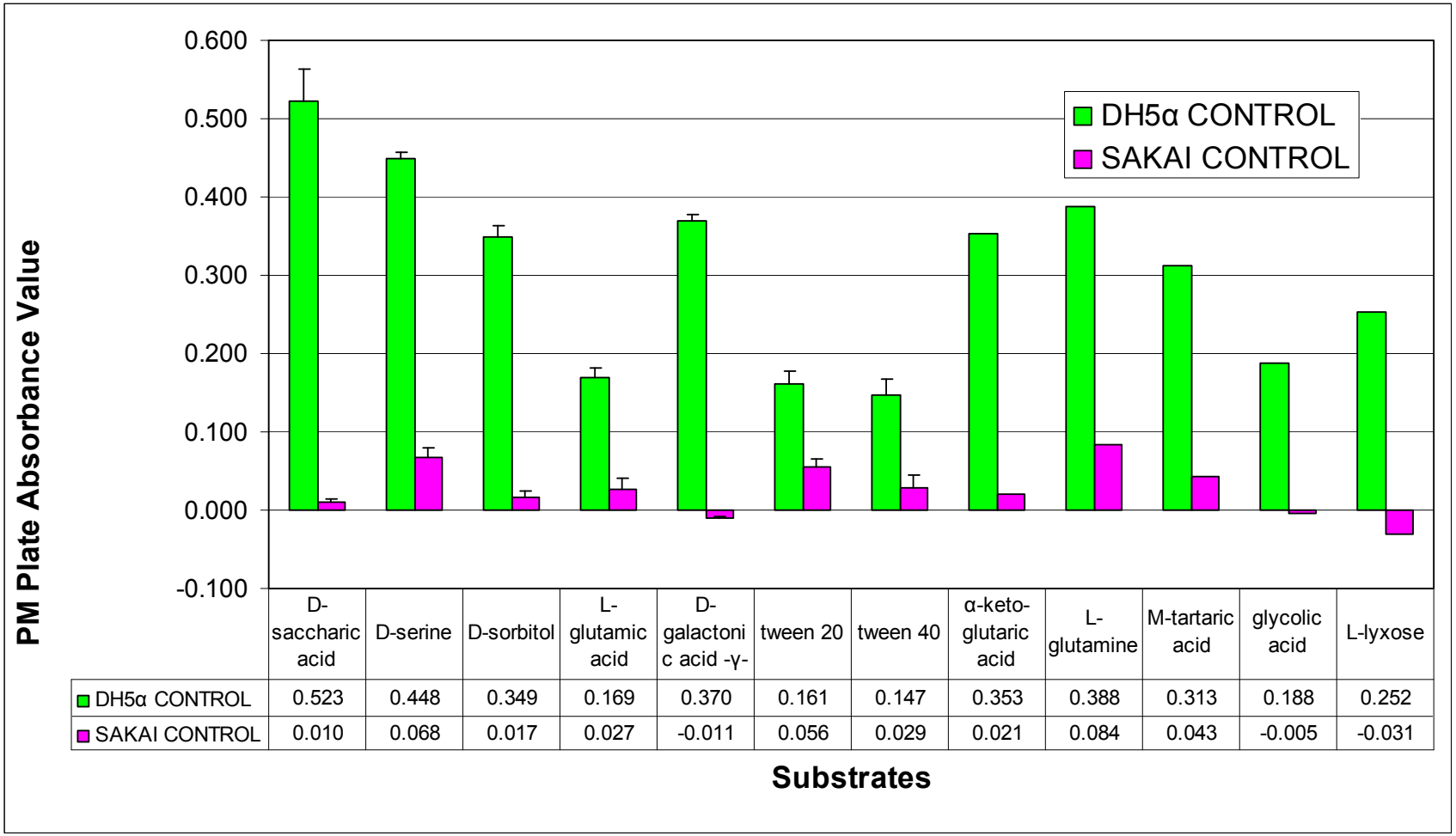
Figure.3.8 Comparison of *E. coli* O157:H7 strain Sakai and the reference *E. coli* K-12 strain DH5α for the substrates, α-keto-butyric acid, α-D-lactose, lactulose, sucrose, α-hydroxy butyric acid and propionic acid. The threshold value for substrate utilization was 0.100. The Sakai strain is above the threshold value and the DH5α strain is below in each case.

threshold value of 0.100) for these substrates but, positive for D-saccharic acid, D-serine, D-sorbitol, L-glutamic acid, D- galactonic-acid-γ-lactone, tween 20, tween 40, α-keto-glutaric acid, L-glutamine, M-tartaric acid, glycolic acid and L-lyxose (Figure.3.9) while Sakai was negative.

Significant differences ($P < 0.05$) in levels of substrate utilization also were seen for 25 other substrates (Table 3.2). *E. coli* O157:H7 strain Sakai utilized significantly higher levels of N-acetyl-D-glucosamine, succinic acid, D-alanine, D-trehalose, glycerol, L-fucose, D,L-α-glycerol phosphate, L-lactic acid, D-ribose, acetic acid, maltose, thymidine, L-asparagine, α-methyl-D-galactoside, β-methyl-D-glucoside, maltotriose, 2-deoxy adenosine, adenosine, fumaric acid, bromo succinic acid, inosine, L-alanine, L-alanyl-glycine, methyl pyruvate and pyruvic acid than *E. coli* DH5α. Percentage differences in utilization for each of these substrates were 30.9%, 26.3%, 38.2%, 48.4%, 40.8%, 37.2%, 42.3%, 42.9%, 44.3%, 20.3%, 20.5%, 40.7%, 26.0%, 29.0%, 22.7%, 28.1%, 21.0%, 38.3%, 26.8%, 56.7%, 35.8%, 46.0%, 42.8%, 33.7% and 60.5% respectively (Table. 3.2).

3.3.6 Comparison of substrate utilization patterns in *E. coli* O157:H7 strains EDL933 and Sakai

Substrate utilization profiles for the two O157:H7 strains EDL933 and Sakai were similar. Only one difference in substrate utilization ($P < 0.05$) was noted. The EDL933 strain was positive for L-glutamine (above the threshold of 0.100) while Sakai was negative for this substrate (below the threshold value) (Figure 3.10). Both EDL933 and Sakai had nearly identical substrate utilization profiles. The main difference between the



	D-saccharic acid	D-serine	D-sorbitol	L-glutamic acid	D-galactonic acid -γ-	tween 20	tween 40	α-keto-glutaric acid	L-glutamine	M-tartaric acid	glycolic acid	L-lyxose
DH5α CONTROL	0.523	0.448	0.349	0.169	0.370	0.161	0.147	0.353	0.388	0.313	0.188	0.252
SAKAI CONTROL	0.010	0.068	0.017	0.027	-0.011	0.056	0.029	0.021	0.084	0.043	-0.005	-0.031

Figure. 3.9 Comparison of *E. coli* O157:H7 strain Sakai and the *E. coli* K-12 reference strain DH5α for the substrates, D-saccharic acid, D-serine, D-sorbitol, L-glutamic acid, D-galactonic-acid-γ-lactone, tween 20, tween 40, α-keto-glutaric acid, L-glutamine, M-tartaric acid, glycolic acid and L-lyxose. The threshold value for substrate utilization was 0.100. The DH5α strain is above the threshold value in each case and the Sakai strain is below the threshold value.

Table 3.2. Percentage difference* in levels of substrate utilization for the pathogenic *E. coli* O157:H7 Sakai strain and the reference *E. coli* K-12 strain DH5α where all substrates were used by both bacteria.

# of Substrate	SOURCE	Sakai Control	DH5α Control	Percentage difference (%)
3	N-acetyl-D-glucosamine	0.394	0.272	30.9
5	Succinic acid	0.573	0.422	26.3
9	D-alanine	0.625	0.386	38.2
10	D-Trehalose	0.585	0.302	48.4
15	glycerol	0.593	0.351	40.8
16	L-fucose	0.518	0.325	37.2
19	D,L-α-glycerol-phosphate	0.453	0.261	42.3
21	L-lactic acid	0.556	0.318	42.9
28	D-ribose	0.520	0.290	44.3
32	acetic acid	0.347	0.276	20.3
34	maltose	0.474	0.377	20.5
36	thymidine	0.781	0.464	40.7
37	L-asparagine	0.541	0.400	26.0
44	α-methyl-D-galactoside	0.491	0.348	29.0
56	β-methyl-D-glucoside	0.389	0.300	22.7
58	maltotriose	0.576	0.414	28.1
59	2-deoxy adenosine	0.709	0.560	21.0
60	adenosine	0.740	0.456	38.3
65	fumaric acid	0.383	0.280	26.8
66	bromo succinic acid	0.527	0.228	56.7
72	inosine	0.664	0.426	35.8
77	L-alanine	0.563	0.304	46.0
78	L-alanyl-glycine	0.592	0.339	42.8
82	methyl pyruvate	0.491	0.326	33.7
92	pyruvic acid	0.597	0.236	60.5

*All values were significantly different ($P < 0.05$).

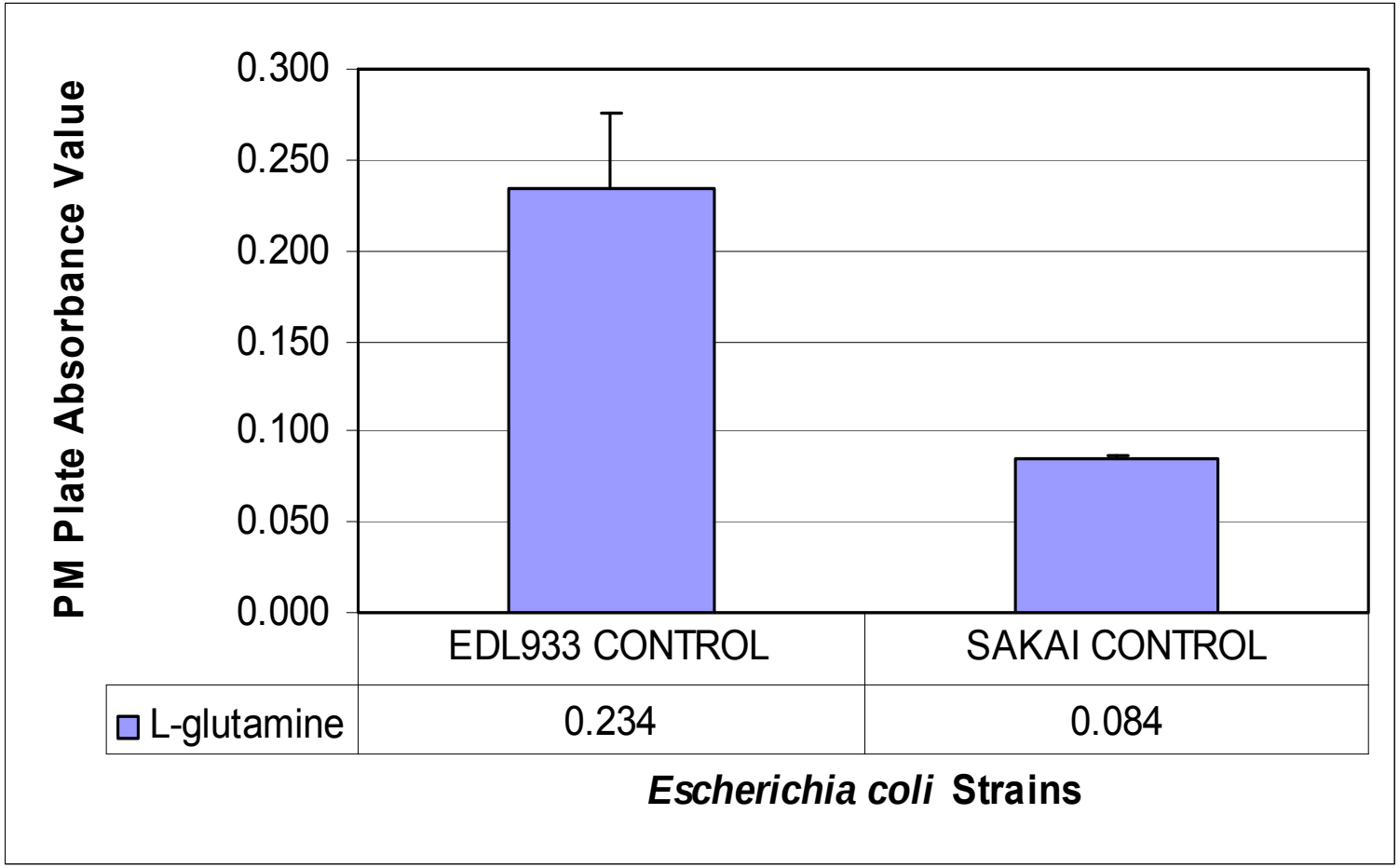


Figure. 3.10 Comparison of *E. coli* O157:H7 strains EDL933 and Sakai for the substrate L-glutamine. The threshold value for substrate utilization was 0.100. EDL933 is above the threshold value in each case and Sakai is below the threshold value

two was in levels of utilization where both strains were positive for a substrate (Table 3.3). Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were seen where EDL933 utilized significantly higher levels ($P < 0.05$) of 12 substrates than Sakai; *i.e.*, EDL933 utilized significantly more L-arabinose, N-acetyl-D-glucosamine, L- aspartic acid, L-proline, α-D-glucose, maltose, sucrose, 2-deoxy adenosine, glycyl-L-aspartic acid, fumaric acid, glycyl-L-proline and D-galacturonic acid than Sakai. Percentage differences in utilization for each of these substrates were 26.0%, 25.5%, 29.3%, 31.6%, 19.7%, 12.3%, 40.4%, 21.6%, 24.1%, 33.3%, 49.6% and 17.7% respectively.

3.3.7 Metabolism in O157:H7 strain EDL933 and its OI deletion mutants

3.3.7.1 Comparison of O157:H7 strain EDL933 with the deletion mutant OI-135

The OI-135 island contains two open reading frames (ORFs) of unknown function. The OI-135 deletion mutant was the only mutant that did not show either a loss or gain in substrate utilization when compared to the parental strain, O157:H7 strain EDL933; *i.e.*, no significant differences in levels of substrate utilization were observed between the OI-135 mutant and the EDL933 parental strain. Consequently, under the growth conditions used in these experiments, no role for the OI-135 island was identified.

3.3.7.2 Comparison of deletion mutants OI-55, OI-59, OI-87 and OI-108 with EDL933.

The OI 55, 59, 87 and 108 contain ORFs for various functions (Perna *et al,* 2001). The OI 55 contains ORFs of unknown function and ORFs that have a transport role. The

Table 3.3. Percentage difference* in levels of substrate utilization for the pathogenic *E. coli* O157:H7 strains EDL933 and Sakai.

# of Substrate	SOURCE	EDL933 Control	Sakai Control	Percentage Difference (%)
2	L-arabinose	0.461	0.341	26.0
3	N-acetyl-D-glucosamine	0.529	0.394	25.5
7	L-aspartic acid	0.655	0.463	29.3
8	L-proline	0.629	0.430	31.6
33	α-D-glucose	0.436	0.350	19.7
34	maltose	0.540	0.474	12.3
47	sucrose	0.617	0.367	40.4
59	2-deoxy adenosine	0.904	0.709	21.6
61	glycyl-L-aspartic acid	0.607	0.461	24.1
65	fumaric acid	0.574	0.383	33.3
85	glycyl-L-proline	0.347	0.175	49.6
94	D-galacturonic acid	0.793	0.652	17.7

*All values were significantly different ($P < 0.05$).

OI-59 has an ORF of unknown function and an ORF thought to code for a regulator. The OI 87 has an unclassified ORF and an ORF of unknown function. The OI-108 has unknown ORFs that are phage or prophage related; *i.e.*, ORFs that code for proteins that are phage or prophage related and ORFs similar to transposases. The deletion mutants corresponding to each of these OI (55, 59, 87 and 108) were compared to the parental strain EDL933. The patterns of substrate utilization (+ or -) for OI-55, 59, 87 and 108 deletion mutants and the O157:H7 parental strain were similar in most cases; *i.e.*, the same substrates were either used or not used in each of these mutant strains as well as in the parental strain EDL933. However, differences were observed in levels of substrate utilization. Two substrates that appeared to show differences in substrate utilization were tween 20 and tween 40 (Figure. 3.11). Levels of substrate utilization for tween 20 and tween 40 fell below the threshold value of 0.100 in EDL933 while mutant levels of substrate utilization were above the threshold value; this suggests that the four mutants (OI-55, 59, 87 and 108) had gained the ability to use tween 20 and tween 40 when compared to the parental strain. However comparison of numerical values for these levels using a T-test showed that the differences in substrate utilization for tween 20 and tween 40 between most of the mutants and the EDL933 control were not different ($P \geq 0.05$). Only the OI-87 mutant used significantly higher ($P < 0.05$) levels of tween 40 than EDL933 suggesting that the OI-87 mutant did gain the ability to use tween 40 as a carbon source.

Significant differences ($P < 0.05$) in levels of substrate utilization also were seen in the OI-55 deletion mutant and the parental strain (Figure. 3.12). The OI-55 mutant utilized significantly higher levels of the three substrates D,L-α-glycerol-phosphate,

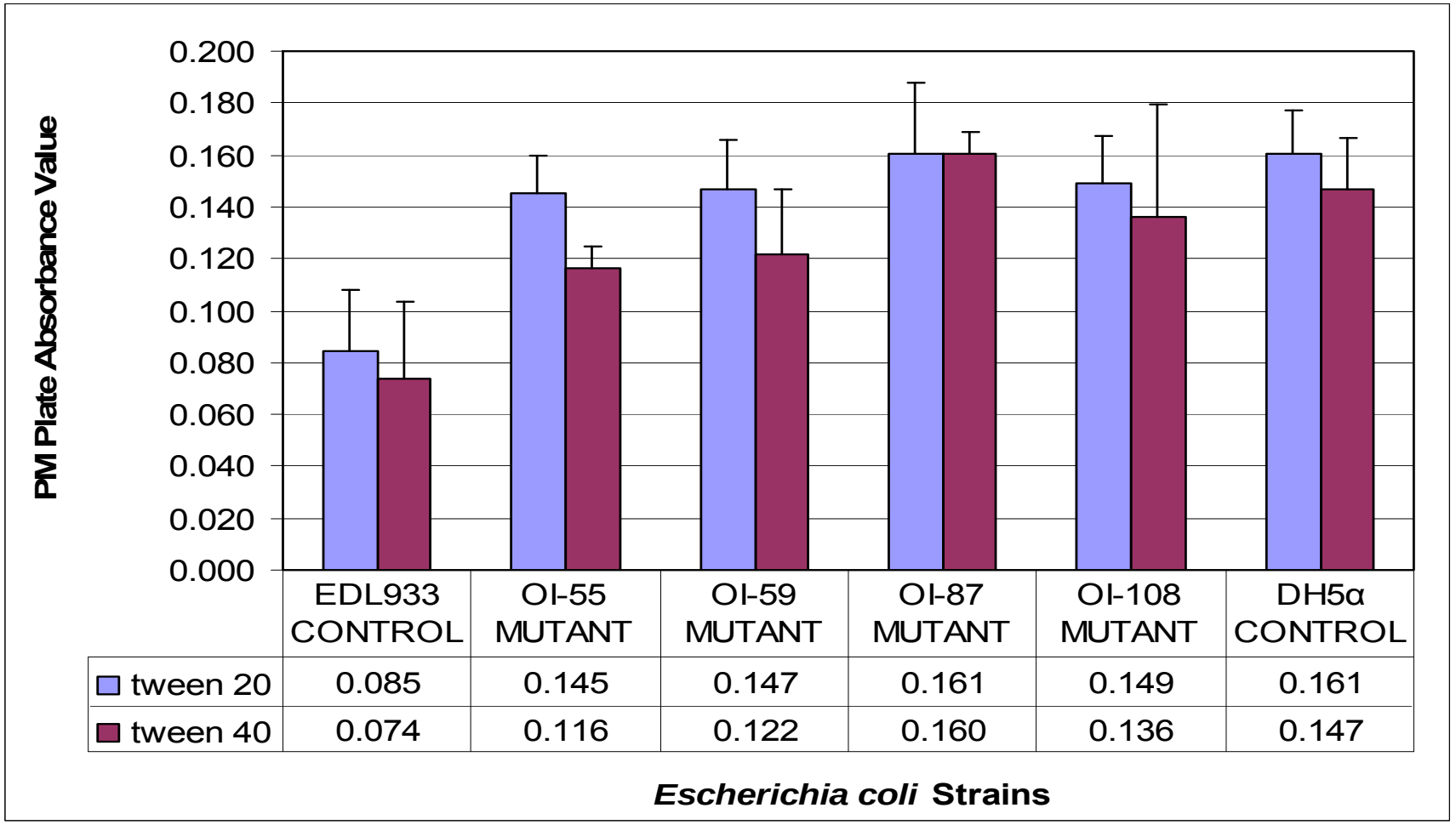
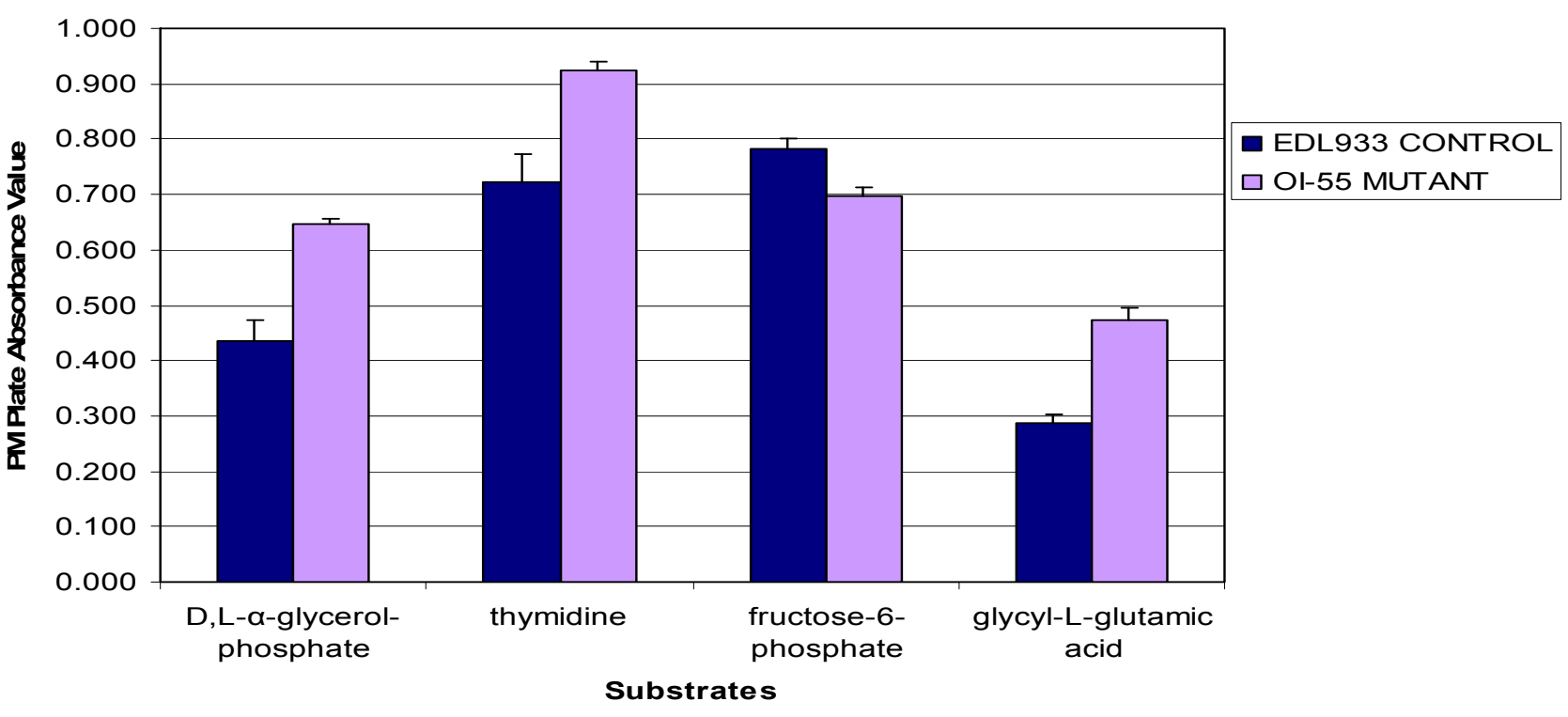


Figure. 3.11. Comparison of *E. coli* O157:H7 parental strain EDL933, the OI-55, 59, 87 and 108 deletion mutants and the *E. coli* K-12 reference strain DH5α for the Substrates tween 20 and tween 40. The threshold value for substrate utilization was < 0.100. The absorbance mean ± S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure. The only significant difference was seen for the OI-87 mutant and the EDL933 parental for tween 40.



SOURCE	D,L-α-glycerol-phosphate	thymidine	fructose-6-phosphate	glycyl-L-glutamic acid
EDL933 CONTROL	0.436	0.722	0.781	0.287
OI-55 MUTANT	0.646	0.925	0.699	0.474
PERCENTAGE DIFFERENCE (%)	32.5	22.0	10.5	39.5

Figure. 3.12. Differences in substrate utilization in the *E. coli* O157:H7 parental strain EDL933 and the OI-55 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization are identified at the bottom of the figure.

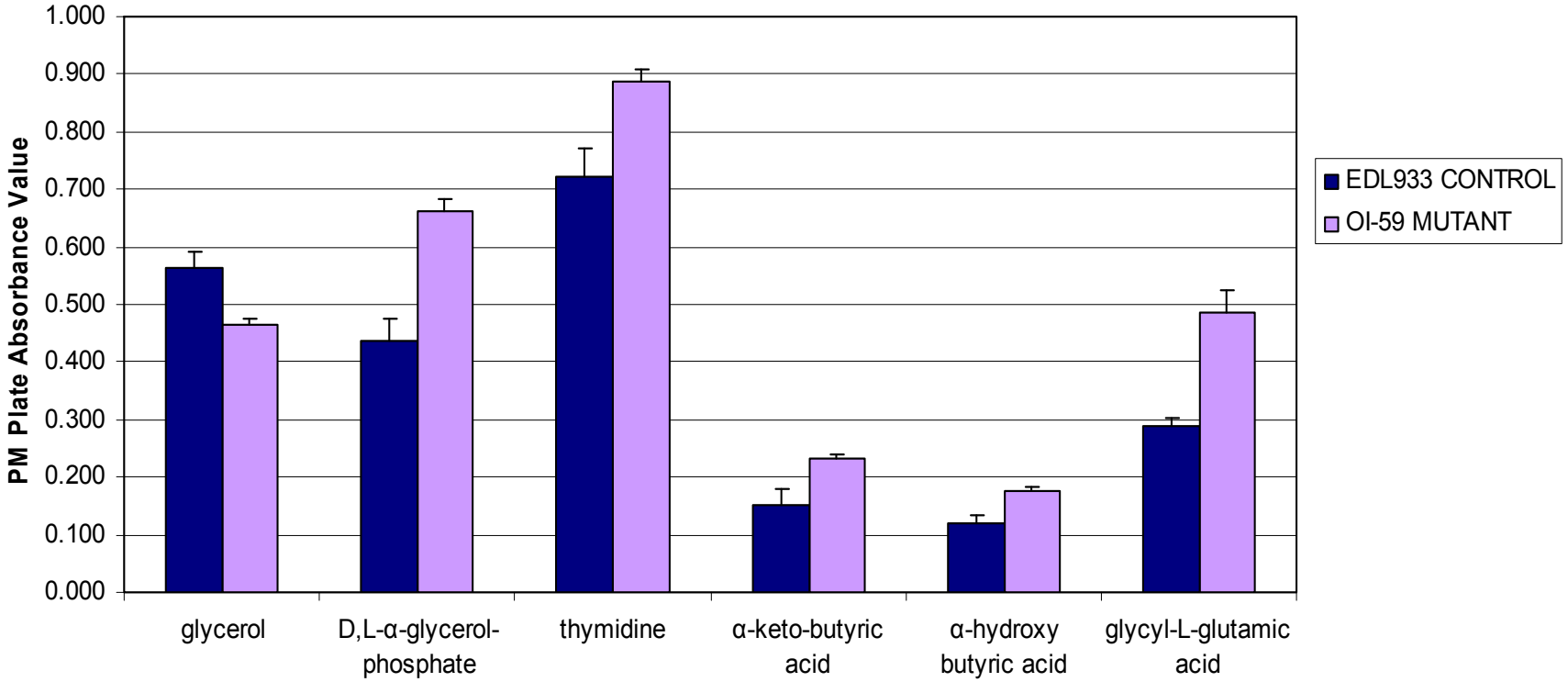
thymidine and glycyl-L-glutamic acid and decreased levels of fructose-6-phosphate compared to the EDL933 parental control ($P < 0.05$). Percentage differences in utilization for each of these substrates were 32.5%, 22.0%, 39.5% and 10.5% respectively.

By comparison the OI-59 mutant (Figure 3.13) used increased levels of five substrates, D,L-α-glycerol-phosphate, thymidine, α-keto-butyric acid, α-hydroxy butyric acid and glycyl-L-glutamic acid and decreased levels of glycerol as carbon sources in comparison to the EDL933 control ($P < 0.05$). The percentage difference in utilization for each of these substrates was 34.1%, 18.8%, 35.8%, 30.4%, 40.9% and 17.6%, respectively.

The OI-87 deletion mutant differed in use of five substrates (Figure. 3.14). In all cases, this mutant used significantly more D,L-α-glycerol-phosphate, D-ribose, thymidine, α-hydroxy butyric acid and glycyl-L-glutamic acid as carbon sources in comparison to the EDL933 control ($P < 0.05$). Percentage differences in utilization for each of these substrates were 29.9%, 7.9%, 21.6%, 31.7% and 41.4% respectively.

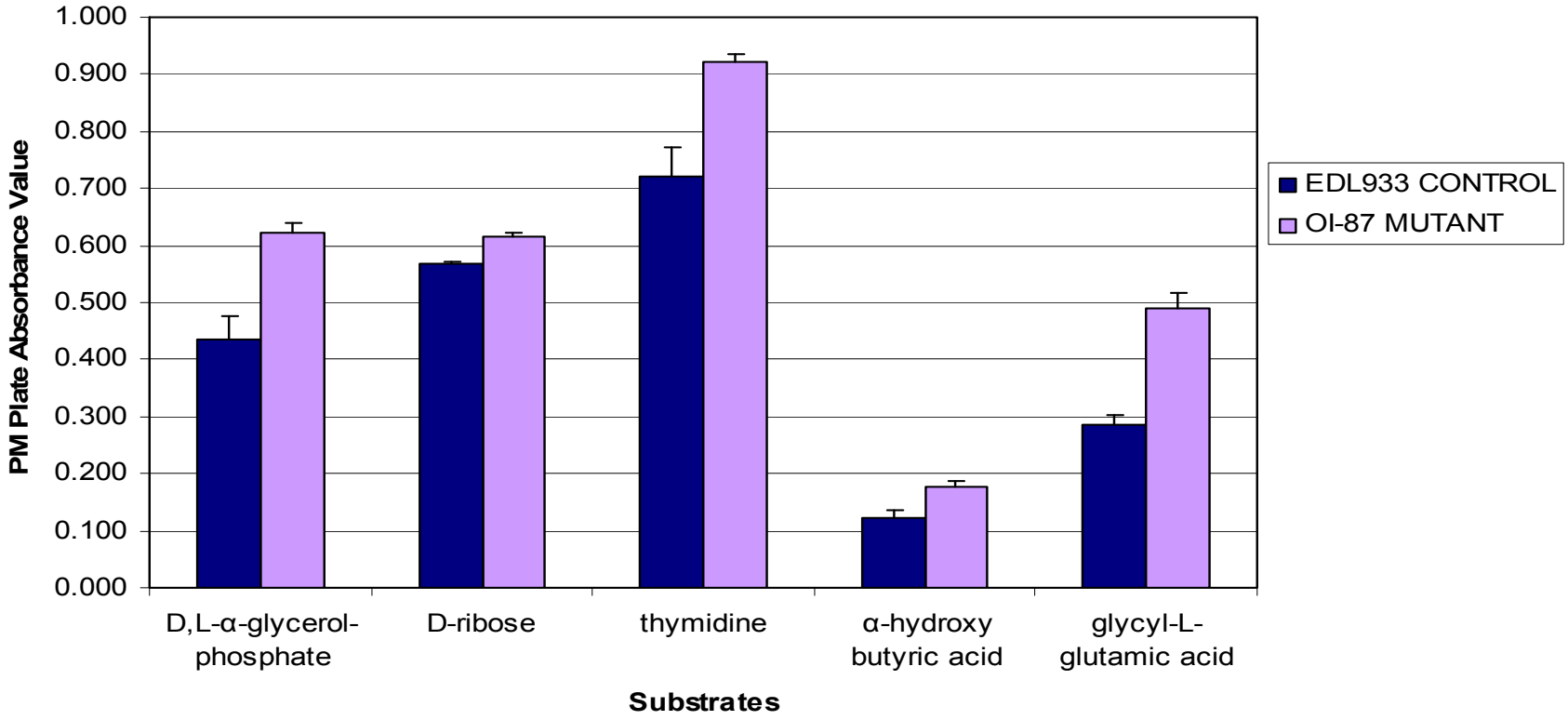
The OI-108 deletion mutant and the EDL933 control differed in use of two different substrates (Figure 3.15). In both cases, the OI-108 mutant used significantly more succinic acid and D-xylose as carbon sources in comparison to the EDL933 control ($P < 0.05$). Percentage differences in utilization for each of these substrates were 24.5% and 15.7% respectively.

In summary, each of the OI-55, OI-59 and OI-87 deletion mutants used significantly higher levels of D, L-α-glycerol-phosphate, thymidine and glycyl-L-glutamic acid control ($P < 0.05$) than EDL933, while the OI-108 deletion mutant did not. Only the OI-87 mutant appeared to gain the ability to use the substrate tween 40, in



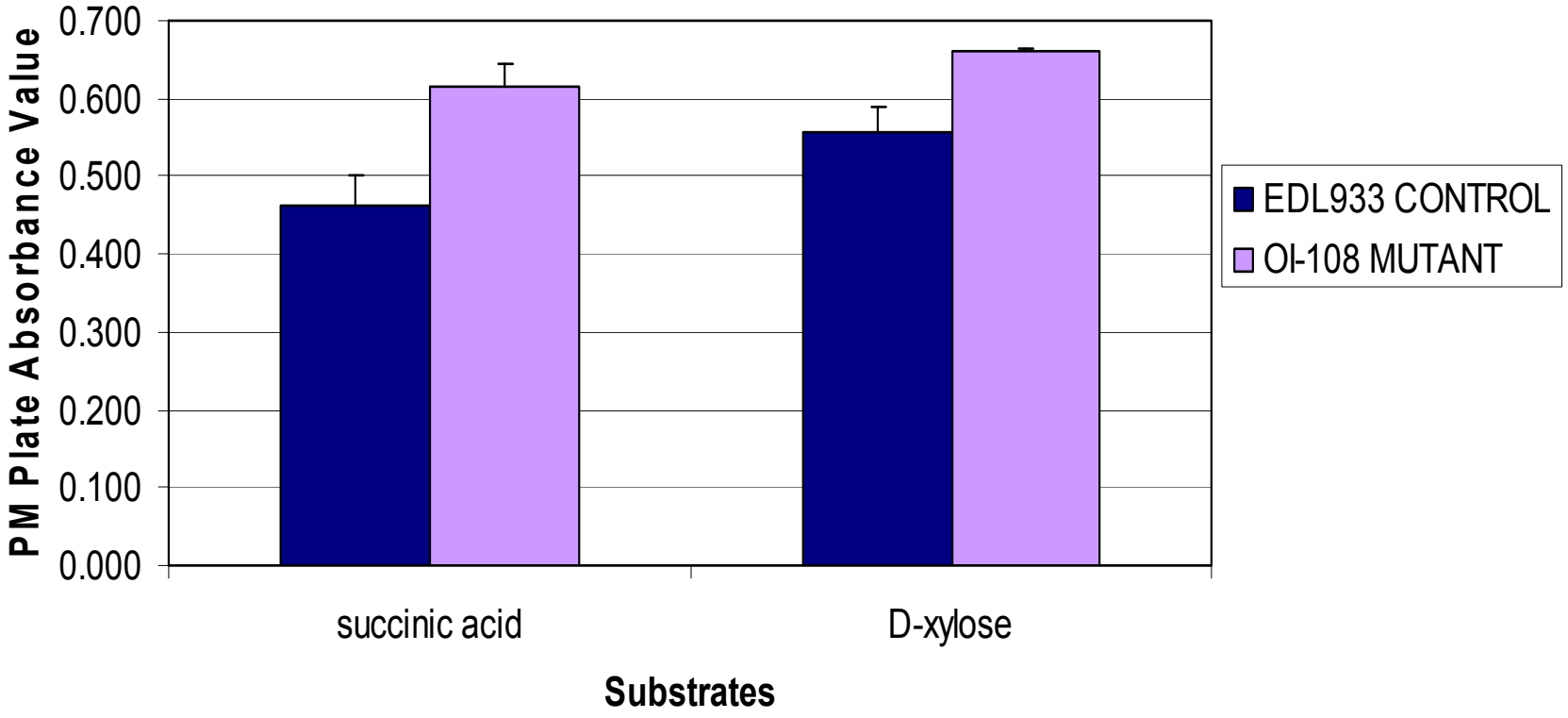
SOURCE	glycerol	D,L-α-glycerol-phosphate	thymidine	α-keto-butyric acid	α-hydroxy butyric acid	glycyl-L-glutamic acid
EDL933 CONTROL	0.564	0.436	0.722	0.150	0.121	0.287
OI-59 MUTANT	0.465	0.661	0.889	0.233	0.174	0.486
PERCENTAGE DIFFERENCE (%)	17.6	34.1	18.8	35.8	30.4	40.9

Figure.3.13. Differences in levels of substrate utilization for the *E. coli* O157:H7 parental control EDL933 and the OI-59 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization are identified at the bottom of the figure.



SOURCE	D,L-α-glycerol-phosphate	D-ribose	thymidine	α-hydroxy butyric acid	glycyl-L-glutamic acid
EDL933 CONTROL	0.436	0.566	0.722	0.121	0.287
OI-87 MUTANT	0.622	0.615	0.921	0.178	0.490
PERCENTAGE DIFFERENCE (%)	29.9	7.9	21.6	31.7	41.4

Figure.3.14. Differences in levels of substrate utilization for the *E. coli* O157:H7 parental strain EDL933 and the OI-87 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization are identified at the bottom of the figure.



SOURCE	succinic acid	D-xylose
EDL933 CONTROL	0.463	0.556
OI-108 MUTANT	0.614	0.660
PERCENTAGE DIFFERENCE (%)	24.5	15.7

Figure.3.15. Differences in levels of substrate utilization for the *E. coli* O157:H7 parental strain EDL933 and the OI-108 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization are identified at the bottom of the figure.

comparison to the EDL933 parental strain. Based on the ORFs found on these OIs use of these substrates may be related to regulation of phage / prophage mobilization which could influence nucleic acid (thymidine), energy (D, L-α-glycerol-phosphate) and protein synthesis precursor (and glycyl-L-glutamic acid) demand and/or utilization within the cells; *i.e.*, regulation of known genes coded for on these OI. Presence of tween 20 or tween 40 in the medium can affect membrane permeability which also might influence phage / prophage mobilization and growth of these mutants.

3.3.7.3 Comparison of the deletion mutant OI-51 with EDL933.

The OI 51 contains ORFs from the prophage CP-933C; most of these ORFs code for unknown proteins. The OI 51 deletion mutant was compared to the parental strain EDL933. The pattern of substrate utilization for the OI-51 deletion mutant and the O157:H7 parental strain was similar. The main differences observed were in levels of substrate utilization. However, two exceptions appeared to occur for utilization of L-glutamic acid and tween 20 (Figure. 3.16); *i.e.*, levels of L-glutamic acid and tween 20 substrate utilization in EDL933 fell below the threshold value of <0.100 used in this study while OI-51 deletion mutant levels of substrate utilization were above the threshold value, suggesting that the mutant gained the ability to use L-glutamic acid and tween 20. Further comparison of these utilization levels using a T-test showed that the differences in substrate utilization for L-glutamic acid and tween 20 between the OI-51 deletion mutant and the EDL933 control were not significantly different ($P \geq 0.05$). This suggests that the mutant did not gain the ability to use L-glutamic acid and tween 20 as carbon sources.

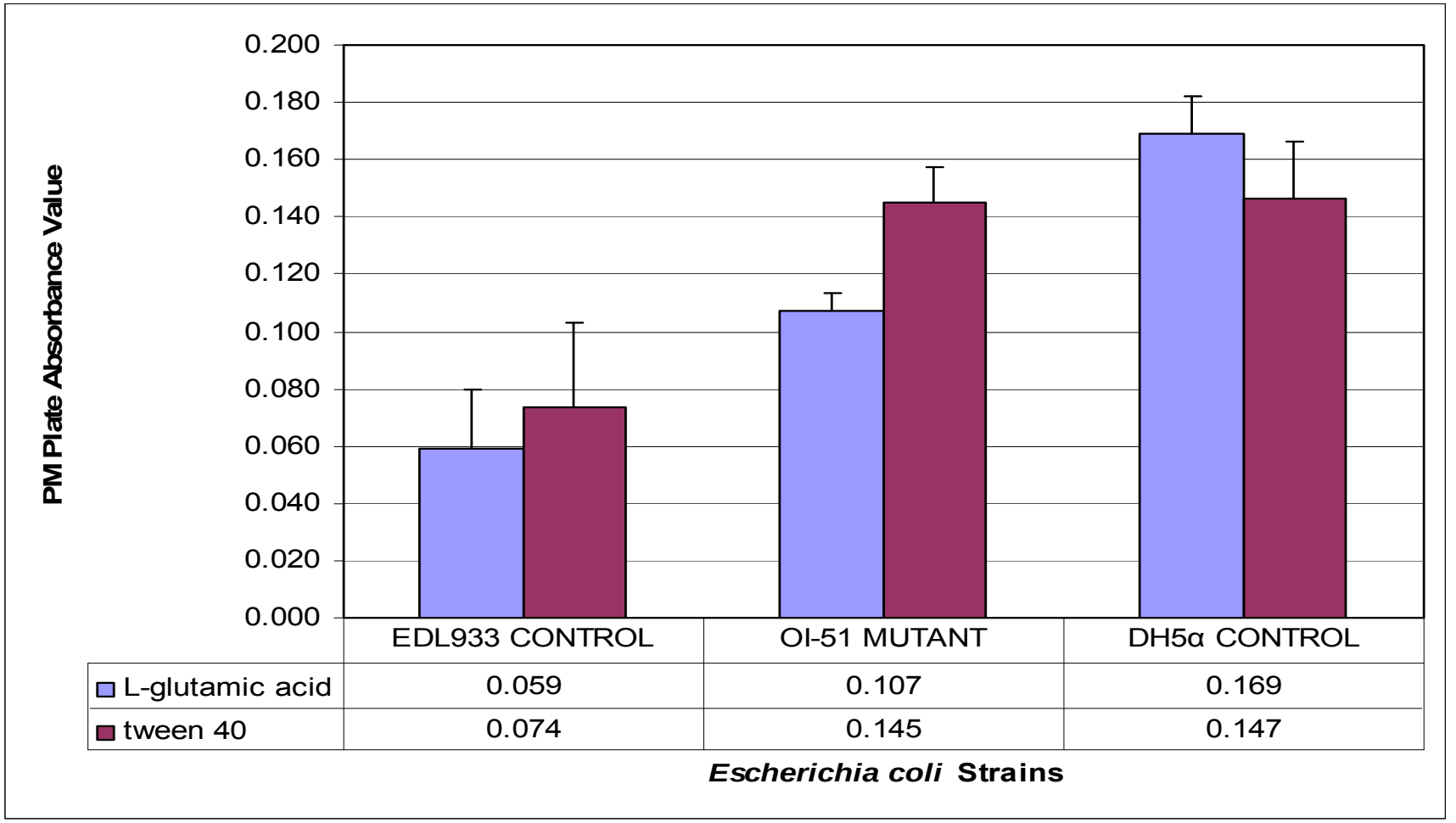


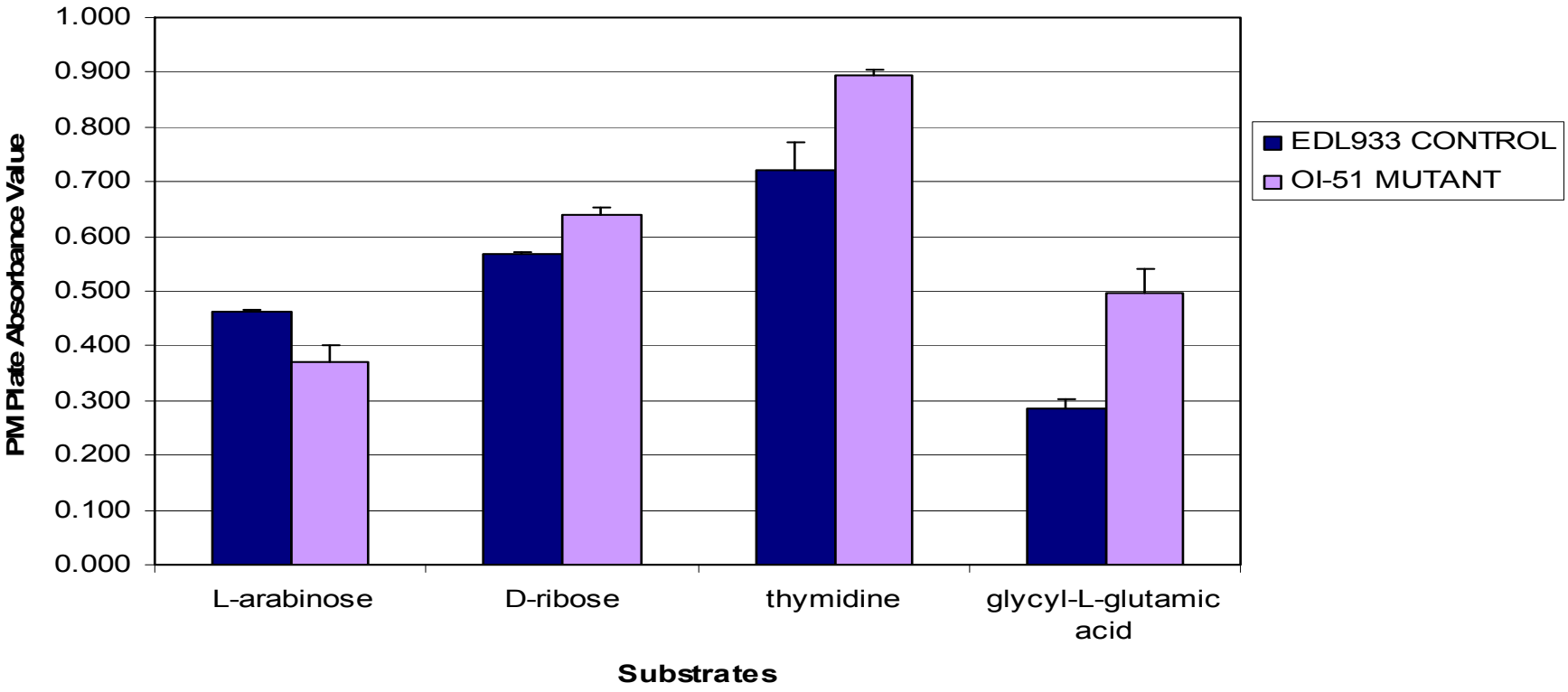
Figure.3.16. Comparison of *E. coli* O157:H7 parental strain EDL933, the OI-51 deletion mutants and the *E. coli* K-12 reference strain DH5α for the substrates L-glutamic acid and tween 20. The threshold value for substrate utilization was <0.100. The absorbance mean ± S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure. There was no significant difference in ability to use this substrate.

Significant differences ($P < 0.05$) in levels of substrate utilization were seen for other substrates where both the mutant and the parental strain were positive (above the threshold) for a given substrate (Figure 3.17). The OI-51 mutant utilized significantly higher levels of the three substrates D-ribose, thymidine and glycyl-L-glutamic acid and decreased levels of L-arabinose compared to the EDL933 parental control. Percentage differences in utilization for each of these substrates were 11.6%, 19.2%, 42.2% and 19.4% respectively.

In summary, despite the OI-51 mutant substrate levels for L-glutamic acid and tween 20 being above the threshold value in each case and the EDL933 parental strain levels being below the threshold value, there was not a significant difference in ability to use these substrates and therefore no gain in function. However, changes in utilization of the substrates D-ribose, thymidine and glycyl-L-glutamic acid may be related to changes in regulation of phage / prophage mobilization in the OI-51 mutant which could result in differences in growth of the mutant relative to the EDL933 parental strain.

3.3.7.4 Comparison of the deletion mutant OI-89 with EDL933.

The OI 89 contains ORFs of unknown function. The OI-89 deletion mutant was compared to the parental strain EDL933 and the pattern of substrate utilization for the OI-89 deletion mutant and the O157:H7 parental strain was similar. The main differences observed were in levels of substrate utilization with one exception (Figure. 3.18); *i.e.*, utilization levels of tween 40 in EDL933 fell below the threshold value of <0.100 while OI-89 deletion mutant levels of substrate utilization were above the threshold value. This suggests that the mutant gained the ability to use tween 40 as a carbon source. However, comparison of these utilization levels using a T-test showed that the difference in



SOURCE	L-arabinose	D-ribose	thymidine	glycyl-L-glutamic acid
EDL933 CONTROL	0.461	0.566	0.722	0.287
OI-51 MUTANT	0.372	0.640	0.893	0.497
PERCENTAGE DIFFERENCE (%)	19.4	11.6	19.2	42.2

Figure.3.17. Differences in levels of substrate utilization for the *E. coli* O157:H7 parental strain EDL933 and the OI-51 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure.

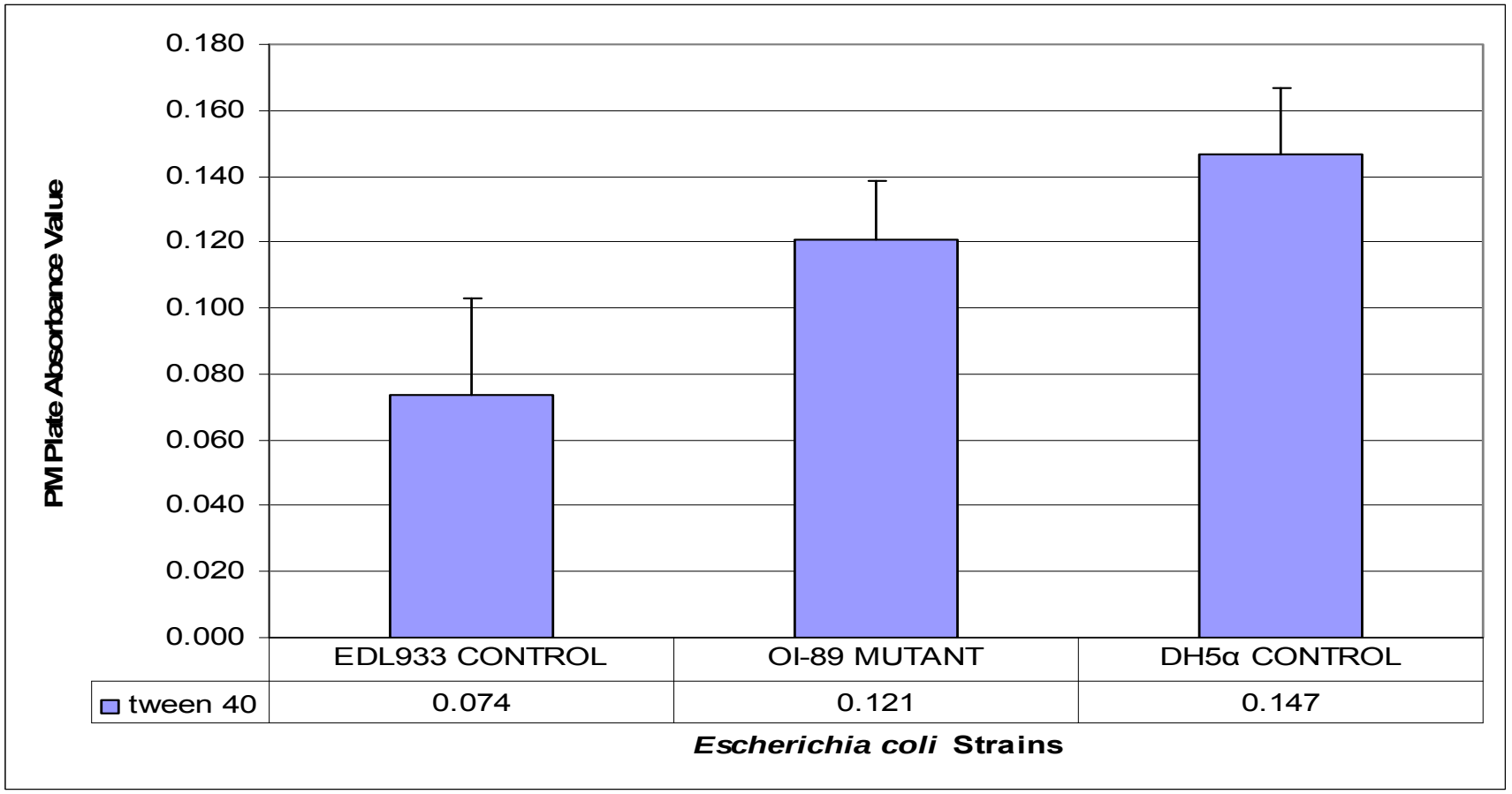


Figure.3.18. Comparison of *E. coli* O157:H7 parental strain EDL933, the OI-89 deletion mutants and the *E. coli* K-12 reference strain DH5α for the substrate tween 40. The threshold value for substrate utilization was 0.100. The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure. There was no significant difference in ability to use this substrate between the OI-89 deletion mutant and the EDL933 parental.

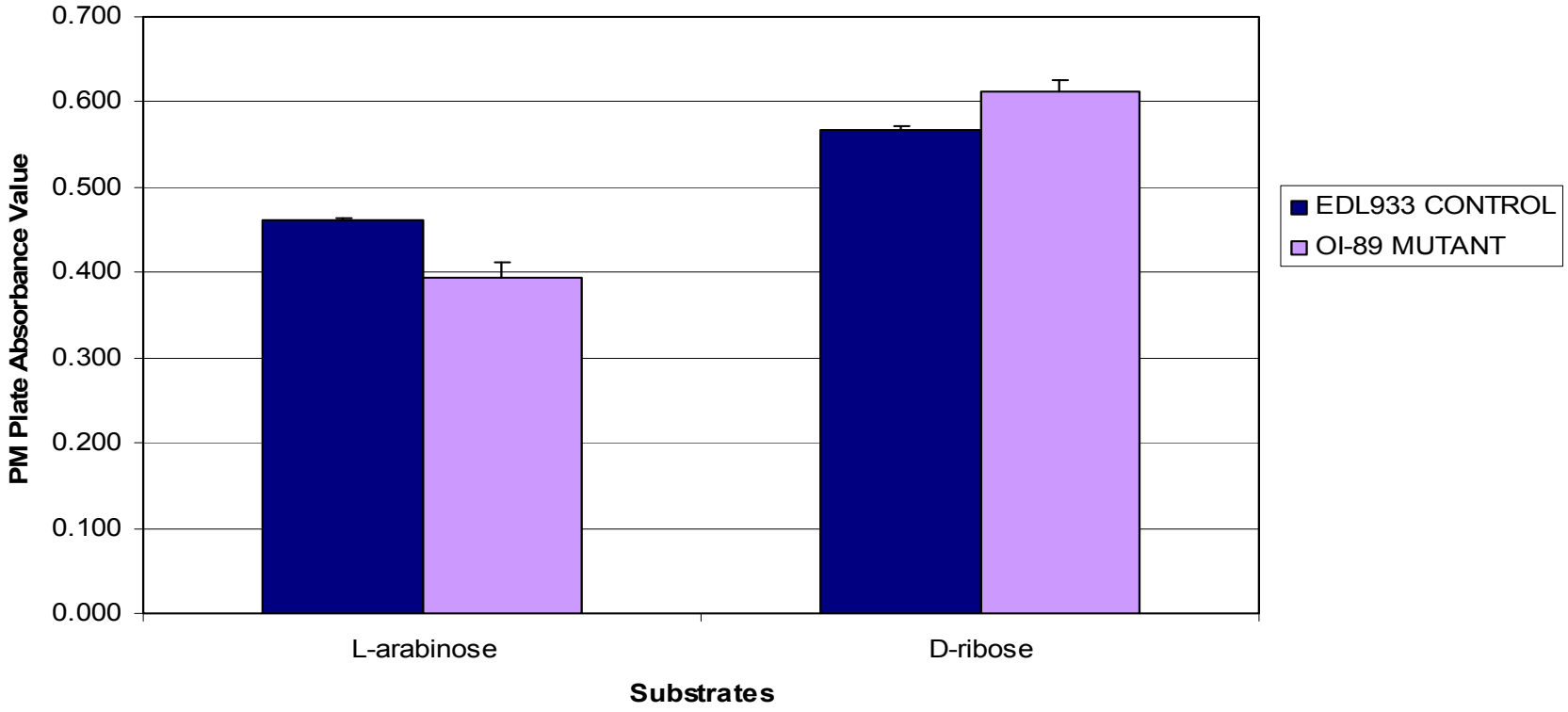
substrate utilization for tween 40 between the OI-89 deletion mutant and the EDL933 control was not different ($P \geq 0.05$) suggesting that this mutant did not gain the ability to use tween 40 as a carbon source.

In contrast, significant differences ($P < 0.05$) in levels of substrate utilization were seen for other substrates where both the mutant and the parental strain were positive (above the threshold) for a given substrate (Figure 3.19). The OI-89 mutant utilized significantly higher levels of D-ribose and decreased levels of L-arabinose compared to the EDL933 parental control. Percentage differences in utilization for each of these substrates were: 14.6% and 7.6% respectively.

In summary, despite the OI-89 mutant substrate levels being above the threshold value for tween 40 and the EDL933 parental strain levels being below the threshold value, there was not a significant difference in ability to use this substrate; therefore no gain in ability to use tween 40 as a carbon source was observed. However, changes in levels of substrate utilization for D-ribose and L-arabinose may be due to changes in regulation of sugar degradation due to the removal of the island which could result in changes in levels of substrate utilization compared to the parental strain EDL933. Also, D-ribose is a precursor in RNA synthesis; this suggests that synthetic activity of the OI-89 deletion mutant may be higher than that of EDL933. This may be related to growth potential of these bacteria.

3.3.7.5 Comparison of the deletion mutant OI-176 with EDL933.

The OI 176 contains an ORF of unknown function. Comparison of the OI-176 deletion mutant and the parental strain EDL933 showed that the pattern of substrate



SOURCE	L-Arabinose	D-ribose
EDL933 CONTROL	0.461	0.566
OI-89 MUTANT	0.393	0.613
PERCENTAGE DIFFERENCE (%)	14.7	7.6

Figure.3.19. Differences in levels of substrate utilization for the *E. coli* O157:H7 parental strain EDL933 and the OI-89 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure.

utilization for the OI-176 deletion mutant and the O157:H7 parental strain was similar. The main differences observed were in levels of substrate utilization; two exceptions appeared to occur for utilization of D-serine and tween 20 (Figure. 3.20). Levels of D-serine and tween 20 substrate utilization in EDL933 fell below the threshold value of 0.100 used in this study while OI-176 deletion mutant levels of substrate utilization were above the threshold value, suggesting that the mutant gained the ability to use D-serine and tween 20 as carbon sources. However, comparison of these utilization levels using a T-test showed that the difference in substrate utilization for D-serine and tween 20 between the OI-176 deletion mutant and the EDL933 control were not different ($P \geq 0.05$); this suggests that this mutant did not gain the ability to use D-serine and tween 20 as carbon sources. However, significant differences ($P < 0.05$) in levels of substrate utilization were seen for other substrates where both the mutant and the parental strain were positive (above the threshold) for a given substrate (Figure 3.21). In all cases the OI-176 mutant utilized significantly less D-xylose, maltotriose, adenosine, N-acetyl-β-D-mannosamine, pyruvic acid and L-galactonic acid-γ-lactone compared to the EDL933 parental control. Percentage differences in utilization for each of these substrates were 16.3%, 26.4%, 9.8%, 20.6%, 16.8% and 14.1% respectively.

In summary, the OI-176 mutant levels of substrate utilization were above the threshold value for D-serine and tween 20, and EDL933 parental strain levels were below the threshold value but there was not a significant difference in substrate utilization for these two substrates. However, changes in levels of substrate utilization for D-xylose, maltotriose, adenosine, N-acetyl-β-D-mannosamine, pyruvic acid and L-galactonic acid-γ-lactone may be due to changes in regulation of sugar degradation or

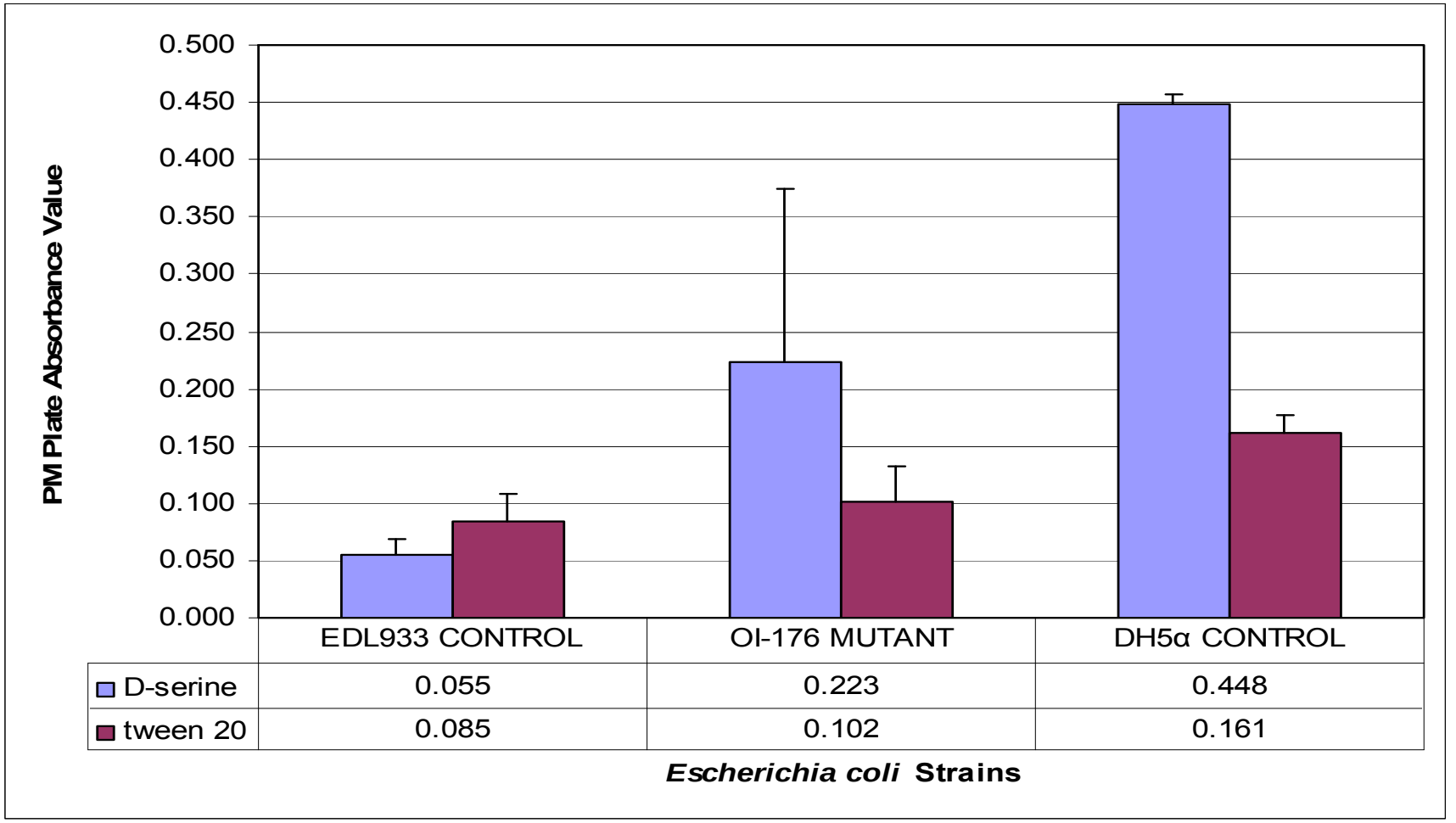
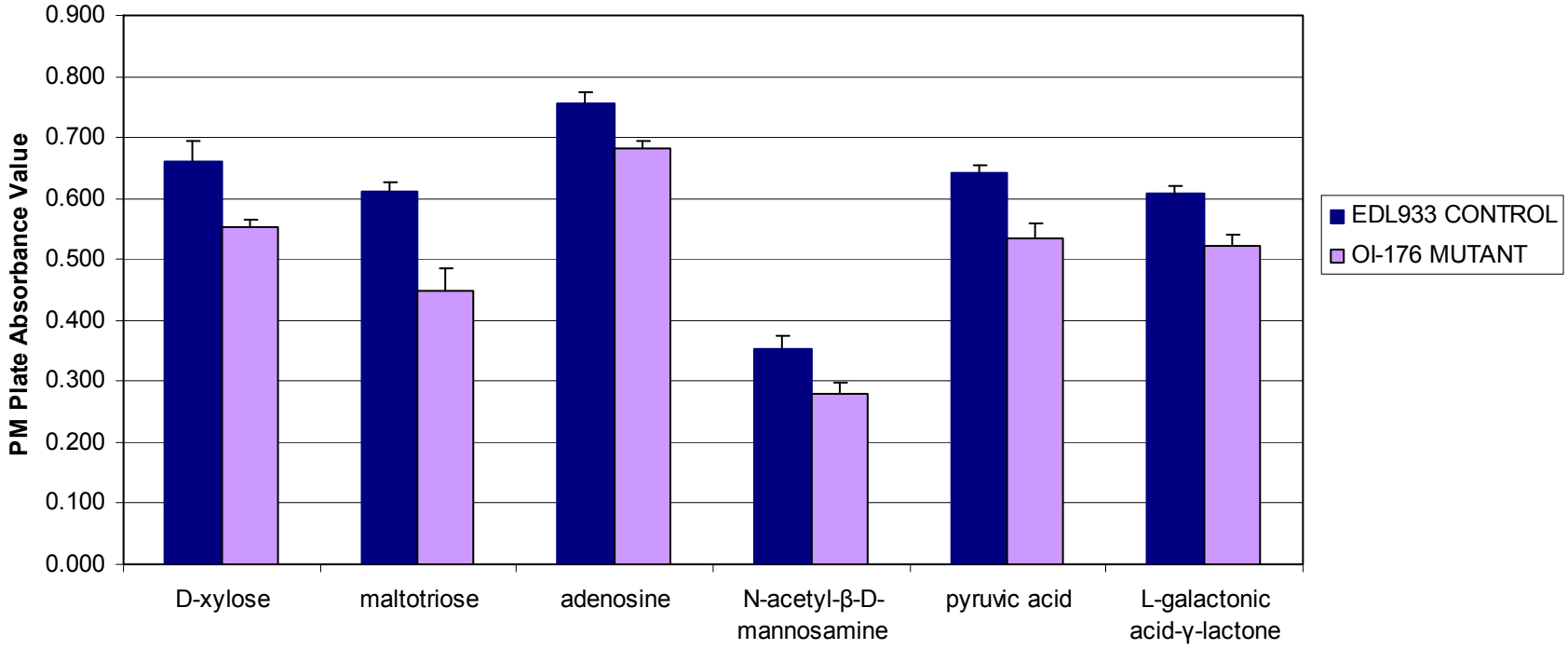


Figure.3.20. Comparison of *E. coli* O157:H7 parental strain EDL933, the OI-176 deletion mutants and the *E. coli* K-12 reference strain DH5α for the Substrates D-serine and tween 20. The threshold value for substrate utilization was 0.100. The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure. There is no significant difference in substrate utilization for D-serine or tween 20 between the OI-176 mutant and the EDL933 parental.



SOURCE	D-xylose	maltotriose	adenosine	N-acetyl-β-D-mannosamine	pyruvic acid	L-galactonic acid-γ-lactone
EDL933 CONTROL	0.660	0.610	0.755	0.354	0.642	0.607
OI-176 MUTANT	0.553	0.449	0.681	0.281	0.534	0.522
PERCENTAGE DIFFERENCE (%)	16.3	26.4	9.8	20.6	16.8	14.1

Figure.3.21. Differences in levels of substrate utilization for the *E. coli* O157:H7 parental strain EDL933 and the OI-176 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure.

nitrogen degradation due to the removal of the genes which are found on this island which could result in changes in levels of substrate utilization compared to the parental strain EDL933. Genes on OI-176 appear to up-regulate utilization of a broad range of sugars. This up-regulation may be a specific adaptation to the human host for EDL933 which is known to often have high levels of sugar in its diet.

3.3.7.6 Comparison of the deletion mutant OI-39 with EDL933.

The OI 39 contains ORFs of unknown function. The OI-39 deletion mutant was compared to the EDL933 parental strain. The pattern of substrate utilization for the OI-39 deletion mutant and the O157:H7 parental strain was similar. The main differences observed were in levels of substrate utilization; *i.e.*, three exceptions appeared to occur for utilization of α-keto-glutaric acid, mono methyl succinate and glucuronamide (Figure. 3.22). Levels of α-keto-glutaric acid and glucuronamide substrate utilization in EDL933 were above the threshold value of <0.100 used in this study while OI-39 deletion mutant levels of substrate utilization were below the threshold value. By contrast, levels of mono methyl succinate were below the threshold level in EDL933 and above the threshold level in the OI-39 deletion mutant, suggesting that the mutant had lost the ability to use α-keto-glutaric acid and glucuronamide but, gained the ability to use mono methyl succinate as a carbon source. However, comparison of these utilization levels using a T-test showed that the difference in substrate utilization for α-keto-glutaric acid, mono methyl succinate and glucuronamide between the OI-39 deletion mutant and the EDL933 control were not significantly different ($P \geq 0.05$) suggesting that this mutant did not lose or gain the

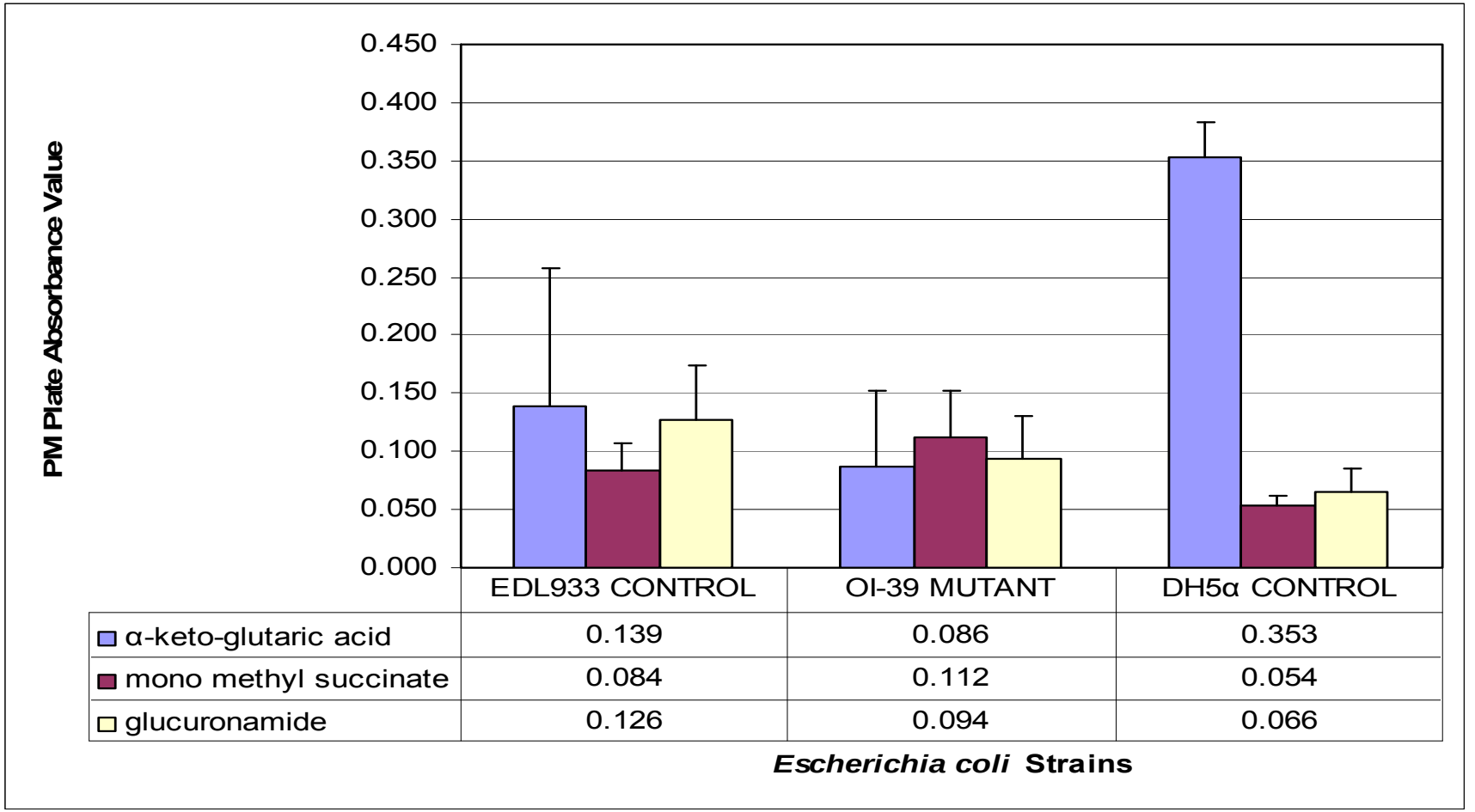
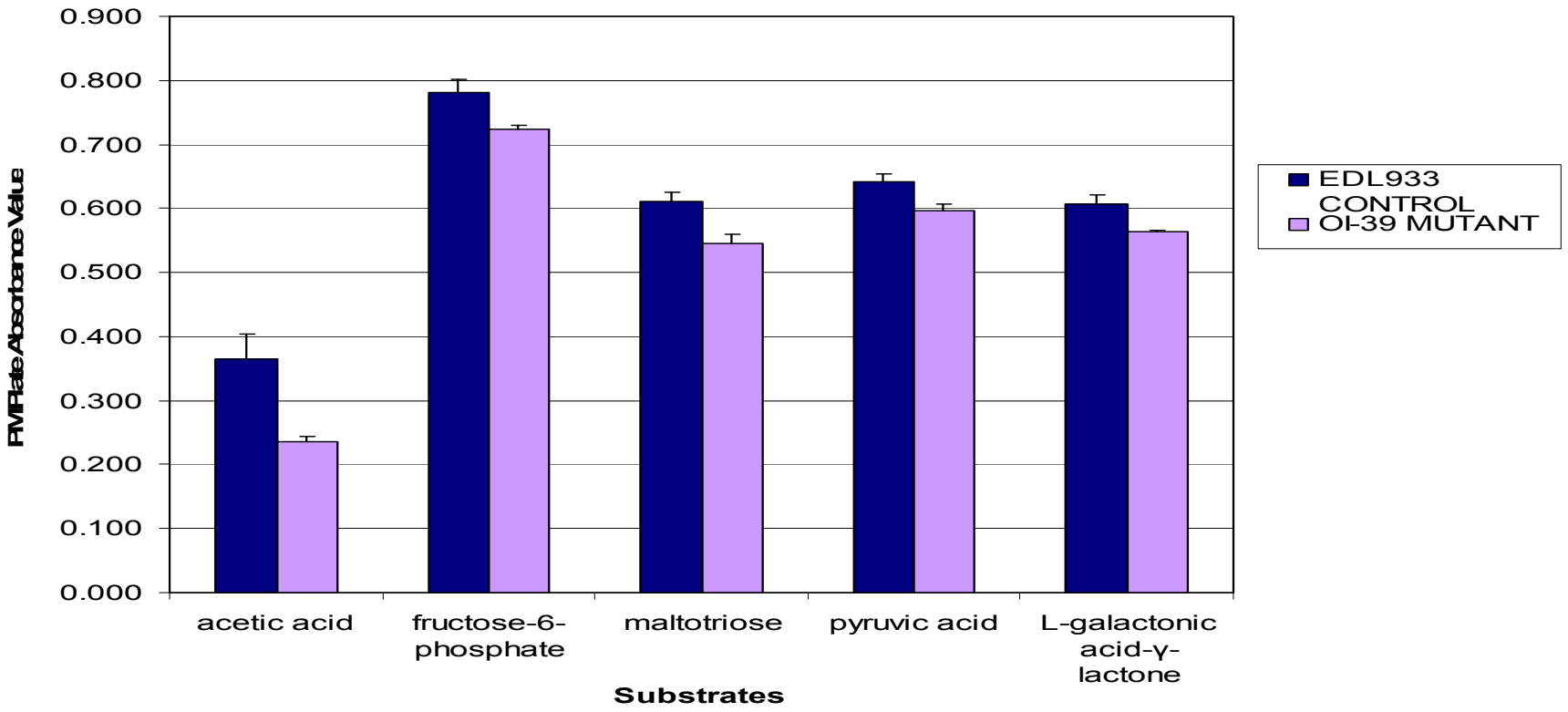


Figure.3.22. Comparison of *E. coli* O157:H7 parental strain EDL933, the OI-39 deletion mutants and the *E. coli* K-12 reference strain DH5α for the Substrates α-keto-glutaric acid, mono methyl succinate and glucuronamide. The threshold value for substrate utilization was 0.100. The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure. There is no significant difference in substrate utilization for any of the substrates when comparing the OI-39 mutant and EDL933.

ability to use of α-keto-glutaric acid, mono methyl succinate and glucuronamide as carbon sources.

By contrast, significant differences ($P < 0.05$) in levels of substrate utilization were seen for other substrates where both the mutant and the parental strain EDL933 were positive (above the threshold) for a given substrate (Figure 3.23). In all cases the OI-39 mutant utilized significantly less acetic acid, fructose-6-phosphate, maltotriose, pyruvic acid and L-galactonic acid-γ-lactone compared to the EDL933 parental control. Percentage differences in utilization for each of these substrates were 35.0%, 7.4%, 10.7%, 7.1%, and 7.2% respectively. Note that acetate, fructose-6-phosphate and pyruvic acid all are substrates and products found in glycolytic and fermentation pathways. Maltotriose is a tri-saccharide consisting of three glucose molecules linked by 1,4-glycosidic bonds, while L- galactonic acid-γ-lactone typically is converted to ascorbate for use in anaerobic biochemical pathways.

In summary, there was no significant difference in substrate utilization for keto-glutaric acid, mono methyl succinate and glucuronamide between the OI-39 deletion mutant and the EDL933 parental strain. However, the differences seen in the level of substrate utilization for acetic acid, fructose-6-phosphate, maltotriose, pyruvic acid and L-galactonic acid-γ-lactone suggest a possible change in sugar degradation due to the removal of this island compared to the levels of substrate utilization for these substrates in EDL933; *i.e.,* genes on this OI appear to promote more efficient utilization of sugars and their metabolites in EDL933.



SOURCE	acetic acid	fructose-6-phosphate	maltotriose	pyruvic acid	L-galactonic acid-γ-lactone
EDL933 CONTROL	0.364	0.781	0.610	0.642	0.607
OI-39 MUTANT	0.237	0.723	0.545	0.597	0.564
PERCENTAGE DIFFERENCE (%)	35.0	7.4	10.7	7.1	7.2

Figure.3.23. Differences in levels of substrate utilization for the *E. coli* O157:H7 parental strain EDL933 and the OI-39 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure.

3.3.7.7 Comparison of the deletion mutant OI-98 with EDL933.

The OI 98 contains an ORF of unknown function and comparison of the OI-98 deletion mutant to the parental strain EDL933 showed that the pattern of substrate utilization for the OI-98 deletion mutant and the O157:H7 parental strain was similar. The main differences observed were in levels of substrate utilization; *i.e*., five exceptions appeared to occur (Figure. 3.24). Levels of D-serine, L-glutamic acid, tween 20, tween 40 and mono methyl succinate substrate utilization in EDL933 fell below the threshold value of 0.100 used in this study while OI-98 deletion mutant levels of substrate utilization were above the threshold value. This suggests that the mutant had gained the ability to use D-serine, L-glutamic acid, tween 20, tween 40 and mono methyl succinate as carbon sources. However, comparison of these utilization levels using a T-test showed that the difference in substrate utilization for D-serine, tween 20, tween 40 and mono methyl succinate between the OI-98 deletion mutant and the EDL933 control were not different ($P \geq 0.05$). In contrast, substrate levels were significantly different ($P < 0.05$) for L-glutamic acid. This suggests that the mutant OI-98 did not gain the ability to use D-serine, tween 20, tween 40 and mono methyl succinate as a carbon sources but did gain the ability to use L-glutamic acid as a carbon source.

Furthermore, a significant difference ($P < 0.05$) in the level of substrate utilization was seen for one other substrate where both the mutant and the parental strain were positive (above the threshold) for this substrate (Figure 3.25). The OI-98 mutant utilized significantly less L-rhamnose compared to the EDL933 parental control. The percentage difference in utilization for this substrate was 20.7%.

In summary, the only significant difference in substrate utilization between the

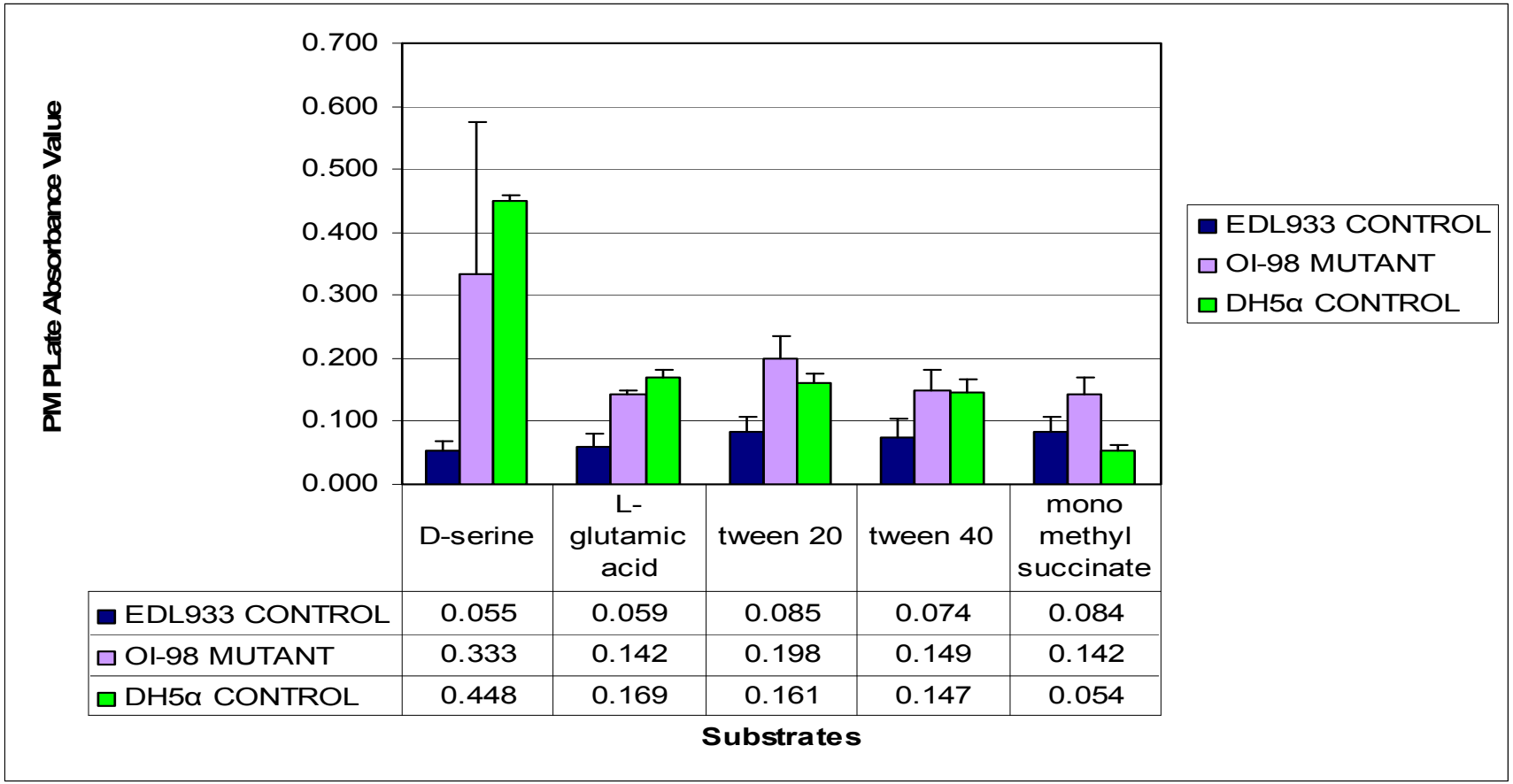
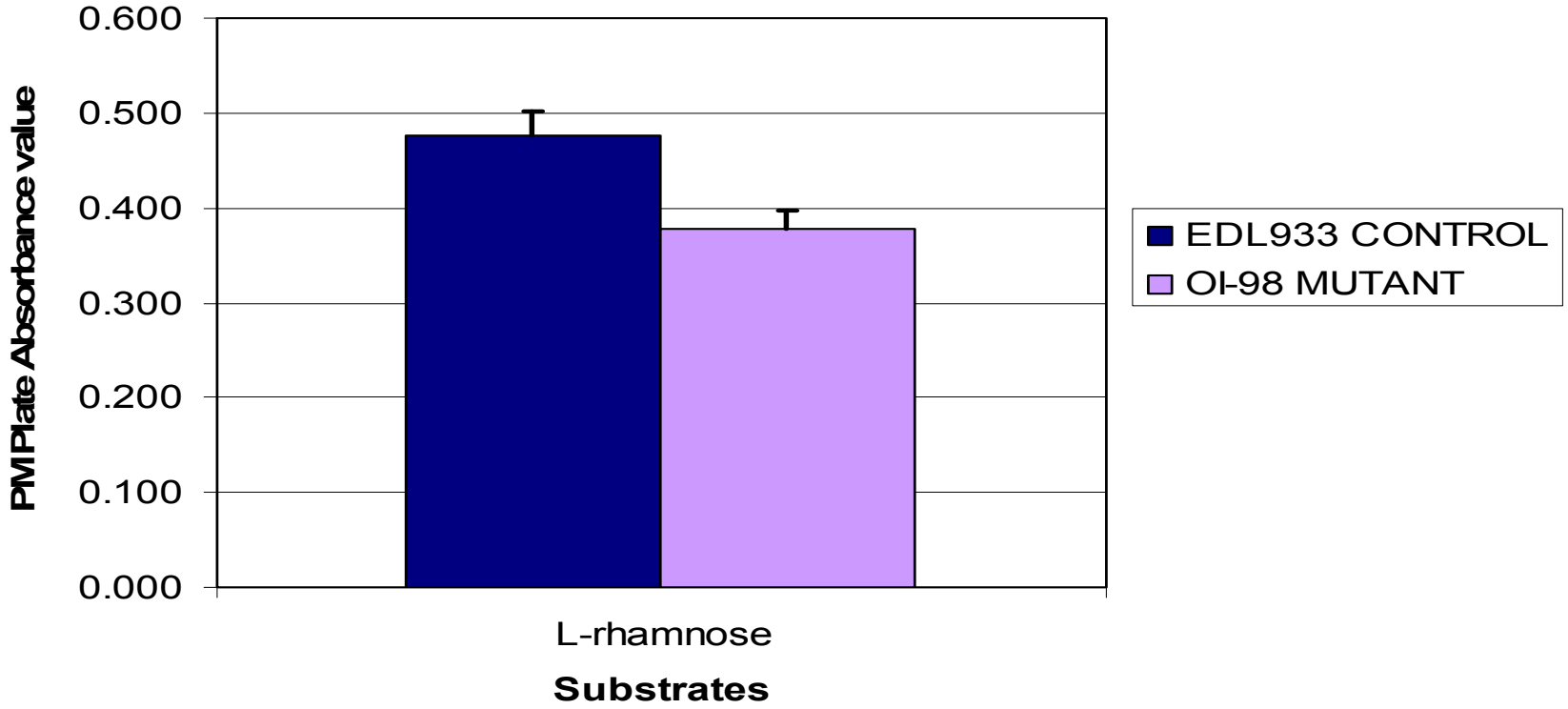


Figure.3.24. Comparison of *E. coli* O157:H7 parental strain EDL933, the OI-98 deletion mutants and the *E. coli* K-12 reference strain DH5α for the Substrates D-serine, L-glutamic acid, tween 20, tween 40 and mono methyl succinate. The threshold value for substrate utilization was <0.100. The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure. The only significant difference in substrate utilization between the OI-98 mutant and the EDL933 parental is for L-glutamic acid.



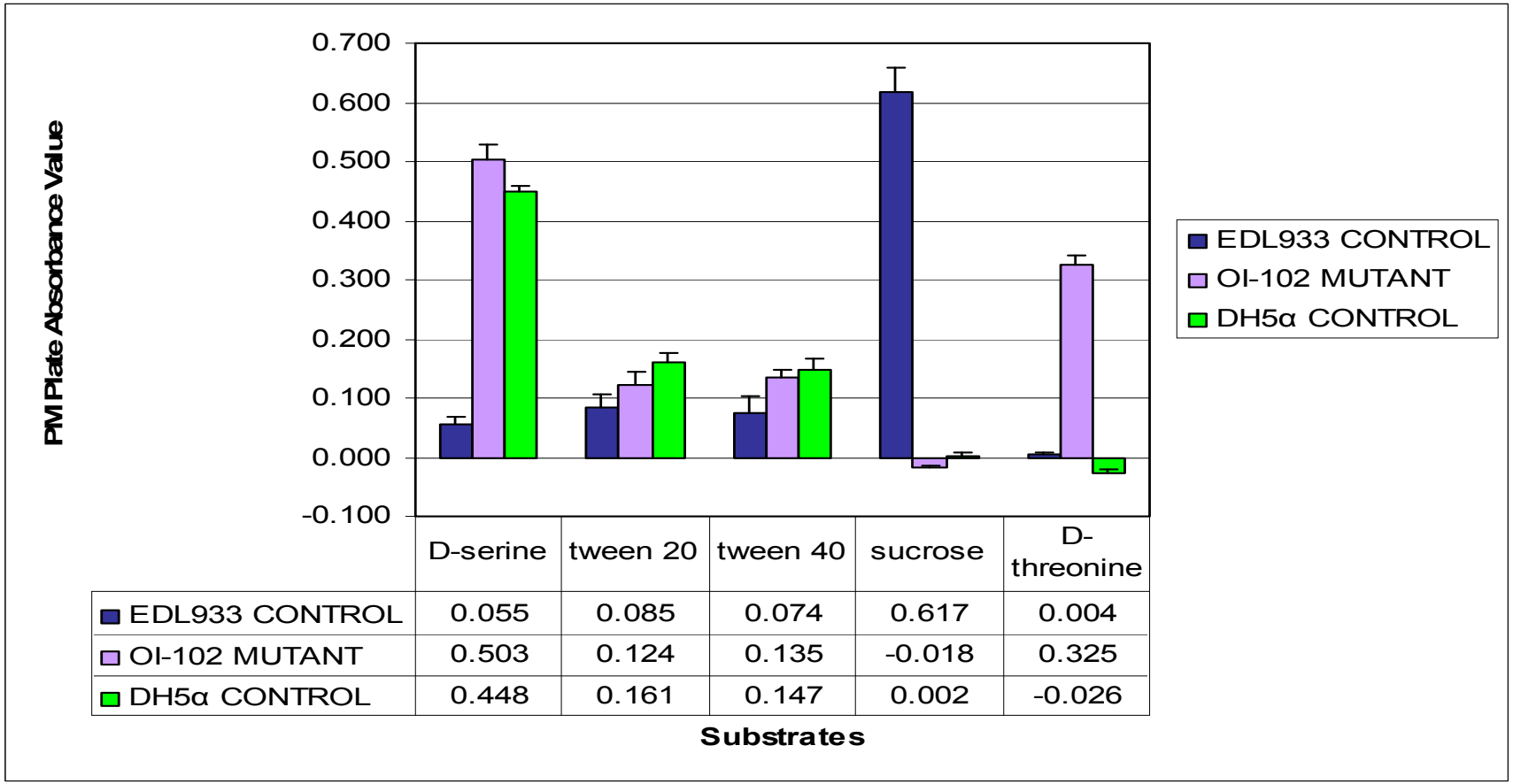
SOURCE	L-Rhamnose
EDL933 control	0.377
OI-98 mutant	0.475
Percentage Difference (%)	20.7

Figure.3.25. Differences in levels of substrate utilization for the *E. coli* O157:H7 parental strain EDL933 and the OI-98 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization are identified at the bottom of the figure.

OI-98 mutant and the EDL933 was for L-glutamic acid, where the mutant was above the threshold value and the EDL933 parental strain was below the threshold value. Use of L-glutamic acid as a carbon source may be related to metabolism of the bacteria suggesting this island could have a role in regulating/suppressing metabolism. Also the increase in the level of substrate utilization for L-rhamnose compared to the parental strain EDL933 suggests the ORF found on this island may also have a role in sugar utilization, which suggests that this island may play a role in regulation of glutamic acid and L-rhamnose utilization in the bacterium.

3.3.7.8 Comparison of the deletion mutant OI-102 with EDL933.

The OI 102 also contains ORFs of unknown function, as well as ORFs coding for putative prophage DNA injection proteins, an ORF coding for a putative resolvase, a sucrose permease, a D-fructokinase, a sucrose hydrolase and a sucrose specific transcriptional regulator (Appendix I). The deletion mutant corresponding to OI 102 was compared to the parental strain EDL933. The pattern of substrate utilization for the OI-102 deletion mutant and the O157:H7 parental strain was similar. The main differences observed were in levels of substrate utilization; *i.e*., five exceptions appeared to occur (Figure. 3.26). Levels of D- serine, tween 20, tween 40 and D-threonine substrate utilization in EDL933 fell below the threshold value of 0.100 used in this study while OI-102 deletion mutant levels of substrate utilization were above the threshold value. For sucrose the opposite was seen. This suggests that the mutant gained the ability to use D-serine, tween 20, tween 40 and D-threonine as carbon sources but, lost the ability to use sucrose as a carbon source. Further comparison of these utilization levels



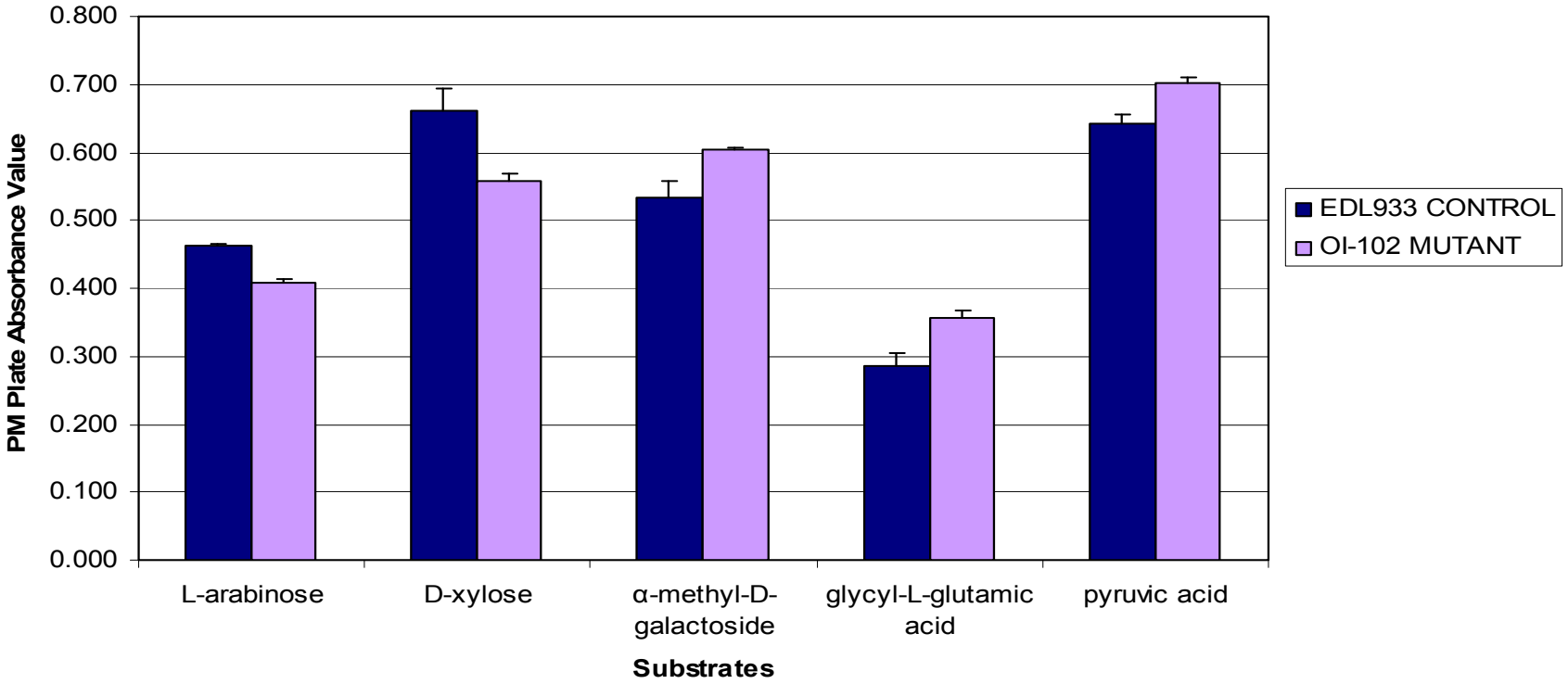
	D-serine	tween 20	tween 40	sucrose	D-threonine
EDL933 CONTROL	0.055	0.085	0.074	0.617	0.004
OI-102 MUTANT	0.503	0.124	0.135	-0.018	0.325
DH5α CONTROL	0.448	0.161	0.147	0.002	-0.026

Figure.3.26. Comparison of *E. coli* O157:H7 parental strain EDL933, the OI-102 deletion mutants and the *E. coli* K-12 reference strain DH5α for the substrates D-serine, tween 20, tween 40, sucrose and D-threonine. The threshold value for substrate utilization was <0.100. The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure. The only significant differences in substrate utilization between the OI-102 mutant and the EDL933 parental were seen for D-serine, D-threonine and sucrose.

using a T-test showed that the difference in substrate utilization for tween 20 and tween 40 between the OI-102 deletion mutant and the EDL933 control were not different ($P \geq 0.05$). However the substrate levels were significantly different ($P < 0.05$) for D-serine, D-threonine and sucrose. This suggests that this mutant did not gain the ability to use tween 20 and tween 40 as carbon sources but, gained the ability to use D-serine and D-threonine as carbon sources and lost the ability to use sucrose as a carbon source.

Furthermore, significant differences ($P < 0.05$) in levels of substrate utilization were seen for five other substrates where both the mutant and the parental strains were positive (above the threshold) for a given substrate (Figure 3.27). The OI-102 mutant utilized significantly less L-arabinose and D-xylose and used significantly more α-methyl-D-galactoside, glycyl-L-glutamic acid and pyruvic acid compared to the EDL933 parental control. Percentage differences in utilization for these substrates were 11.6%, 15.4%, 11.5%, 19.8% and 8.5% respectively.

In summary, the only significant differences in substrate utilization between the OI-102 mutant and the EDL933 parental strain were for D-serine, D-threonine and sucrose. Mutant values were above the threshold level for D-serine and D-threonine but, below the threshold value for sucrose. The EDL933 parental strain was below the threshold value for D-serine and D-threonine but, above the threshold for sucrose. The loss in ability of the deletion mutant to use sucrose is most likely due to the removal of this OI containing various ORFs coding for genes relating to sucrose utilization. The gain in ability to use D-serine and D-threonine may be due to the removal of transcriptional regulators which could be suppressing the bacteria's ability to use these substrates due to a preference for sucrose and loss of this regulation has restored ability to use D-serine



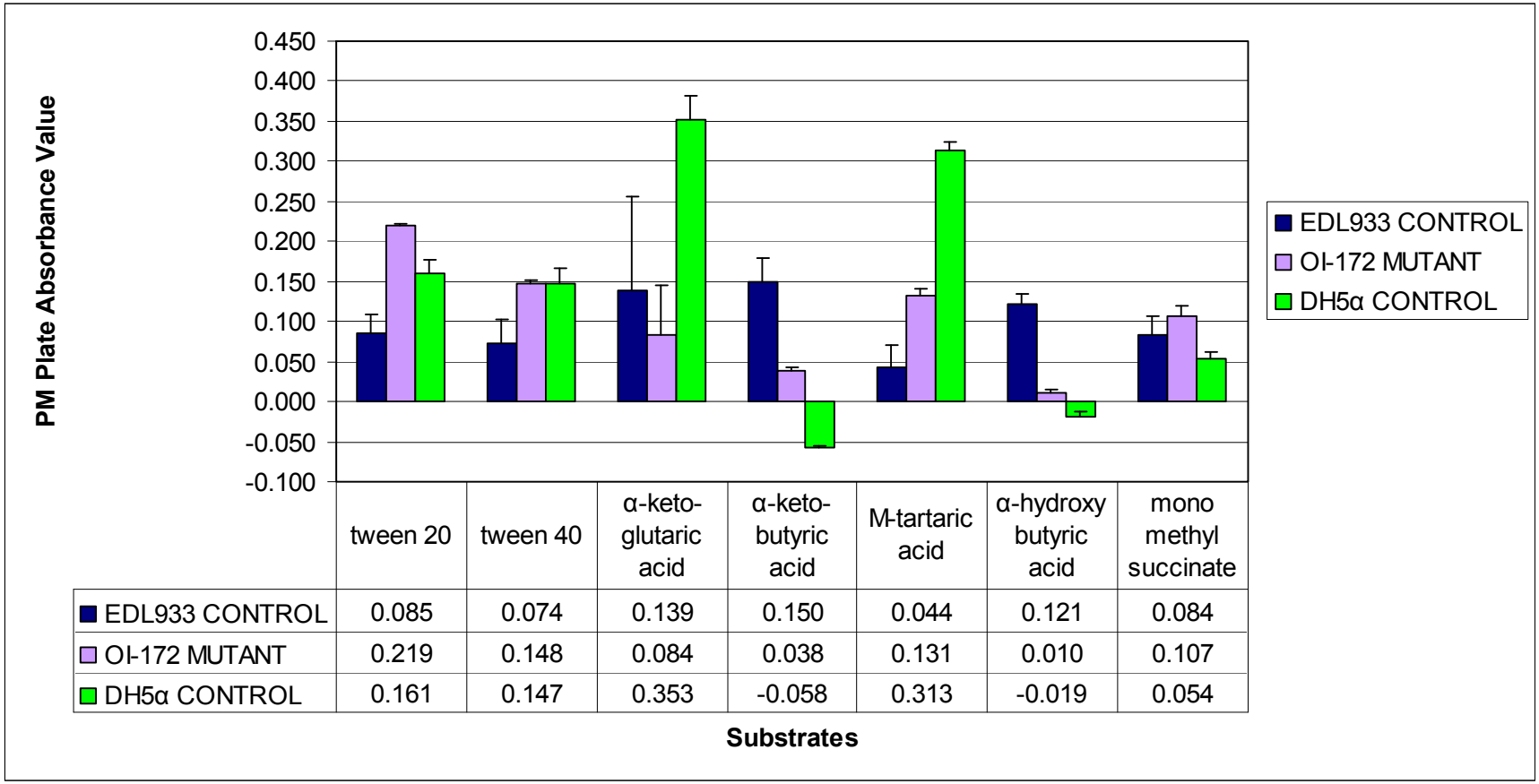
SOURCE	L-arabinose	D-xylose	α-methyl-D-galactoside	glycyl-L-glutamic acid	pyruvic acid
EDL933 CONTROL	0.461	0.660	0.534	0.287	0.642
OI-102 MUTANT	0.408	0.559	0.604	0.358	0.702
PERCENTAGE DIFFERENCE (%)	11.6	15.4	11.5	19.8	8.5

Figure.3.27. Differences in levels of substrate utilization for the *E. coli* O157:H7 parental strain EDL933 and the OI-102 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure.

and D-threonine as carbon sources. Also the increase in the level of substrate utilization for α-methyl-D-galactoside, glycyl-L-glutamic acid and pyruvic acid and decrease in level of utilization for L-arabinose and D-xylose compared to EDL933 suggests the ORFs found on this island on the parental line may also have a role in sugar consumption.

3.3.7.9 Comparison of the deletion mutant OI-172 with EDL933.

The OI 172 contains ORFs unknown function, ORFs coding for two putative intergrases, a transposase, a putative resolvase, a putative ATP-dependent helicase and two putative helicases; *i.e.,* genes likely involved in bacteriophage propagation and DNA synthesis, as well as regulation of cell growth and cell division (Appendix I). The OI-172 deletion mutant was compared to the parental strain EDL933. The pattern of substrate utilization for the OI-172 deletion mutant and the O157:H7 parental strain was similar. The main differences observed were in levels of substrate utilization; seven exceptions appeared to occur in substrate utilization (Figure. 3.28). Levels of tween 20, tween 40, M-tartaric acid and mono methyl succinate substrate utilization in EDL933 fell below the threshold value of <0.100 used in this study while OI-172 deletion mutant levels of substrate utilization were above the threshold value. The opposite was seen where mutant levels of α-keto-glutaric acid, α-keto-butyric acid and α-hydroxy butyric acid fell below the threshold level while levels in EDL933 were above the threshold value, suggesting that the mutant had gained the ability to use tween 20, tween 40, M-tartaric acid and mono methyl succinate as carbon sources but, lost the ability to use α-keto-glutaric acid, α-keto-butyric acid and α-hydroxy butyric acid as carbon sources. Further comparison of these utilization levels using a T-test showed that the difference in substrate utilization



	tween 20	tween 40	α-keto-glutaric acid	α-keto-butyric acid	M-tartaric acid	α-hydroxy butyric acid	mono methyl succinate
EDL933 CONTROL	0.085	0.074	0.139	0.150	0.044	0.121	0.084
OI-172 MUTANT	0.219	0.148	0.084	0.038	0.131	0.010	0.107
DH5α CONTROL	0.161	0.147	0.353	-0.058	0.313	-0.019	0.054

Figure.3.28. Comparison of *E. coli* O157:H7 parental strain EDL933, the OI-172 deletion mutants and the *E. coli* K-12 reference strain DH5α for the substrates tween 20, tween 40, α-keto-glutaric acid, α-keto-butyric acid, M-tartaric acid, α-hydroxy butyric acid and mono methyl succinate. The threshold value for substrate utilization was <0.100. The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure. The only significant differences in substrate utilization were for tween 20, α-keto-butyric acid, M-tartaric acid and α-hydroxy butyric acid.

for tween 40, α-keto-glutaric acid and mono methyl succinate between the OI-172 deletion mutant and the EDL933 control were not different ($P \geq 0.05$). However the substrate levels were significantly different ($P < 0.05$) for tween 20, α-keto-butyric acid, M-tartaric acid and α-hydroxy butyric acid. This suggests that this mutant did not gain the ability to use tween 40 and mono methyl succinate or lose the ability to use α-keto-glutaric acid as carbon sources but, did gain the ability to use tween 20 and M-tartaric acid as carbon sources and lost the ability to use α-keto-butyric acid and α-hydroxy butyric acid as carbon sources.

Furthermore, significant differences ($P < 0.05$) in levels of substrate utilization were seen for forty other substrates where both the mutant and the parental strain were positive (above the threshold) for a given substrate. In all cases the OI-172 mutant used significantly less L-arabinose, N-acetyl-D-glucosamine, succinic acid, D-galactose, L-aspartic acid, L-proline, D-trehalose, glycerol, D-glucuronic acid, D,L-α-glycerol phosphate, D-xylose, glucose-6-phosphate, D,L,-malic acid, D-ribose, acetic acid, α-D-glucose, D-melibiose, L-asparagine, α-methyl-D-galactoside, lactulose, sucrose, uridine, glucose-1-phosphate, fructose-6-phosphate, β-methyl-D-glucoside, maltotriose, 2-deoxy adenosine, adenosine, glycyl-L-aspartic acid, fumaric acid, bromo succinic acid, propionic acid, inosine, L-serine, L-alanine, N-acetyl-β-D-mannosamine, L-malic acid, pyruvic acid, L-galactonic acid-γ-lactone and D-galacturonic acid compared to the EDL933 parental control. Percentage differences in level of utilization for these substrates were 24.4%, 15.8%, 38.3%, 22.9%, 49.6%, 28.2%, 27.8%, 21.4%, 27.8%, 34.9%, 30.2%, 11.8%, 41.2%, 26.4%, 36.0%, 15.6%, 21.5%, 49.7%, 22.8%, 31.3%, 24.3%, 21.5%, 21.9%, 20.1%, 30.9%, 14.7%, 19.9%, 19.7%, 31.5%, 44.3%, 39.9%,

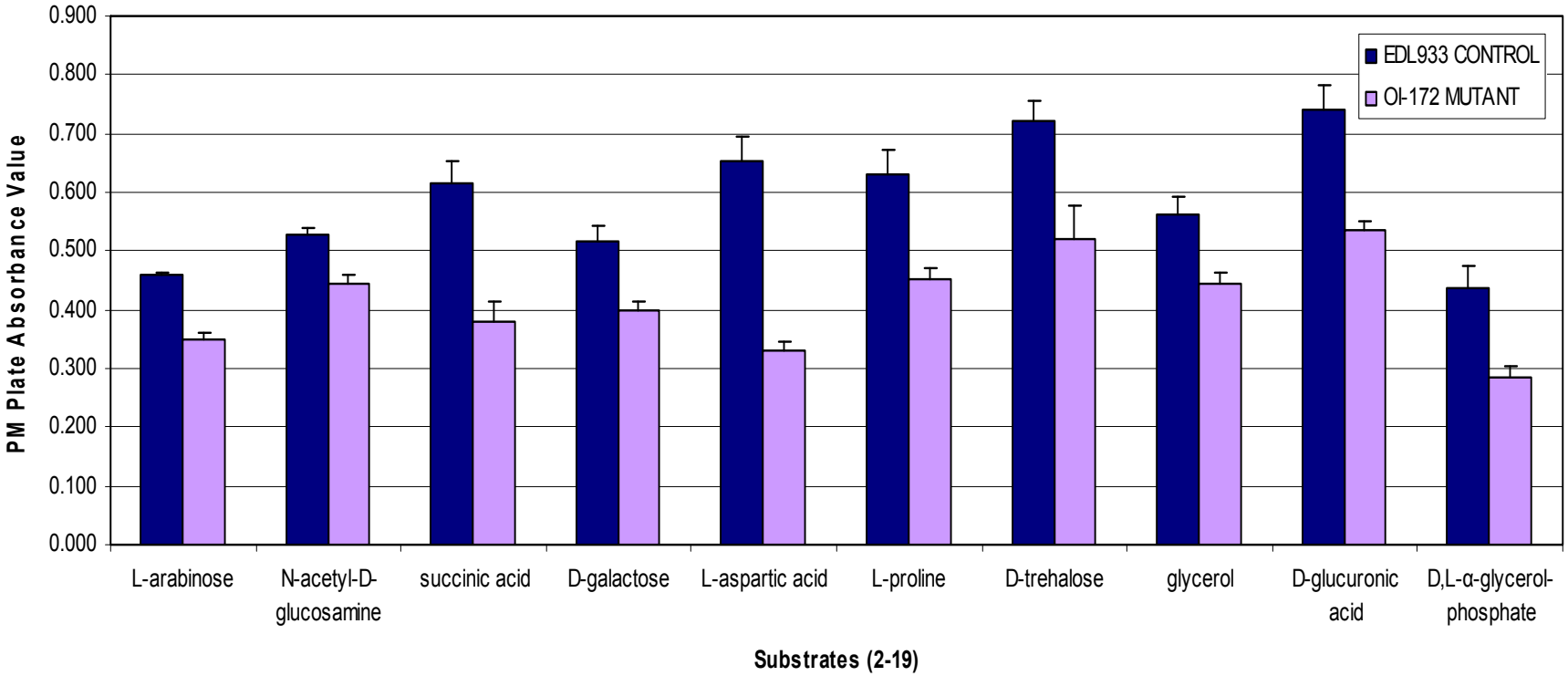
66.8%, 31.6%, 21.0%, 19.5%, 23.1%, 40.9%, 26.6%, 27.1% and 13.0% respectively (Figure 3.29 - 3.32 and Table 3.4).

In summary, the only significant differences in substrate utilization were for the substrates tween 20, M-tartaric acid , α-keto-butyric acid and α-hydroxy butyric acid. The mutant was above the threshold value for tween 20 and M-tartaric acid and below the threshold value for α-keto-butyric acid and α-hydroxy butyric acid. The EDL933 parental strain showed the opposite result for these substrates. The use of tween 20 suggests that this OI could have a role in membrane integrity. If the island does have a role in membrane function it could help explain why the mutant had so many differences in level of substrate utilization compared to EDL933 and why in each case the mutant had a lowered ability to use a substrate compared to the parental strain.

3.3.8 Metabolism in the *E. coli* K-12 reference strain DH5α and the O157:H7 OI deletion mutants

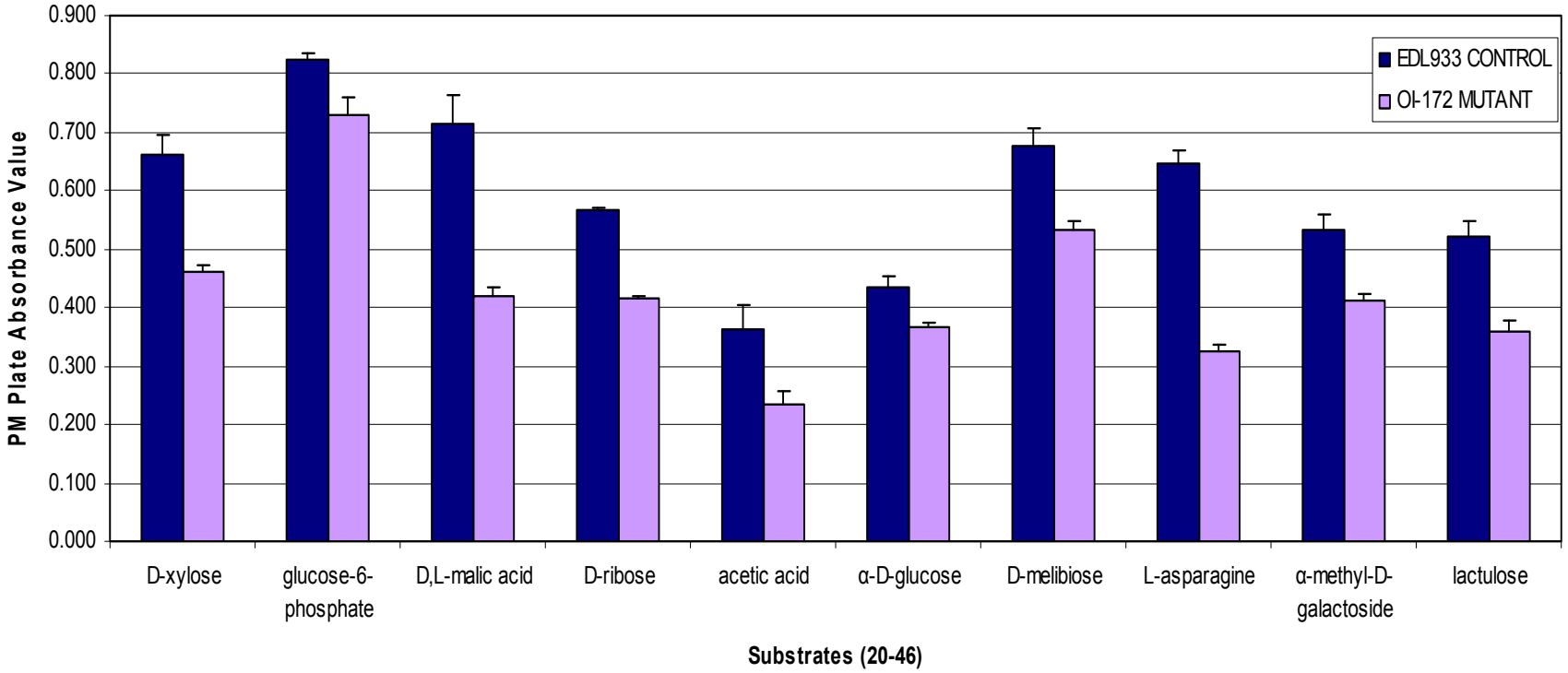
3.3.8.1 Comparison of DH5α with the OI-39 deletion mutant

Comparison of the OI-39 deletion mutant and the DH5α strain showed many of the same differences seen when the parental O157 strain EDL933 was compared with DH5α. There were 15 cases where differences were seen between the two strains using a T-test. Seven of these cases were no longer significantly different ($P > 0.05$) in level of substrate utilization in the DH5α and OI-39 comparison but, were in the DH5α and EDL933 comparison. The substrates involved were succinic acid, L-proline, dulcitol, glucose-6-phosphate, fructose-6-phosphate, glycyl-L-aspartic acid and L-serine. This suggests that the mutant behaved more like DH5α for these substrates than the parental strain EDL933.



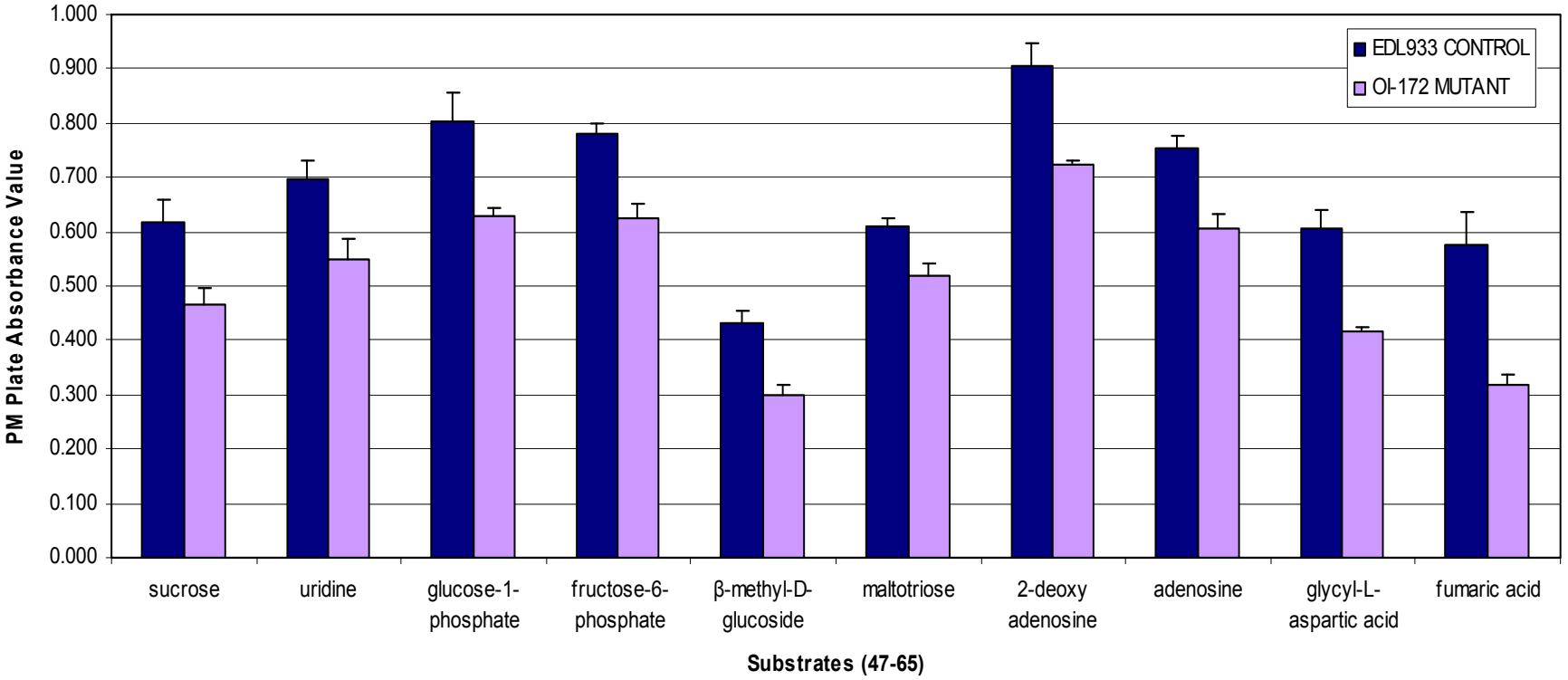
SOURCE	L-arabinose	N-acetyl-D-glucosamine	succinic acid	D-galactose	L-aspartic acid	L-proline	D-trehalose	glycerol	D-glucuronic acid	D,L-α-glycerol-phosphate
EDL933 CONTROL	0.461	0.529	0.614	0.517	0.655	0.629	0.721	0.564	0.741	0.436
OI-172 MUTANT	0.349	0.445	0.379	0.399	0.330	0.451	0.520	0.443	0.535	0.284
PERCENTAGE DIFFERENCE (%)	24.4	15.8	38.3	22.9	49.6	28.2	27.8	21.4	27.8	34.9

Figure.3.29. Differences in levels of substrate utilization (substrates 2- 19) for the *E. coli* O157:H7 parental strain EDL933 and the OI-172 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure.



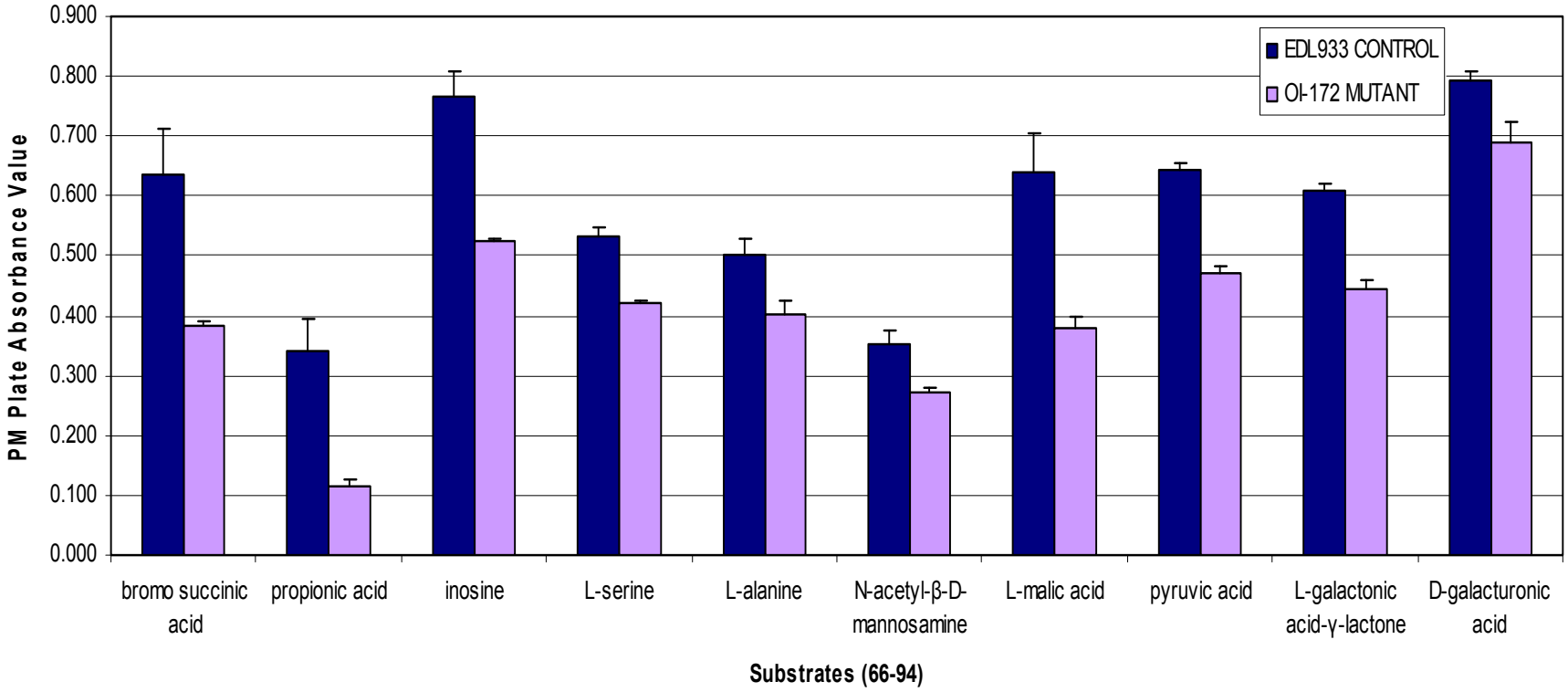
SOURCE	D-xylose	glucose-6-phosphate	D,L-malic acid	D-ribose	acetic acid	α-D-glucose	D-melibiose	L-asparagine	α-methyl-D-galactoside	lactulose
EDL933 CONTROL	0.660	0.826	0.714	0.566	0.364	0.436	0.678	0.647	0.534	0.521
OI-172 MUTANT	0.461	0.728	0.420	0.417	0.233	0.368	0.532	0.326	0.412	0.358
PERCENTAGE DIFFERENCE (%)	30.2	11.8	41.2	26.4	36.0	15.6	21.5	49.7	22.8	31.3

Figure.3.30. Differences in levels of substrate utilization (substrates 20- 46) for the *E. coli* O157:H7 parental strain EDL933 and the OI-172 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure.



SOURCE	sucrose	uridine	glucose-1-phosphate	fructose-6-phosphate	β-methyl-D-glucoside	maltotriose	2-deoxy adenosine	adenosine	glycyl-L-aspartic acid	fumaric acid
EDL933 CONTROL	0.617	0.698	0.805	0.781	0.433	0.610	0.904	0.755	0.607	0.574
OI-172 MUTANT	0.467	0.548	0.628	0.624	0.299	0.520	0.724	0.606	0.415	0.320
PERCENTAGE DIFFERENCE (%)	24.3	21.5	21.9	20.1	30.9	14.7	19.9	19.7	31.5	44.3

Figure.3.31. Differences in levels of substrate utilization (substrates 47-65) for the *E. coli* O157:H7 parental strain EDL933 and the OI-172 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure.



SOURCE	bromo succinic acid	propionic acid	inosine	L-serine	L-alanine	N-acetyl-β-D-mannosamine	L-malic acid	pyruvic acid	L-galactonic acid-γ-lactone	D-galacturonic acid
EDL933 CONTROL	0.636	0.342	0.766	0.532	0.501	0.354	0.641	0.642	0.607	0.793
OI-172 MUTANT	0.383	0.114	0.524	0.420	0.404	0.272	0.379	0.471	0.443	0.689
PERCENTAGE DIFFERENCE (%)	39.9	66.8	31.6	21.0	19.5	23.1	40.9	26.6	27.1	13.0

Figure.3.32. Differences in levels of substrate utilization (substrates 66-94) for the *E. coli* O157:H7 parental strain EDL933 and the OI-172 deletion mutant. All values were significantly different ($P < 0.05$).The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure.

Table 3.4. Percentage difference* in levels of substrate utilization for the OI-172 deletion mutant and the EDL933 parental.

SOURCE	EDL933 control	OI-172 mutant	Percentage Difference (%)
L-arabinose	0.461	0.349	24.4
N-acetyl-D-glucosamine	0.529	0.445	15.8
succinic acid	0.614	0.379	38.3
D-galactose	0.517	0.399	22.9
L-aspartic acid	0.655	0.330	49.6
L-proline	0.629	0.451	28.2
D-trehalose	0.721	0.520	27.8
Glycerol	0.564	0.443	21.4
D-glucuronic acid	0.741	0.535	27.8
D,L-α-glycerol-phosphate	0.436	0.284	34.9
D-xylose	0.660	0.461	30.2
glucose-6-phosphate	0.826	0.728	11.8
D,L-malic acid	0.714	0.420	41.2
D-ribose	0.566	0.417	26.4
acetic acid	0.364	0.233	36.0
α-D-glucose	0.436	0.368	15.6
D-melibiose	0.678	0.532	21.5
L-asparagine	0.647	0.326	49.7
α-methyl-D-galactoside	0.534	0.412	22.8
Lactulose	0.521	0.358	31.3
Sucrose	0.617	0.467	24.3
Uridine	0.698	0.548	21.5
glucose-1-phosphate	0.805	0.628	21.9
fructose-6-phosphate	0.781	0.624	20.1
β-methyl-D-glucoside	0.433	0.299	30.9
Maltotriose	0.610	0.520	14.7
2-deoxy adenosine	0.904	0.724	19.9
Adenosine	0.755	0.606	19.7
glycyl-L-aspartic acid	0.607	0.415	31.5
fumaric acid	0.574	0.320	44.3
bromo succinic acid	0.636	0.383	39.9
propionic acid	0.342	0.114	66.8
Inosine	0.766	0.524	31.6
L-serine	0.532	0.420	21.0
L-alanine	0.501	0.404	19.5
N-acetyl-β-D-mannosamine	0.354	0.272	23.1
L-malic acid	0.641	0.379	40.9
pyruvic acid	0.642	0.471	26.6
L-galactonic acid-γ-lactone	0.607	0.443	27.1
D-galacturonic acid	0.793	0.689	13.0

*All values were significantly different ($P < 0.05$).

The remaining eight cases were for substrates where there were significant differences in either substrate utilization (+ or -) or level of substrate utilization.

Significant differences ($P < 0.05$) in substrate utilization were found where the OI-39 deletion mutant was negative (below threshold of <0.100) and DH5α strain was positive (above threshold) for three substrates, tween 20, tween 40 and α-keto-glutaric acid (Figure 3.33). It appears that DH5α has the ability to use tween 20, tween 40 and α-keto-glutaric acid as a carbon source and the OI-39 deletion mutant does not.

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were also seen (Table 3.5). The OI-39 deletion mutant used significantly higher levels of D-mannitol, D-fructose, mucic acid and D-malic acid while DH5α used significantly higher levels of acetic acid. Percentage differences in utilization for each of these substrates were 26.0%, 25.5%, 29.3%, 31.6% and 17.7% respectively (Table 3.5).

3.3.8.2 Comparison of DH5α with the OI-51 deletion mutant

Comparison of the OI-51 deletion mutant from EDL933 and the DH5α strain showed many of the same differences in substrate utilization (+ or -) and levels of substrate utilization seen when the parental strain EDL933 was compared with DH5α. There were 10 cases where significant differences were seen using a T-test. Half of those cases were no longer significantly different ($P \geq 0.05$) in level of substrate utilization in the DH5α and OI-51 comparison but were in the DH5α and EDL933 comparison. They were for L-arabinose, α-D-glucose, L-glutamine, fructose-6-phosphate and L-serine. This

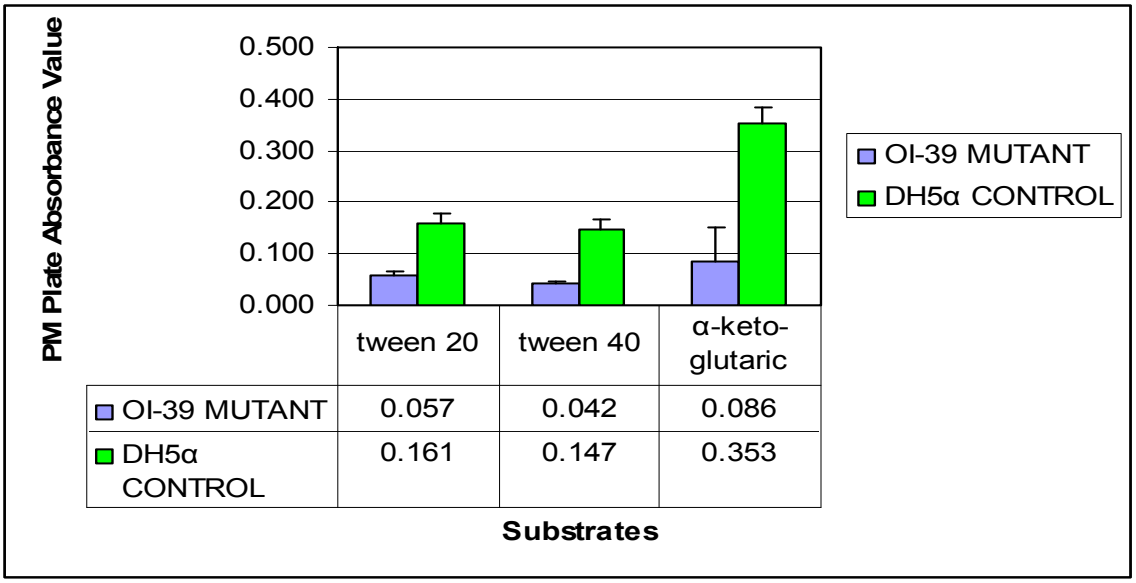


Figure. 3.33 Comparison of *E. coli* OI deletion mutant OI-39 and *E. coli* DH5α for the substrates tween 20, tween 40 and α-keto-glutaric acid. The threshold value for substrate utilization was <0.100. DH5α is above the threshold value in each case and the OI-39 deletion mutant is below the threshold value.

Table 3.5. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-39 and the *E. coli* DH5α strain.

# of Substrate	Source	OI-39 mutant	DH5α Control	Percentage difference* (%)
23	D-mannitol	0.585	0.457	21.9
31	D-fructose	0.588	0.420	28.5
32**	**acetic acid**	**0.237**	**0.276**	**14.4**
68	mucic acid	0.548	0.420	23.3
83	D-malic acid	0.556	0.328	41.0

*All values were significantly different ($P < 0.05$).
** Bolded value is the case where DH5α has a higher level of substrate utilization.

suggests that the mutant behaved more like DH5α for these substrates than the parental strain EDL933. The remaining five cases were for substrates where there were significant differences in either substrate utilization (+ or -) or the level of substrate utilization.

A significant difference ($P < 0.05$) in substrate utilization was found for one substrate. The OI-51 deletion mutant was positive (above threshold of 0.100) and DH5α negative (below the threshold) for glucuronamide utilization (Figure 3.34). It appears that the OI-51 mutant has the ability to use glucuronamide as a carbon source while DH5α does not.

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were also seen (Table 3.6). The OI-51 deletion mutant used significantly higher levels of D-mannitol, acetic acid, glycyl-L-glutamic acid and D-malic acid compared to DH5α. Percentage differences in utilization for each of these substrates were 28.6%, 17.2%, 51.5% and 42.4% respectively (Table. 3.6).

3.3.8.3 Comparison of DH5α with the OI-55 deletion mutant

Comparison of the OI-55 deletion mutant and DH5α strain show many of the same differences in either substrate utilization or levels of substrate utilizations as seen when comparing the parental O157 strain EDL933 with the *E. coli* K-12 reference strain DH5α. There were nine cases where differences were seen between the two bacterial strains using a T-test. In two cases significant differences ($P \geq 0.05$) in level of substrate utilization seen in DH5α and EDL933 were no longer seen when DH5α and the OI-55 deletion mutant were compared; *i.e.*, use of L-arabinose and fructose-6-phosphate changed, suggesting that the mutant behaved more like DH5α for these substrates than the parental strain EDL933. The remaining seven cases were for substrates where there

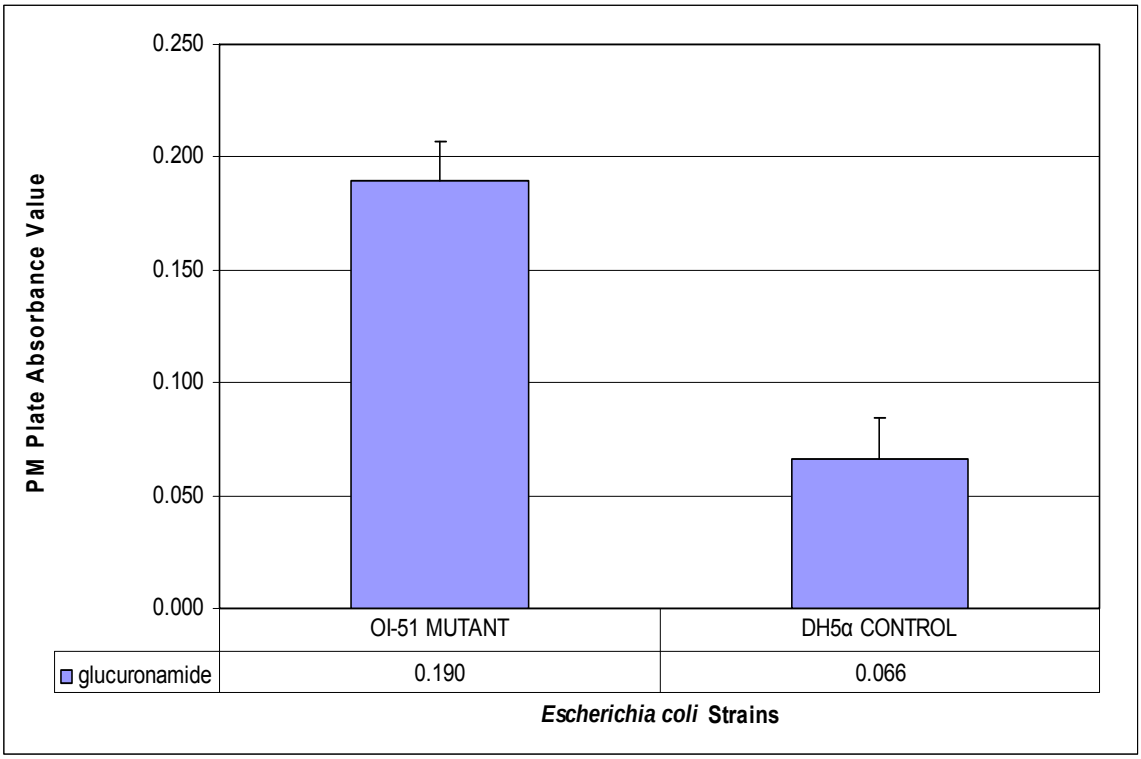


Figure. 3.34 Comparison of *E. coli* OI deletion mutant OI-51 and *E. coli* DH5α for the substrate glucuronamide. The threshold value for substrate utilization was <0.100. The OI-135 deletion mutant is above the threshold for glucuronamide and DH5α is below the threshold.

Table 3.6. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-51 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	OI-51 mutant	DH5α Control	Percentage Difference (%)
23	D-mannitol	0.640	0.457	28.6
32	acetic acid	0.334	0.276	17.2
73	glycyl-L-glutamic acid	0.497	0.241	51.5
83	D-malic acid	0.570	0.328	42.4

*All values were significantly different ($P < 0.05$).

were significant differences in either substrate utilization (+ or -) or level of substrate utilization. Significant differences ($P < 0.05$) in substrate utilization were found for one substrate. The OI-55 deletion mutant was positive (above threshold of ≥ 0.100) and DH5α was negative (below threshold) for glucuronamide (Figure 3.35). It appears that the OI-55 mutant had the ability to use glucuronamide as a carbon source while DH5α did not.

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were also seen (Table 3.7). The OI-55 deletion mutant used significantly higher levels of D-mannitol, acetic acid, glycyl-L-glutamic acid, L-alanyl-glycine and D-malic acid while DH5α used significantly higher levels of mucic acid. Percentage differences in utilization for each of these substrates were 25.6%, 16.8%, 49.3%, 29.4% and 76.1% respectively (Table. 3.7).

3.3.8.4 Comparison of DH5α with the OI-59 deletion mutant

Comparison of the OI-59 deletion mutant with the DH5α strain showed many of the same differences in substrate utilization (+ and -) and levels of substrate utilization as seen when the parental strain EDL933 was compared with DH5α. There were nine cases where differences were seen using a T-test. In three of these cases levels of substrate utilization for L-arabinose, α-D-glucose and fructose-6-phosphate were no longer significantly different ($P \geq 0.05$) when DH5α and the OI-59 mutant were compared but, were different when DH5α and EDL933 were compared. This suggests that the OI-59 mutant behaved more like DH5α for these substrates than like the parental strain EDL933; interestingly two of these substrates are part of the glycolysis pathway. A

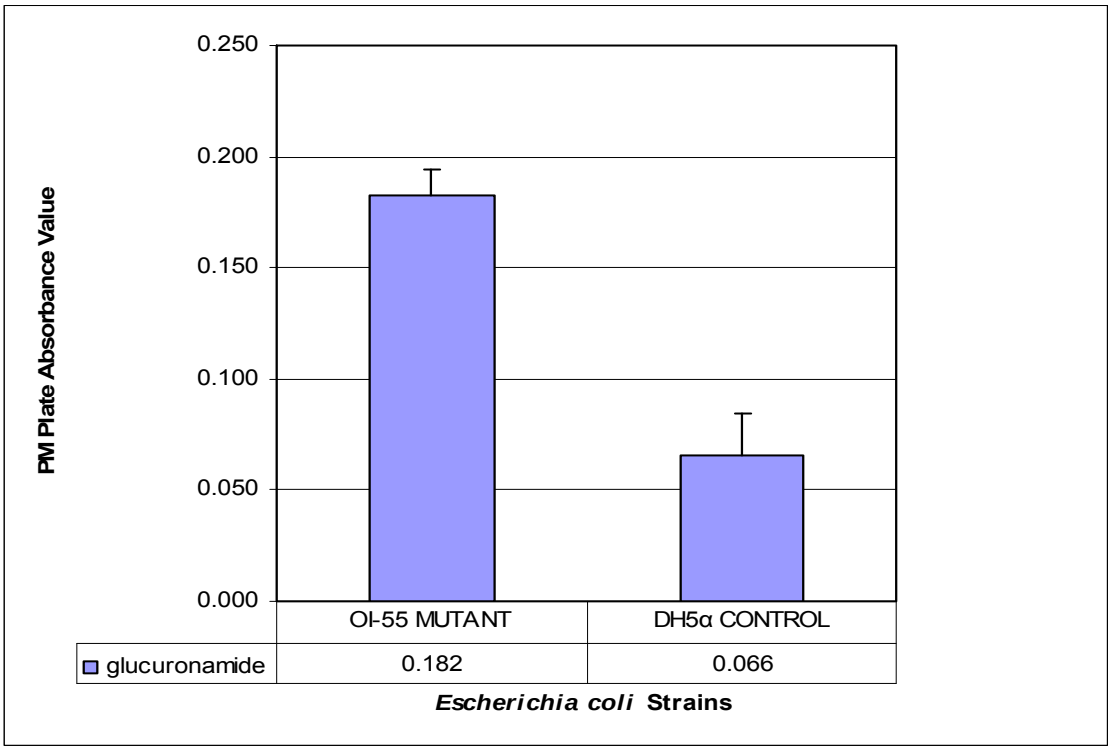


Figure. 3.35. Comparison of *E. coli* OI deletion mutant OI-55 and *E. coli* DH5α for the substrate glucuronamide. The threshold value for substrate utilization was <0.100. The OI-135 deletion mutant is above the threshold for glucuronamide and DH5α is below the threshold.

Table 3.7. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-55 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	OI-55 mutant	DH5α Control	Percentage Difference (%)
23	D-mannitol	0.614	0.457	25.6
32	acetic acid	0.332	0.276	16.8
68	**mucic acid**	**0.100**	**0.420**	**76.1**
73	glycyl-L-glutamic acid	0.474	0.241	49.3
78	L-alanyl-glycine	0.480	0.339	29.4
83	D-malic acid	0.517	0.328	36.5

*All values were significantly different ($P < 0.05$).
** Bolded values are cases where DH5α has a higher substrate utilization level.

significant difference ($P < 0.05$) in substrate utilization also was found where glucuronamide use in the OI-59 deletion mutant was positive (above threshold of ≥ 0.100) while its use in DH5α was negative (below the threshold) (Figure 3.36). It appears that the OI-59 mutant has the ability to use glucuronamide as a carbon source while DH5α does not.

In addition, significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were seen (Table 3.8). The OI-59 deletion mutant used significantly higher levels of D-mannitol, acetic acid, α-keto-glutaric acid, glycyl-L-glutamic acid and D-malic acid in comparison to DH5α. Percentage differences in utilization for each of these substrates were 25.5%, 22..2%, 21.1%, 50.4% and 42..2% respectively (Table. 3.8).

3.3.8.5 Comparison of DH5α with the OI-87 deletion mutant

Comparison of the OI-87 deletion mutant with the DH5α strain showed many of the same differences in substrate utilization (+ and -) and levels of substrate utilization seen when the parental strain EDL933 was compared with DH5α. There were nine cases where differences were seen using a T-test. In three of these cases levels of substrate utilization for L-arabinose, fructose-6-phosphate and L-serine were no longer significantly different ($P \geq 0.05$) when DH5α and the OI-87 mutant were compared but, were different when DH5α and EDL933 were compared. This suggests that the OI-87 mutant behaved more like DH5α for these substrates than like the parental strain EDL933.

A significant difference ($P < 0.05$) in substrate utilization was found for

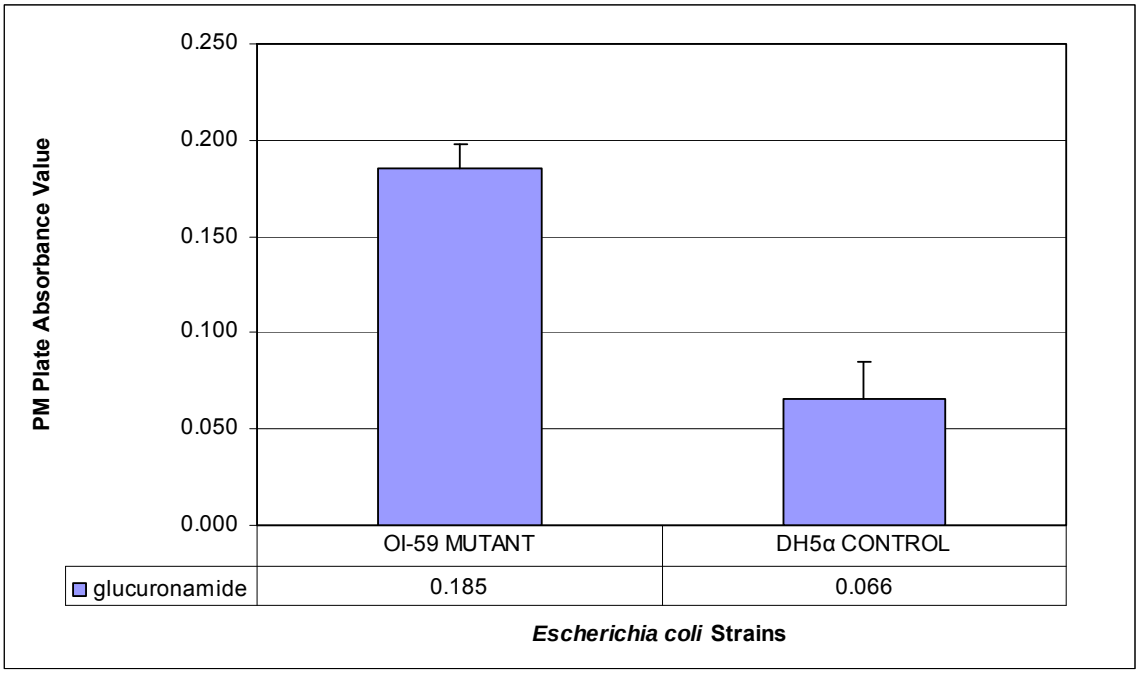


Figure. 3.36. Comparison of *E. coli* OI deletion mutant OI-59 and *E. coli* DH5α for the substrate glucuronamide. The threshold value for substrate utilization was <0.100. The OI-59 deletion mutant is above the threshold for glucuronamide and DH5α is below the threshold.

Table 3.8. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-59 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	OI-59 mutant	DH5α Control	Percentage Difference (%)
23	D-mannitol	0.613	0.457	25.5
32	acetic acid	0.355	0.276	22.2
42	α-keto-glutaric acid	0.447	0.353	21.1
73	glycyl-L-glutamic acid	0.486	0.241	50.4
83	D-malic acid	0.568	0.328	42.2

*All values were significantly different ($P < 0.05$).

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glucuronamide. In this case the OI-87 deletion mutant was positive (above threshold of <0.100) for glucuronamide but, negative (below threshold) in the *E. coli* K-12 reference strain DH5α (Figure 3.37). It appears that the OI-87 mutant had the ability to use glucuronamide as a carbon source while DH5α did not.

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were also seen (Table 3.9). The OI-87 deletion mutant used significantly higher levels of D-mannitol, acetic acid, glycyl-L-glutamic acid, L-alanyl-glycine and D-malic acid in comparison to DH5α. Percentage differences in utilization for each of these substrates was 28.2%, 21.9%, 50.9%, 31.2% and 36.2% respectively (Table. 3.9).

3.3.8.6 Comparison of DH5α with the OI-89 deletion mutant

Comparison of the OI-89 deletion mutant with the DH5α strain showed many of the same differences in substrate utilization (+ and -) and levels of substrate utilization seen when the parental strain EDL933 was compared with DH5α. There were 12 cases where differences were seen using a T-test. In seven of these cases levels of substrate utilization for succinic acid, D-galactose, L-lactic acid, α-D-glucose, fructose-6-phosphate, L-serine and L-malic acid were no longer significantly different ($P \geq 0.05$) when DH5α and the OI-89 mutant were compared but, were different when DH5α and EDL933 were compared. This suggests that the mutant behaved more like DH5α for these substrates than it behaved like the parental strain EDL933.

Significant differences ($P < 0.05$) in substrate utilization were found for the substrate glucuronamide. The OI-89 deletion mutant was positive (above threshold of

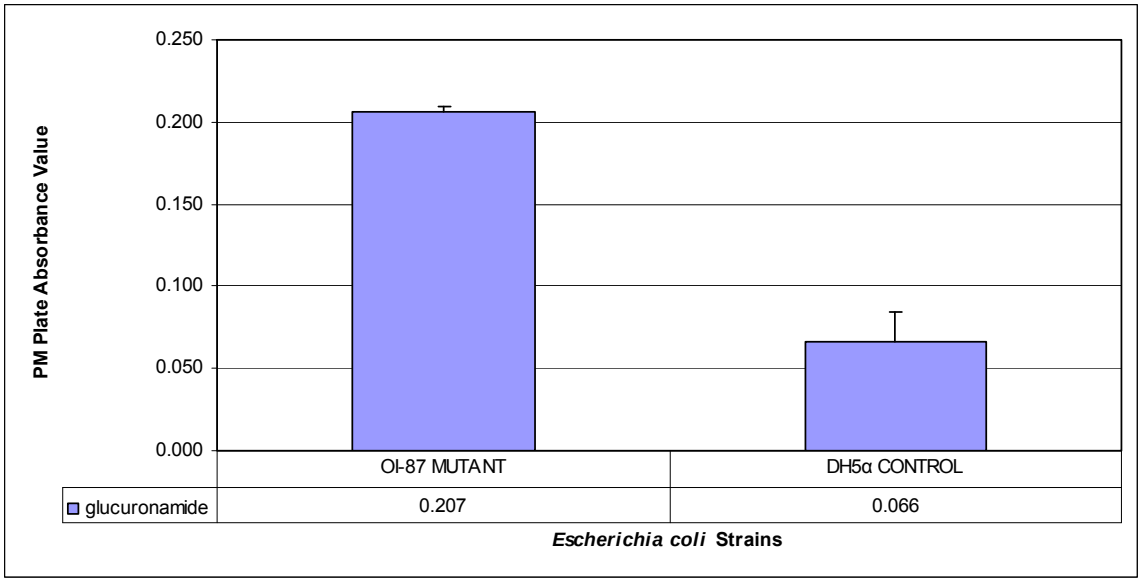


Figure. 3.37. Comparison of *E. coli* OI deletion mutant OI-87 and *E. coli* DH5α for the substrate glucuronamide. The threshold value for substrate utilization was <0.100. The OI-87 deletion mutant is above the threshold for glucuronamide and DH5α is below the threshold.

Table 3.9. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-87 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	OI-87 mutant	DH5α Control	Percentage Difference (%)
23	D-mannitol	0.636	0.457	28.2
32	acetic acid	0.354	0.276	21.9
73	glycyl-L-glutamic acid	0.490	0.241	50.9
78	L-alanyl-glycine	0.492	0.339	31.2
83	D-malic acid	0.515	0.328	36.2

*All values were significantly different ($P < 0.05$).

<0.100) for glucuronamide while, DH5α was negative (below threshold) for this substrate (Figure 3.38). It appears that the OI-89 mutant had the ability to use glucuronamide as a carbon source while DH5α did not.

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were also seen (Table 3.10). The OI-89 deletion mutant used significantly higher levels of D-mannitol, acetic acid, mucic acid and L-alanyl-glycine in comparison to DH5α. Percentage differences in utilization for each of these substrates were 20.1%, 18.2%, 25.7% and 31.7% respectively (Table. 3.10).

3.3.8.7 Comparison of DH5α with the OI-98 deletion mutant

Comparison of the OI-98 deletion mutant with the DH5α strain showed many of the same differences in substrate utilization (+ and -) and levels of substrate utilization seen when the parental strain EDL933 was compared with DH5α. There were 16 cases where differences were seen using a T-test. In nine of these cases levels of substrate utilization for succinic acid, L-proline, dulcitol, D-serine, glycerol, L-glutamic acid, L-glutamine, fructose-6-phosphate and D-galacturonic acid were no longer significantly different ($P \geq 0.05$) when DH5α and the OI-98 mutant were compared but, were different when DH5α and EDL933 were compared. This suggests that the mutant behaved more like the DH5α reference strain for these substrates than it behaved like the parental strain EDL933.

Significant differences ($P < 0.05$) in substrate utilization were found for the two substrates mono methyl succinate and glucuronamide. The OI-98 deletion mutant was positive (above threshold of <0.100) for both mono methyl succinate and glucuronamide but, negative (below threshold) for use of DH5α (Figure 3.39). It appears that the OI-98

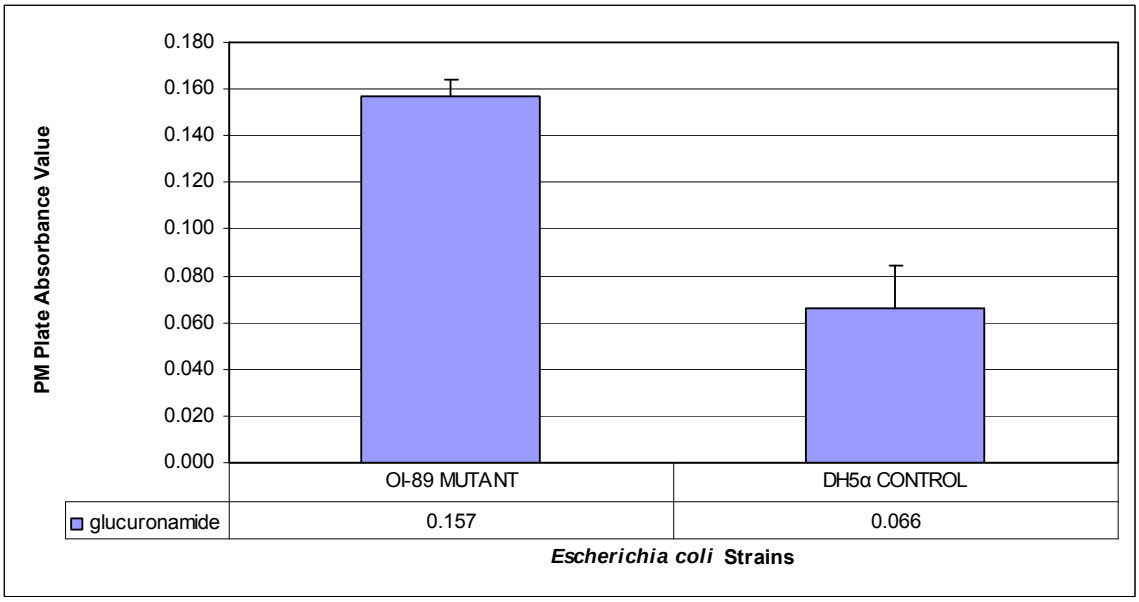


Figure. 3.38. Comparison of *E. coli* OI deletion mutant OI-89 and *E. coli* DH5α for the substrate glucuronamide. The threshold value for substrate utilization was <0.100. The OI-89 deletion mutant is above the threshold for glucuronamide and DH5α is below the threshold.

Table 3.10. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-89 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	OI-89 mutant	DH5α Control	Percentage Difference (%)
23	D-mannitol	0.572	0.457	20.1
32	acetic acid	0.338	0.276	18.2
68	mucic acid	0.565	0.420	25.7
78	L-alanyl-glycine	0.496	0.339	31.7

*All values were significantly different ($P < 0.05$).

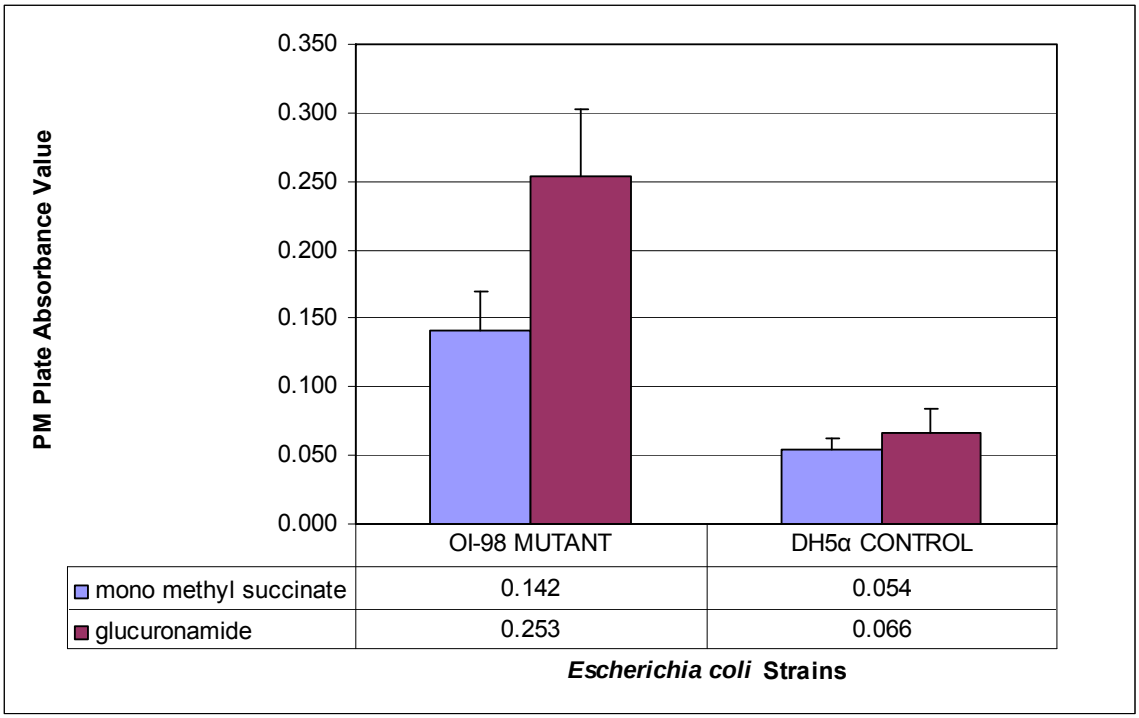


Figure. 3.39. Comparison of *E. coli* OI deletion mutant OI-98 and *E. coli* DH5α for the substrates mono methyl succinate and glucuronamide. The threshold value for substrate utilization was <0.100. The OI-98 deletion mutant is above the threshold for mono methyl succinate and glucuronamide and DH5α is below the threshold.

mutant had the ability to use mono methyl succinate and glucuronamide as a carbon source but, DH5α did not.

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were also seen (Table 3.11). The OI-98 deletion mutant used significantly higher levels of D-mannitol, D-fructose, acetic acid, α-keto-glutaric acid and L-alanyl-glycine in comparison to DH5α. Percentage differences in utilization for each of these substrates were 27.6%, 33.3%, 28.5%, 22.3% and 27.5% respectively (Table. 3.11).

3.3.8.8 Comparison of DH5α with the OI-102 deletion mutant

Comparison of the OI-102 deletion mutant with the DH5α strain showed many of the same differences in substrate utilization (+ and -) and levels of substrate utilization seen when the parental strain EDL933 was compared with DH5α. There were 16 cases where differences were seen using a T-test. In half of these cases levels of substrate utilization for D-alanine, dulcitol, D-serine, glycerol, sucrose, uridine, fructose-6-phosphate and glycyl-L-aspartic acid were no longer significantly different ($P \geq 0.05$) when DH5α and the OI-102 mutant were compared but, were different when DH5α and EDL933 were compared. This suggests that the mutant behaved more like DH5α for these substrates than like the parental strain EDL933.

Significant differences ($P < 0.05$) in substrate utilization also were found for the substrates D-threonine and glucuronamide. The OI-102 deletion mutant was positive (above threshold of <0.100) for D-threonine and glucuronamide but, negative (below threshold) for D-threonine and glucuronamide in DH5α (Figure 3.40). It appears that the

Table 3.11. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-98 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	OI-98 mutant	DH5α Control	Percentage Difference (%)
23	D-mannitol	0.631	0.457	27.6
31	D-fructose	0.630	0.420	33.3
32	acetic acid	0.387	0.276	28.5
42	α-keto-glutaric acid	0.454	0.353	22.3
78	L-alanyl-glycine	0.467	0.339	27.5

*All values were significantly different ($P < 0.05$).

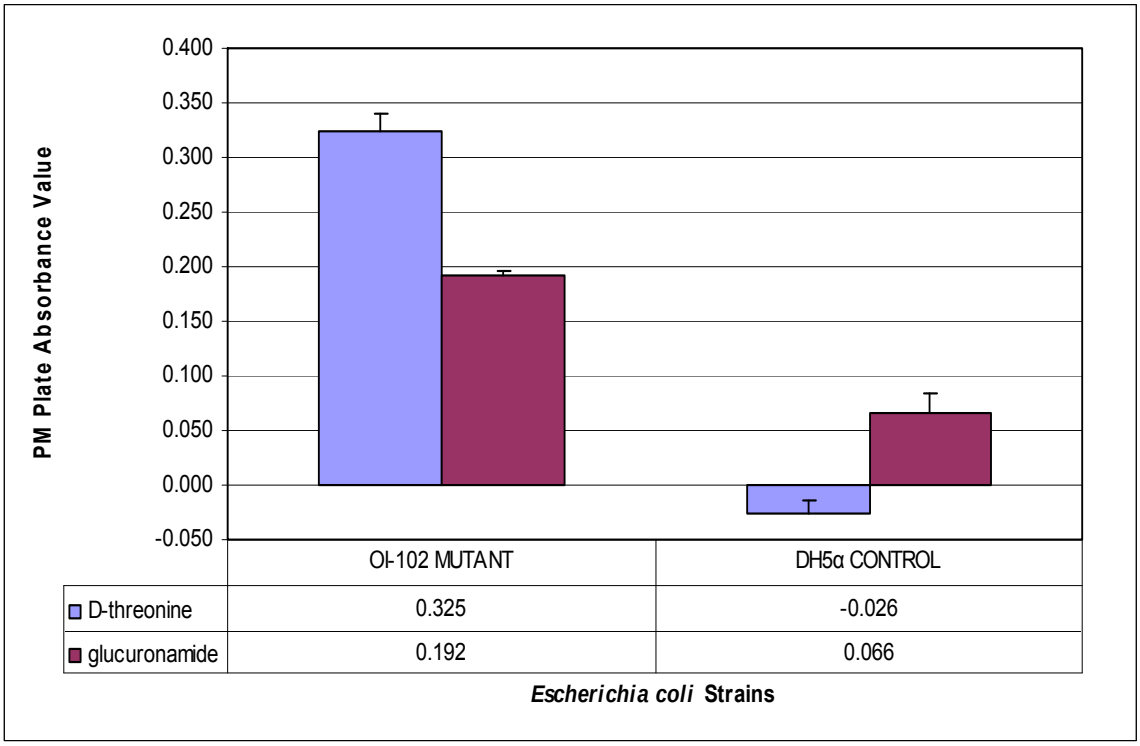


Figure. 3.40. Comparison of *E. coli* OI deletion mutant OI-102 and *E. coli* DH5α for the substrates D-threonine and glucuronamide. The threshold value for substrate utilization was <0.100. The OI-102 deletion mutant is above the threshold for D-threonine and glucuronamide and DH5α is below the threshold.

OI-102 mutant had the ability to use D-threonine and glucuronamide as a carbon source but, that DH5α did not.

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were also seen (Table 3.12). The OI-102 deletion mutant used significantly higher levels of D-mannitol, D-fructose, acetic acid, glycyl-L-glutamic acid, L-alanyl-glycine and D-malic when compared to DH5α. Percentage differences in utilization for each of these substrates were 23.5%, 25.7%, 25.0%, 32.7%, 33.3% and 35.4% respectively (Table. 3.12).

3.3.8.9 Comparison of DH5α with the OI-108 deletion mutant

Comparison of the OI-108 deletion mutant with the DH5α strain showed many of the same differences in substrate utilization (+ and -) and levels of substrate utilization seen when the parental strain EDL933 was compared with DH5α. There were 13 cases where differences were seen using a T-test. In seven of these cases levels of substrate utilization for succinic acid, L-aspartic acid, glycerol, D, L-malic acid, L-asparagine, fructose-6-phosphate and maltotriose were no longer significantly different ($P \geq 0.05$) when DH5α and the OI-108 mutant were compared but, were different when DH5α and EDL933 were compared. This suggests that the mutant behaved more like the *E. coli* K-12 reference strain DH5α for these substrates than like the parental strain EDL933.

Significant differences ($P < 0.05$) in substrate utilization were found for one substrate. The OI-108 deletion mutant was positive (above threshold of <0.100) for use of glucuronamide but, negative (below threshold) for glucuronamide in DH5α (Figure 3.41). It appeared that the OI-108 mutant had the ability to use glucuronamide as a

Table 3.12. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-102 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	OI-102 mutant	DH5α Control	Percentage Difference (%)
23	D-mannitol	0.598	0.457	23.5
31	D-fructose	0.566	0.420	25.7
32	acetic acid	0.368	0.276	25.0
73	glycyl-L-glutamic acid	0.358	0.241	32.7
78	L-alanyl-glycine	0.508	0.339	33.3
83	D-malic acid	0.508	0.328	35.4

*All values were significantly different ($P < 0.05$).

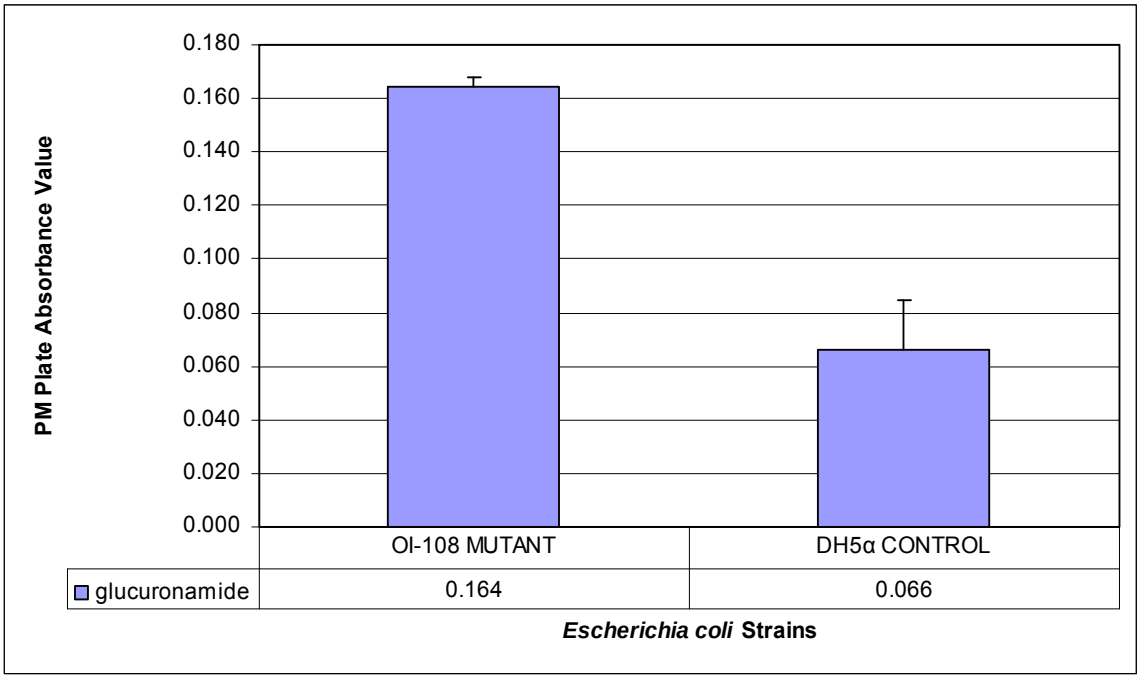


Figure. 3.41. Comparison of *E. coli* OI deletion mutant OI-108 and *E. coli* DH5α for the substrate glucuronamide. The threshold value for substrate utilization was 0.100. The OI-102 deletion mutant is above the threshold for glucuronamide and DH5α is below the threshold.

carbon source while DH5α did not.

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were also seen (Table 3.13). The OI-108 deletion mutant used significantly higher levels of D-mannitol, D-fructose, acetic acid, glycyl-L-glutamic acid and L-alanyl-glycine in comparison to DH5α. Percentage differences in utilization for each of these substrates were 27.5%, 25.5%, 21.6%, 40.7% and 33.4% respectively (Table. 3.13).

3.3.8.10 Comparison of DH5α with the OI-135 deletion mutant

Comparison of the OI-135 deletion mutant with the DH5α strain showed many of the same differences in substrate utilization (+ and -) and levels of substrate utilization seen when the parental strain EDL933 was compared with DH5α. There were 11 cases where differences were seen using a T-test. In six of these cases levels of substrate utilization for D-alanine, glycerol, α-D-glucose, fructose-6-phosphate, glycyl-L-aspartic acid and N-acetyl-β-D-mannosamine were no longer significantly different ($P \geq 0.05$) when DH5α and the OI-135 mutant were compared but, were different when DH5α and EDL933 were compared. This suggests that the OI-135 mutant behaved more like DH5α for these substrates than like the parental strain EDL933.

Significant differences ($P < 0.05$) in substrate utilization were found for two substrates. The OI-135 deletion mutant was positive (above threshold of <0.100) for use of glucuronamide but, negative (above threshold) for use of glucuronamide in DH5α; the opposite was seen for the substrate tween 20 (Figure 3.42). It appears that the OI-135 deletion mutant had the ability to use glucuronamide but, not tween 20 as a carbon

Table 3.13. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-108 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	OI-108 mutant	DH5α Control	Percentage Difference (%)
23	D-mannitol	0.630	0.457	27.5
31	D-fructose	0.564	0.420	25.5
32	acetic acid	0.352	0.276	21.6
73	glycyl-L-glutamic acid	0.406	0.241	40.7
78	L-alanyl-glycine	0.508	0.339	33.4

*All values were significantly different ($P < 0.05$).

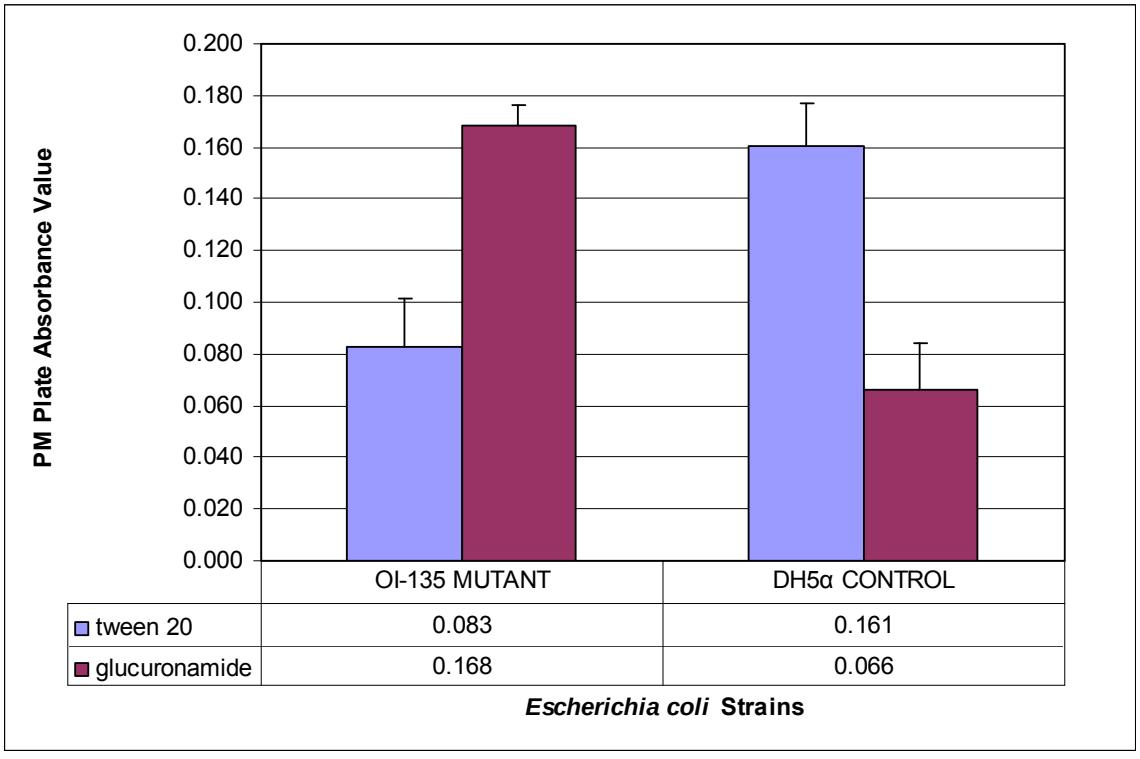


Figure.3.42 Comparison of *E. coli* OI deletion mutant OI-135 and *E. coli* DH5α for the substrates tween 20 and glucuronamide. The threshold value for substrate utilization was <0.100. DH5α is above the threshold value in for tween 20 and the OI-135 deletion mutant is above the threshold for glucuronamide.

source.

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were also seen (Table 3.14). The OI-135 deletion mutant used significantly higher levels of D-mannitol, acetic acid and L-alanyl glycine compared to DH5α. Percentage differences in utilization for each of these substrates were 25.5%, 24.7% and 35.4% respectively (Table. 3.14).

3.3.8.11 Comparison of DH5α with the OI-172 deletion mutant

Comparison of the OI-172 deletion mutant and DH5α strain show many of the same differences seen when comparing the parental EDL933 and DH5α; however there were more differences between these two strains than any other mutant. There were 24 cases where differences were seen using a T-test. In 20 of these cases, levels of substrate utilization for succinic acid, D-galactose, L-aspartic acid, L-proline, D-alanine, glycerol, D,L-α-glycerol-phosphate, L-lactic acid, glucose-6-phosphate, D,L-malic acid, α-D-glucose, uridine,glucose-1-phosphate, fructose-6-phosphate, β-methyl-D-glucoside, maltotriose, glycyl-L-aspartic acid, fumaric acid, N-acetyl-β-D-mannosamine and L-malic acid were no longer significantly different ($P \geq 0.05$) when DH5α and the OI-172 mutant were compared but, were different when DH5α and EDL933 were compared. This suggests that the OI-172 mutant behaved more like DH5α for these substrates than the parental strain EDL933.

Significant differences ($P < 0.05$) in substrate utilization were found for three substrates. The OI-172 deletion mutant was positive (above threshold of <0.100) for use of mono methyl succinate and glucuronamide but, negative (above threshold) in DH5α;

Table 3.14. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-135 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	OI-135 mutant	DH5α Control	Percentage Difference (%)
23	D-mannitol	0.614	0.457	25.5
32	acetic acid	0.367	0.276	24.7
78	L-alanyl-glycine	0.524	0.339	35.4

*All values were significantly different ($P < 0.05$).

the opposite was seen for use of α-keto glutaric acid (Figure 3.43). It appears that the OI-172 deletion mutant has the ability to use mono methyl succinate and glucuronamide but, not α-keto glutaric acid as a carbon source.

A significant difference ($P < 0.05$) in the "level" of substrate utilization where both strains were positive for a substrate was also seen (Table 3.15). The OI-172 deletion mutant used significantly higher levels of tween 20 compared to DH5α. The percentage difference in utilization of tween 20 was 26.6% (Table. 3.15).

3.3.8.12 Comparison of DH5α with the OI-176 deletion mutant

Comparison of the OI-176 deletion mutant with the DH5α strain showed many of the same differences in substrate utilization (+ and -) and levels of substrate utilization seen when the parental strain EDL933 was compared with DH5α. There were 15 cases where differences were seen using a T-test. In eleven of these cases levels of substrate utilization for D-galactose, D-alanine, dulcitol, D-serine, glucose-6-phosphate, α-D-glucose, uridine, fructose-6-phosphate, maltotriose, glycyl-L-aspartic acid and N-acetyl-β-D-mannosamine were no longer significantly different ($P \geq 0.05$) when DH5α and the OI-176 mutant were compared but, were different when DH5α and EDL933 were compared. This suggests that the mutant behaved more like DH5α for these substrates than it behaved like the parental strain EDL933.

The remaining four cases were for substrates where there were significant differences in either substrate utilization (+ or -) or level of substrate utilization. Significant differences ($P < 0.05$) in substrate utilization were found for three substrates. The OI-176 deletion mutant was positive (above threshold of <0.100) for use of

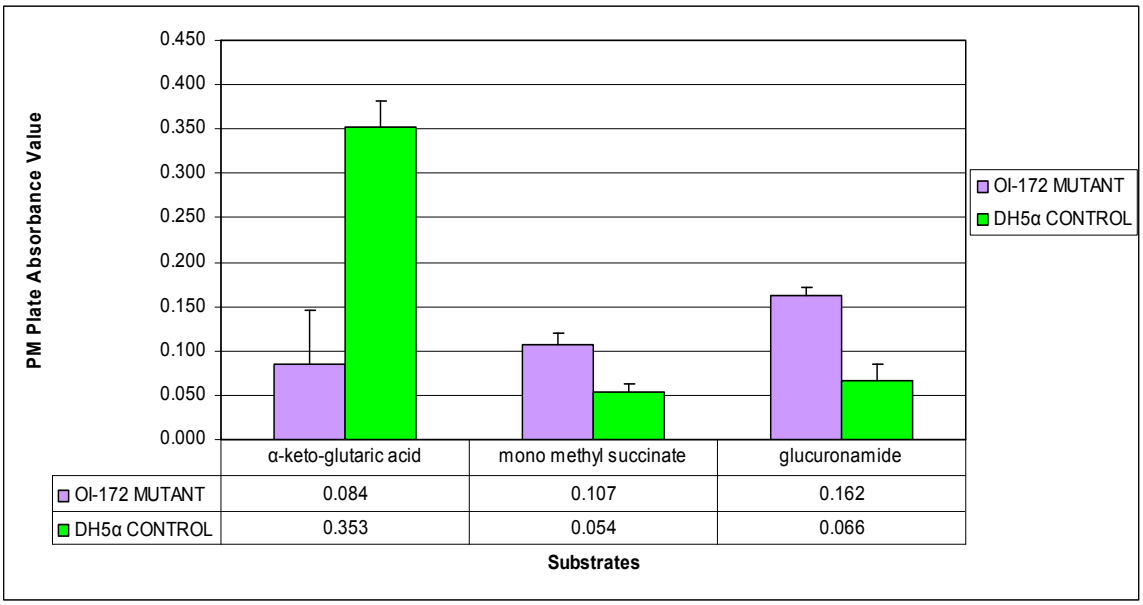


Figure. 3.43 Comparison of *E. coli* OI deletion mutant OI-172 and *E. coli* DH5α for the substrates α-keto-glutaric acid, mono methyl succinate and glucuronamide. The threshold value for substrate utilization was <0.100. DH5α is above the threshold value for α-keto glutaric acid and the OI-172 deletion mutant is above the threshold for mono methyl succinate and glucuronamide.

Table 3.15. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-172 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	OI-172 mutant	DH5α Control	Percentage Difference (%)
29	tween 20	0.219	0.161	26.6

*All values were significantly different ($P < 0.05$).

glucuronamide but, negative (below threshold) for use of glucuronamide in DH5α (Figure 3.44). It appears that the OI-176 mutant had the ability to use glucuronamide as a carbon source but, DH5α did not.

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were also seen (Table 3.16). The OI-176 deletion mutant used significantly higher levels of D-fructose, α-keto-glutaric acid and D-malic acid compared to DH5α. Percentage differences in utilization for each of these substrates were 20.6%, 25.5% and 33.1% respectively (Table. 3.16).

3.3.9 Metabolism in O157:H7 strain Sakai and its SL deletion mutant

The SL 72 contains ORFs coding for hypothetical proteins, tellurium resistance, and urease synthesis, as well as ORFs coding for transposases and adhesins, synthesis of membrane proteins, regulatory proteins and transcriptional regulators. The deletion mutant (SL-72) corresponding to the SL removed was compared to the parental strain Sakai. The pattern of substrate utilization (+ or -) for the SL 72 deletion mutant and the O157:H7 parental strain was similar in most cases; *i.e*., the same substrates were either used or not used in each of these mutant strains as well as in the parental Sakai strain. However, four exceptions appeared to occur for utilization of tween 20, α-keto-glutaric acid, L-glutamine and mono methyl succinate. The SL 72 mutant was above the threshold value of <0.100 used in this study for tween 20, α-keto-glutaric acid and L-glutamine, while the Sakai parent was below the threshold of <0.100 for these substrates. The opposite result was seen for mono methyl succinate where the mutant fell below the threshold but, Sakai was above the threshold (Figure. 3.45). This suggests that the mutant

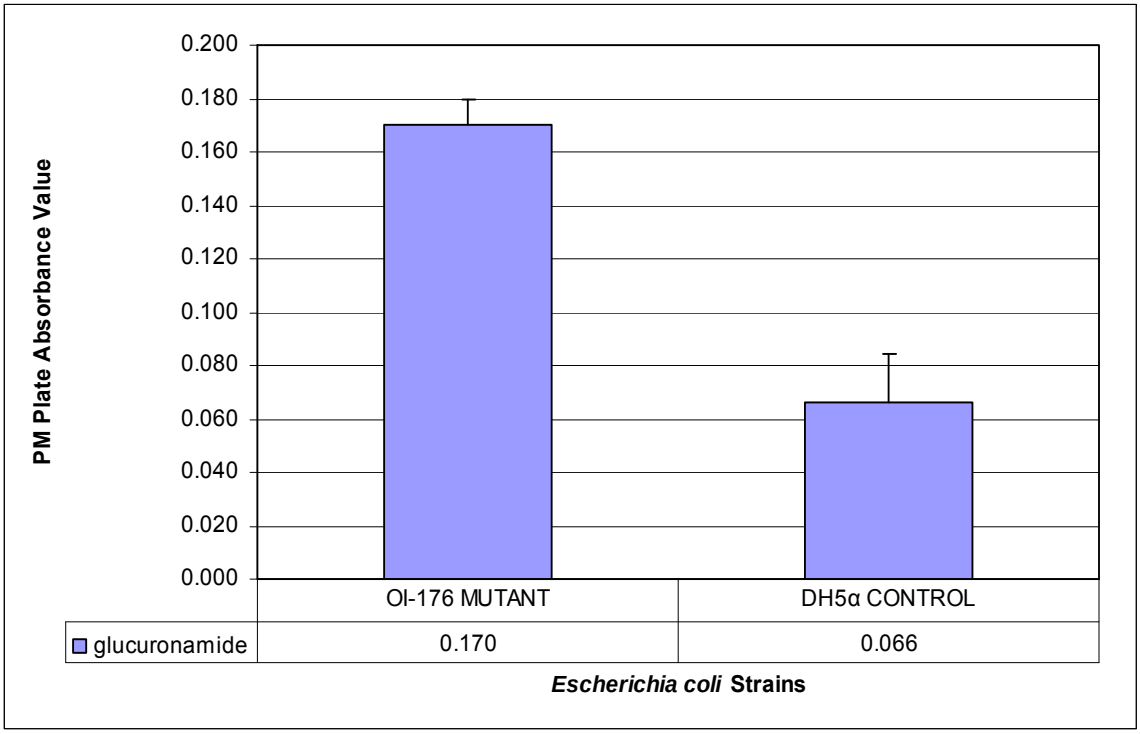
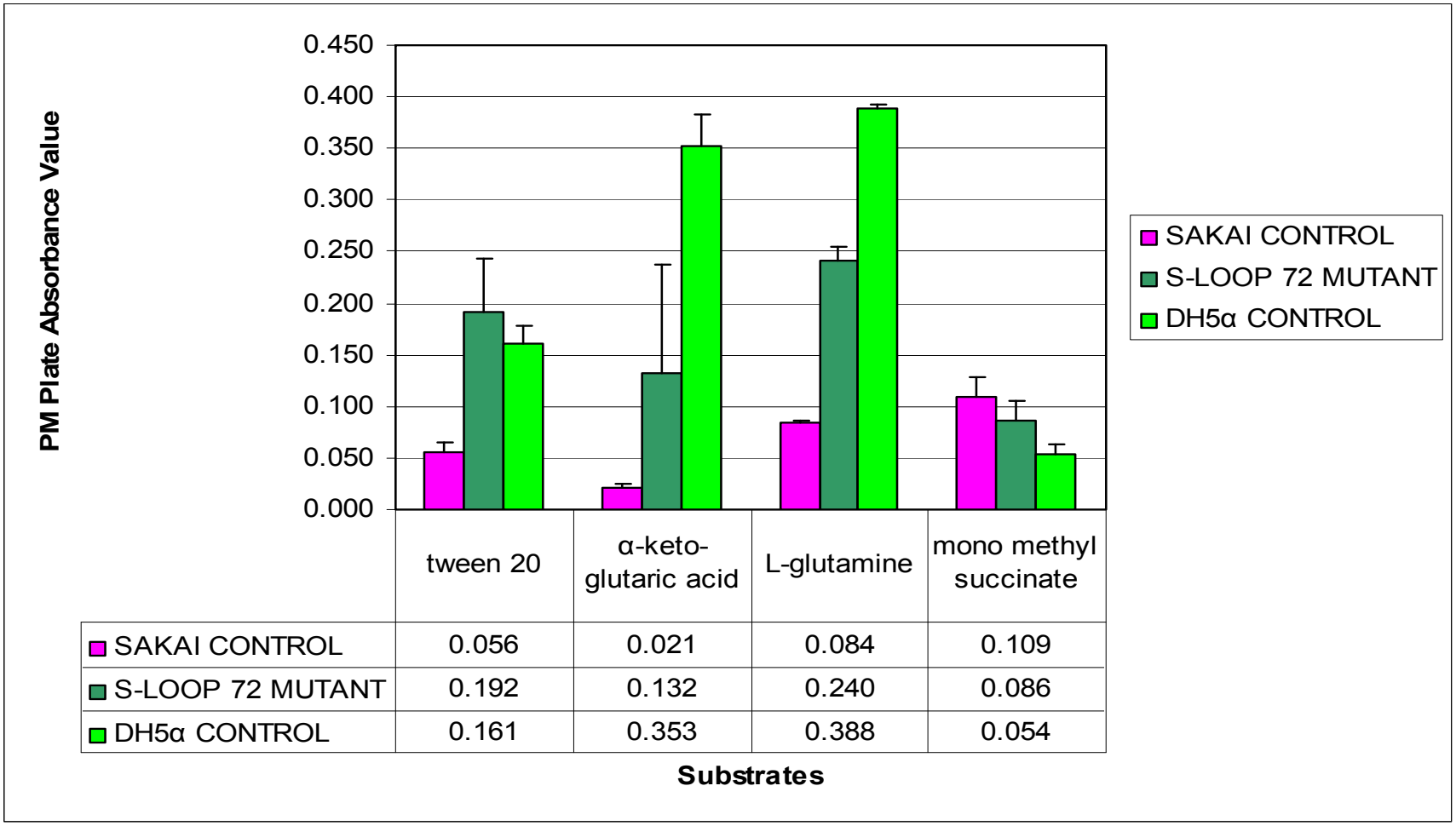


Figure. 3.44 Comparison of *E. coli* OI deletion mutant OI-176 and *E. coli* DH5α for the substrate glucuronamide. The threshold value for substrate utilization was <0.100. The OI-176 mutant is above the threshold value for glucuronamide and DH5α below the threshold.

Table 3.16. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant OI-176 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	OI-176 mutant	DH5α Control	Percentage Difference (%)
31	D-fructose	0.529	0.420	20.6
42	α-keto-glutaric acid	0.473	0.353	25.5
83	D-malic acid	0.491	0.328	33.1

*All values were significantly different ($P < 0.05$).



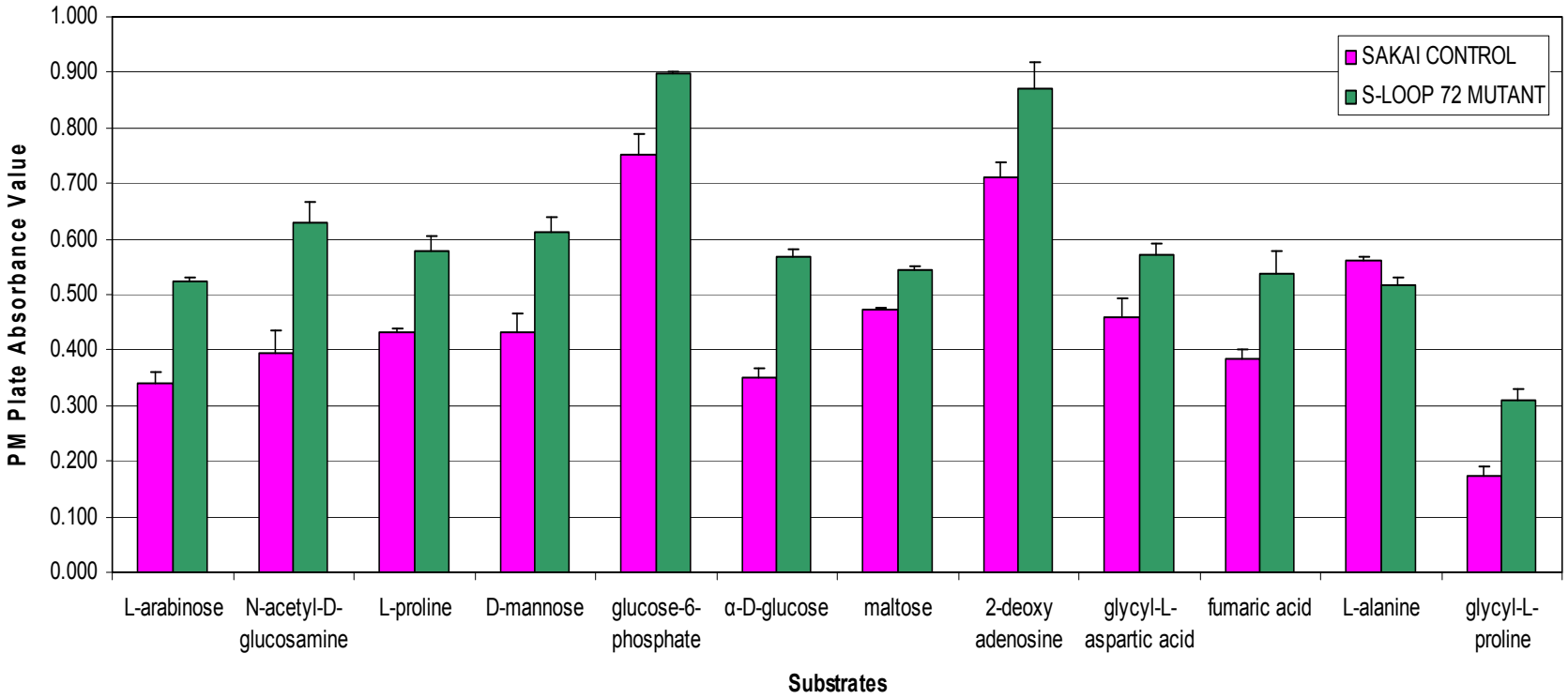
	tween 20	α-keto-glutaric acid	L-glutamine	mono methyl succinate
SAKAI CONTROL	0.056	0.021	0.084	0.109
S-LOOP 72 MUTANT	0.192	0.132	0.240	0.086
DH5α CONTROL	0.161	0.353	0.388	0.054

Figure.3.45. Comparison of *E. coli* O157:H7 parental strain Sakai, the SL 72 deletion mutant and the *E. coli* K-12 reference strain DH5α for the Substrates tween 20, α-keto-glutaric acid, L-glutamine and mono methyl succinate. The threshold value for substrate utilization was <0.100. The absorbance mean ± S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure. The only significant difference in substrate utilization is for L-glutamine.

had gained the ability to use tween 20, α-keto-glutaric acid and L-glutamine and lost the ability to use mono methyl succinate as carbon sources compared to Sakai. Further, analysis of these differences using a T-test showed that the differences in substrate utilization for tween 20, α-keto-glutaric acid and mono methyl succinate between the S-Loop 72 mutant and the Sakai control were not significant ($P \geq 0.05$). This suggests that the mutant did not gain or lose the ability to use these substrates as carbon sources. In the case of L-glutamine, the SL 72 mutant did use significantly higher ($P < 0.05$) levels of L-glutamine than Sakai suggesting that the SL 72 mutant did gain the ability to use L-glutamine as a carbon source.

Significant differences ($P < 0.05$) in levels of substrate utilization also were seen between the SL 72 mutant and the Sakai parent strain (Figure. 3.46). The SL 72 mutant utilized significantly higher levels of 11 of the 12 substrates; *i.e*., L-arabinose, N-acetyl-D-glucosamine, L-proline, D-mannose, glucose-6-phosphate, α-D-glucose, maltose, 2-deoxy adenosine, glycyl-L-aspartic acid, fumaric acid and glycyl-L-proline and decreased levels of L-alanine compared to the Sakai parental control ($P < 0.05$). Percentage differences in utilization for each of these substrates were 34.7%, 37.5%, 25.4%, 29.3%, 16.0%, 38.4%, 12.8%, 18.6%, 19.5%, 28.9%, 43.5% and 7.8% respectively.

In summary, the only significant difference in substrate utilization was for the substrate L-glutamine. The SL 72 deletion mutant was above the threshold value for L-glutamine and the parental strain Sakai was below the threshold for this substrate. The use of L-glutamine suggests that this island could have a role in metabolism, most likely in regulating metabolism. Also the differences seen in level of substrate utilization where



SOURCE	L-arabinose	N-acetyl-D-glucosamine	L-proline	D-mannose	glucose-6-phosphate	α-D-glucose	maltose	2-deoxy adenosine	glycyl-L-aspartic acid	fumaric acid	L-alanine	glycyl-L-proline
SAKAI CONTROL	0.341	0.394	0.430	0.433	0.753	0.350	0.474	0.709	0.461	0.383	0.563	0.175
S-LOOP 72 MUTANT	0.522	0.631	0.577	0.612	0.896	0.567	0.543	0.871	0.572	0.538	0.519	0.309
PERCENTAGE DIFFERENCE (%)	34.7	37.5	25.4	29.3	16.0	38.4	12.8	18.6	19.5	28.9	7.8	43.5

Figure.3.46. Differences in levels of substrate utilization for the *E. coli* O157:H7 parental strain Sakai and the SL 72 deletion mutant. All values were significantly different ($P < 0.05$). The absorbance mean $\pm$ S.E. for each substrate is shown. Mean values for substrate utilization also are identified at the bottom of the figure.

in almost all the cases the deletion mutant had a higher level of substrate utilization compared to the parental strain Sakai also suggests that this island has a role in regulation/suppression of various processes such as sugar degradation.

3.3.10 Metabolism in *E. coli* DH5α and the O157:H7 SL 72 deletion mutant

Comparison of the SL 72 deletion mutant and the DH5α strain resulted in several of the same differences seen when the Sakai parent and DH5α strains were compared. There were 28 cases where differences were seen between the two comparisons using a T-test. In five cases use of tween 20, tween 40, α-keto-glutaric acid, β-methyl-D-glucoside and maltotriose was not significantly different ($P \geq 0.05$) in DH5α as compared to the SL 72 mutant but, was different when substrate levels in DH5α and Sakai were compared. This suggests that the SL 72 mutant behaved more like DH5α for these substrates than like the parental strain Sakai. The remaining 23 substrates looked at exhibited significant differences in either substrate utilization (+ or -) or level of substrate utilization.

Significant differences ($P < 0.05$) in substrate utilization were found for the two substrates, dulcitol and glucuronamide. The SL 72 deletion mutant was positive (above threshold of <0.100) for use of dulcitol and glucuronamide but, negative (above threshold) for dulcitol and glucuronamide in DH5α (Figure 3.47). It appears that the SL 72 deletion mutant had the ability to use dulcitol and glucuronamide as a carbon source.

Significant differences ($P < 0.05$) in levels of substrate utilization where both strains were positive for a substrate were also seen (Table 3.17). The SL 72 deletion mutant used significantly higher levels L-arabinose, D-galactose, L-aspartic acid, L-proline, D-mannose, D-xylose, D-mannitol, glucose-6-phosphate, D, L-malic acid, D-

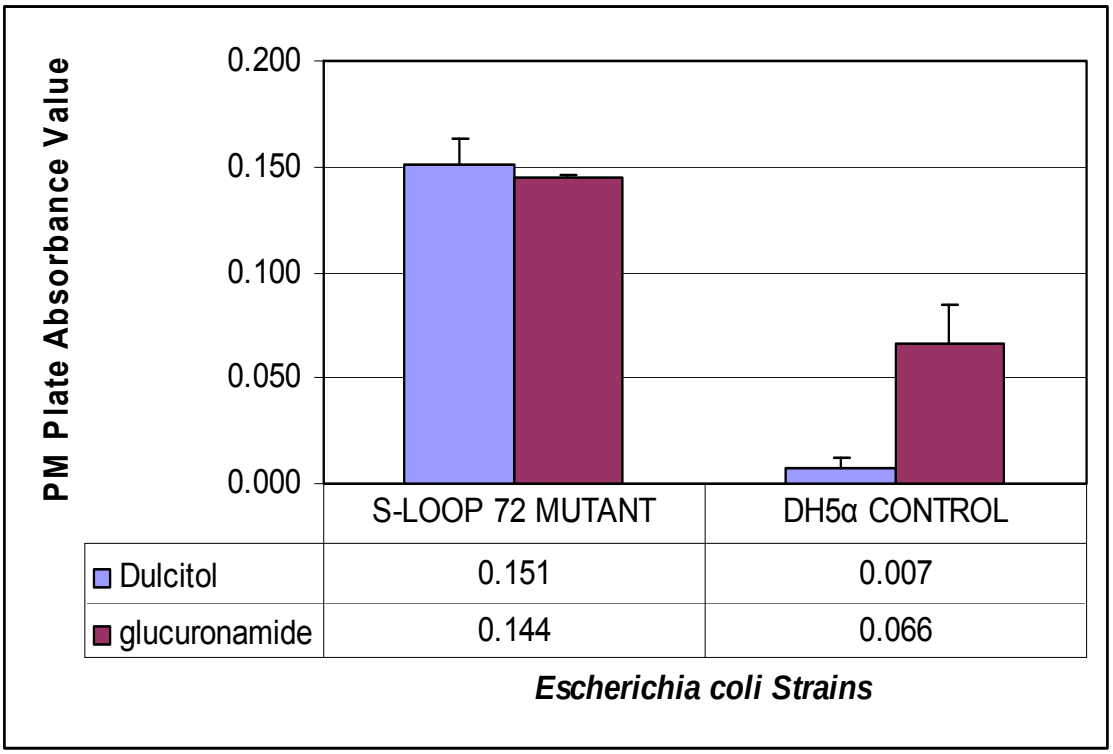


Figure. 3.47 Comparison of *E. coli* O157:H7 SL 72 deletion mutant and *E. coli* DH5α for the substrates dulcitol and glucuronamide. The threshold value for substrate utilization was <0.100. The SL 72 deletion mutant is above the threshold value for dulcitol and glucuronamide and DH5α is below the threshold for these substrates.

Table 3.17. Percentage difference* in levels of substrate utilization for the O157:H7 deletion mutant SL 72 and the *E. coli* DH5α strain.

# of Substrate	SOURCE	SL 72 mutant	DH5α Control	Percentage Difference (%)
2	L-arabinose	0.522	0.289	44.7
6	D-galactose	0.523	0.297	43.2
7	L-aspartic acid	0.616	0.412	33.2
8	L-proline	0.577	0.265	54.1
11	D-mannose	0.612	0.350	42.7
20	D-xylose	0.642	0.303	52.8
23	D-mannitol	0.635	0.457	28.0
25	glucose-6-phosphate	0.896	0.607	32.2
27	D,L-malic acid	0.583	0.382	34.4
31	D-fructose	0.647	0.420	35.0
33	α-D-glucose	0.567	0.290	48.9
35	D-melibiose	0.688	0.405	41.2
48	Uridine	0.706	0.527	25.4
51	glucose-1-phosphate	0.823	0.545	33.8
52	fructose-6-phosphate	0.786	0.615	21.7
61	glycyl-L-aspartic acid	0.572	0.415	27.5
68	mucic acid	0.655	0.420	35.9
75	L-serine	0.573	0.373	34.9
80	N-acetyl-β-D-mannosamine	0.363	0.242	33.4
93	L-galactonic acid-γ-lactone	0.581	0.221	61.9
94	D-galacturonic acid	0.750	0.582	22.4

*All values were significantly different ($P < 0.05$).

fructose, α-D-glucose, melibiose, uridine, glucose-1-phosphate, fructose-6-phosphate, glycyl-L-aspartic acid, mucic acid, L-serine, N-acetyl-β-D-mannosamine, L-galactonic acid-γ-lactone and D-galacturonic acid compared to DH5α. Percentage differences in utilization for each of these substrates were 44.7%, 43.2%, 33.2%, 54.1%, 42.7%, 52.8%, 28.0%, 32.2%, 34.4%, 35.0%, 48.9%, 41.2%, 25.4%, 33.8% 21.7%, 27.5%, 35.9%, 34.9%, 33.4%, 61.9% and 22.4% respectively (Table. 3.17).

3.4 Discussion

Identification of microorganisms traditionally has been done by assessment of the phenotypic properties of species, in particular for their ability to grow on selective media containing nutrients needed for growth. Broad scale phenotypic profiling of bacteria allows refinement of this approach so that even sub-groups of a species can be indentified based upon groups of nutrients utilized; *i.e.,* identification of their metabolic profile (Bochner *et al.* 2008; Bochner 2009). The metabolic profile of a bacteria is determined by the DNA that bacteria possesses and that in turn is influenced by the environment that bacteria is isolated from and the selective pressures that bacteria has encountered (Lang, 2005). Research looking at metabolic profiles from different bacteria has shown that most individuals within a species utilize a narrow group of core substrates specific to the species which can allow for differentiation between strains of a species or between mutants of a specific strain (Funchain *et al.* 2000; Tracy *et al.* 2002; Lang 2005). These differences in species or between strains of the same species could be niche specific or due to selective pressures that species has encountered (Lang , 2005).

Core substrates utilized by *E. coli* K-12 MG1655 for PM plate #1 (PM1) have been identified (www.biolog.com). The PM1 plate contains 95 substrates and MG1655 utilizes 65 (68.4%) of the substrates. In this thesis study, the *E. coli* strains used 84.8% $\pm$ 3.0% of the same substrates as MG1655 (Table 3.18). In a different study by Cooper *and* Lenski (2000), they compared a strain of *E. coli* B to 12 populations generated from this original strain using Biolog ES plates after specific generation numbers were reached (2,000, 10,000 and 20,000). They looked at differences in catabolic function of 64 informative substrates and found that after 10,000 generations there was a decay in the

Table 3.18. The percentage of substrates on the PM1 plate utilized by each of the *E. coli* strains (13 O157:H7 deletion mutants and 3 controls O157:H7 EDL933 and Sakai and K-12 DH5α) in this study in comparison to the 65 core substrates utilized on the PM1 plate by the *E. coli* strain MG1655.

STRAINS	Percentage Substrate Utilization for Core Substrates on PM1 Compared to MG1655
DH5α	87.7% (57)*
EDL933	84.6% (55)
SAKAI	83.1% (54)
OI-39	84.6% (55)
OI-51	84.6% (55)
OI-55	84.6% (55)
OI-59	84.6% (55)
OI-87	84.6% (55)
OI-89	84.6% (55)
OI-98	87.7% (57)
OI-102	86.2% (56)
OI-108	84.6% (55)
OI-135	84.6% (55)
OI-172	81.5% (53)
OI-176	86.2% (56)
SL 72	83.1% (54)

*note: The number in brackets is the number of substrates out of the possible 65 core substrates utilized by MG1655 that each of the strains in this study utilized which was used to calculate the percentage of substrate utilization by the strains in comparison to MG1655.

ability to use 16 of the 64 informative substrates in the 12 populations in comparison to the ancestral strain (*E. coli* B). Tracy *et al.* (2002) used PM1 and PM3 plates to compare 21 archival strains of *Salmonella typhimurium* to nine non-archival *S. typhimurium* strains including strain 2004 that was sequenced. The strain 2004 utilized 86 of the 95 substrates of the PM1 plate and the archival strains were found to have differences in utilization for some of these specific 86 substrates; e.g., sucrose was found to be utilized by only five archival strains and α-D-lactose was only utilized by 6 archival strains. These studies show the same type of results as my findings since in both cases there are differences in substrate utilization between either populations generated from an ancestral strain or between strains compared to an ancestral strain of the same species.

Glycolysis is a metabolic pathway known to be active in enteric bacteria such as *E. coli* (Lodish *et al*. 2000) and is thought to be involved in colonization of the intestine of animals by these bacteria (Miranda *et al*. 2004). In my thesis, three substrates and the product of the glycolytic pathway were examined for their ability to be used by both a laboratory control, *E. coli* DH5α as well as by clinical isolates and deletion mutants of the human pathogen *E. coli* O157:H7: *i.e*., utilization of glucose-6-phosphate, α-D-glucose, fructose-6-phosphate and pyruvic acid were examined. All four of these substrates were used by each of the O157:H7 mutants, the O157:H7 controls and the *E. coli* K-12 reference strain DH5α **(**Figure. 3.5, results**).**

Verification of this well known substrate utilization pattern with the Biolog^{TM} system was important because it validates the approach used in this study; e.g., differences in substrate utilization in the O157:H7 deletion mutants and controls were compared.

Additional research into substrate utilization in bacteria suggests that in addition to core substrates, bacterial isolates from unique environments are likely to use a specialized group of substrates highly specific to the environment that they have been isolated from (Bochner 1989). Most *E. coli* O157:H7 are sorbitol negative after 24 h, dulcitol positive, B-glucuronidase negative, and raffinose positive (Ratnam *et al.* 1988). In this study all the O157:H7 mutants (OI and SL) and both controls, EDL933 and Sakai were tested for two of the biochemical characteristics, sorbitol and dulcitol. All of the O157:H7 mutants and both of the O157:H7 controls were sorbitol negative (Figure. 3.3, results) and dulcitol positive (Figure. 3.2, results) confirming the identified characteristics of O157:H7. The *E. coli* DH5α reference strain had the opposite substrate utilization pattern compared to the O157:H7 strains (sorbitol positive and dulcitol negative).

3.4.1 Comparison of the substrate utilization in laboratory and clinical isolates of *E. coli* DH5α and *E. coli* O157:H7 strains EDL933 and Sakai.

Overall substrate utilization in *E. coli* O157:H7 strain EDL933, Sakai and in *E. coli* K-12 DH5α was similar. There were only 15 cases where differences in substrate utilization occurred in EDL933 and DH5α (Figure. 3.6 and 3.7, results) and only 18 cases in Sakai and DH5α (Figure. 3.8 and 3.9, results). However, there were 68 cases where substrate utilization was significantly increased or decreased relative to the controls, suggesting that despite using similar substrates, how these three strains utilize these substrates was different (Table 3.1 and 3.2, results). In cases where differences in substrate utilization occurred, three are well recognized; *i.e*., D-sorbitol, sucrose and D-serine. When use of the sugar alcohol, sorbitol was looked at in *E. coli* O157:H7, the bacteria were not able to utilize it (Ratnam *et al*. 1988). However, the majority of other

E. coli isolates including K-12 are able to use sorbitol (Mukherjee *et al*. 2006). The PM results for sorbitol confirm this, since *E. coli* O157:H7 strain EDL933 and Sakai were both negative (below the threshold value) for use of D-sorbitol while *E. coli* DH5α was positive (above the threshold value).

Use of sucrose and D-serine is also important since ability of *E. coli* to use these substrates is thought to be environment based (Moritz *and* Welch 2006). It is speculated that because hosts of intestinal pathogens such as O157:H7 have a diet rich in sugars such as sucrose, these pathogens have evolved to use sucrose, but appear to have lost the ability to use other nutrients such as serine. Mukherjee *et al.* (2006) studied expression of these two phenotypes in the O157:H7 strain EDL933 and in *E. coli* K-12; they found that EDL933 was unable to use serine as a carbon source while *E. coli* K-12 could and the reverse was seen for sucrose. In the thesis, results for both of these substrates and the parental strains reflect these findings; *i.e*., both EDL933 and Sakai were positive for sucrose utilization but, negative for serine utilization while the DH5α control was negative for sucrose utilization and positive for use of serine. These results follow the predicted outcomes and also support the theory that intestinal pathogens have evolved to use sucrose but, have lost the ability to use serine since both strains can use sucrose as a carbon source but not serine. Durso *et al.* (2004) compared commensal strains of *E. coli* and various strains of *E. coli* O157:H7 for carbon source utilization. They found differences in utilization between commensal strains and O157:H7. For example, *E. coli* O157:H7 were more likely to use dulcitol, and sucrose than the commensal strains. These results also support my results since it was previously shown that my O157:H7 strains

were positive for both dulcitol and sucrose and DH5α was shown to be negative for these substrates.

The laboratory control, DH5α and the clinical isolates for both EDL933 and Sakai also showed significant differences in levels of substrate utilization supporting the hypothesis that environmental differences between commensal strains and pathogenic bacteria of the same species will result in selection for phenotypic differences among the bacteria that will facilitate their survival within a specific niche. Fabich *et al.* (2008) studied substrate utilization in EDL933 and *E. coli* K-12 and found that there was a difference in sugar affinity between these two strains when they colonized the mouse intestine. EDL933 used three sugars that were not used in *E. coli* strain MG1655. They speculated that these differences allow the O157:H7 pathogen EDL933 to colonize the intestine in the presence of other commensal bacteria. Of the six sugars that EDL933 used for colonization based on the assays performed by Fabich *et al.* (2008) five were tested in this study; *i.e.*, arabinose, fucose, galactose, mannose and ribose. Two of above mentioned sugars, arabinose and fucose, *E. coli* K-12 also uses for colonization. When DH5α and EDL933 were compared in this study, the EDL933 strain used significantly higher levels of each of these five sugars (Table 3.1, results). When DH5α and Sakai were compared, Sakai was also positive for each of the five sugars but, only showed a significant difference in level of utilization for two of the sugars, fucose and ribose (Table 3.2, results). The percentage difference between EDL933 and DH5α for arabinose and fucose was 37.4% and 47.1% respectively, suggesting that EDL933 would outcompete K-12 for use of both these sugars when placed in the same environment due to a higher utilization rate for these sugars than DH5α. Sakai also had a higher level of

utilization for fucose than DH5α with a percentage difference of 37.2%. These results also support the theory put forth by Fabich *et al.* (2008) which suggests that invading pathogens such as O157:H7 adopt a "slash and burn" approach to colonization where maximum growth is achieved by being inflexible and greedy; *i.e.,* both EDL933 and Sakai showed increased levels of utilization for certain sugars relative to the laboratory strain DH5α. This was especially true for the O157:H7 strain EDL933.

3.4.2 Comparison of substrate utilization patterns in different clinical isolates of *E. coli* O157:H7

Substrate utilization in the different clinical isolates of *E. coli* O157:H7, strains EDL933 and Sakai was qualitatively similar. The only difference in substrate utilization between the two strains was for L-glutamine (Figure. 3.10, results); *i.e*., the EDL933 strain was positive (above threshold) while Sakai was negative (below threshold). However, there were 12 cases where significant differences in the quantitative level of substrate utilization were observed (Table 3.3, results).

The PM system is able to distinguish between two strains of the same serotype by finding singular differences in profiles. Mukherjee *et al.* (2008) also identified some variation in substrate utilization in different strains of *E. coli* O157:H7; *i.e*., clinical isolates that were N-acetyl-D-galactosamine (Aga) negative from a spinach outbreak. The majority of O157:H7's are Aga positive and in the reference collection of 120 strains tested only 2 were Aga negative but all the isolates tested from the spinach outbreak were Aga negative, a rare phenotype in O157:H7. The PM system allowed for characterization and identification of this group isolated from the outbreak.

Comparison of the clinical strains also identified cases where there were significant differences in level of utilization of 12 substrates. In each case, the EDL933 strain had a higher level of utilization than Sakai. Three cases where EDL933 had a higher utilization were for sucrose, L-arabinose and α-D-glucose. The ability to use sucrose is thought to be an acquired trait due to selection pressures of the hosts of these bacteria that have sugar rich diets (Moritz *and* Welch 2006). Moritz *and* Welch (2006) found that the O157:H7 strain EDL933 was an avid sucrose fermentor compared to other O157:H7's. L-arabinose and α-D-glucose were shown to be important in colonization studies of the intestine, where L-arabinose was shown to be important in colonizing mouse intestine for both EDL933 and K-12 (Fabich *et al*. 2008); it was also shown that glycolysis is an important pathway involved in colonization, and α-D-glucose is one of the substrates utilized in this pathway (Miranda *et al*. 2004). The percentage difference for these substrates, sucrose, L-arabinose and α-D-glucose was 40.4%, 26.0% and 19.7% respectively. These increases in levels of substrate utilization could confer an advantage to EDL933 and in theory allow the pathogen to reach maximum growth by being more inflexible and greedy; then EDL933 should outcompete Sakai despite both having the same serotype and could possibly be a better pathogen than Sakai. Lim *et al*. (2010) compared 3 wild type strains of *E. coli* O157:H7 as part of their study looking at the effect of the removal of the plasmid O157. They found that there was a difference in substrate utilization for 3 carbon substrates when comparing the 3 wild type O157:H7 strains. Their study showed that O157:H7 strain Sakai grew 3-fold more than the negative control for α-keto-butyric acid, α-hydroxy-butyric acid and L-galactonic acid-g-lactone while the O157:H7 strain WSU180 grew 2-fold more and the O157:H7 strain 43894 only

grew 1-fold more thnn the negative control. This showed that these 3 wildtype strains had differences in level of substrate utilization for the above mentioned 3 substrates. Their results support the results seen in my thesis since in their study they also found differences in level of substrate utilization between O157:H7 strains. Further studies looking at competition between EDL933 and Sakai should be undertaken to elucidate other differences between these two strains and other strains of *E. coli* O157:H7.

3.4.3 Comparison of substrate utilization patterns in *E. coli* O157:H7 strains EDL933 and Sakai with their respective OI and SL deletion mutants for loss or gains in substrate utilization.

The majority of the OI deletion mutants (OI-39, 51,55,59,89, 108, 135 and 176) did not have any gain or loss of function in substrate utilization compared to the EDL933 parental control. Lim *et al*. (2010) created O157:H7 mutants missing the pO157 plasmid. In their study they did not find any differences in carbon source utilization using the PM plates between the mutants and the parental strains. They did find differences using different PM plates. This suggests that looking at these mutants with other PM plates may elucidate possible roles for these mutants. The remaining mutants OI-87, 98, 102 and 172 OI deletion mutants and the SL 72 deletion mutant did have a gain or loss of function compared to the parental strains EDL933 (OI) or Sakai (SL).

The OI-87 deletion mutant gained the ability to use tween 40 as a substrate (Figureure 3.11, results). Tween 40 a detergent, is also known as polysorbate 40 and is a substrate that is known to be a solubilizing agent for membranes and is used in protein chemistry to stabilize proteins (Ayorinde *et al*. 2000; Yao *et al*. 2009). Looking at Table 8.1 (Appendix I), the OI-87 mutant has two ORFs of unknown function; these results do

not specifically clarify the role of this island but suggest areas for further study. This gain in function for utilization of tween 40 suggests that the island could have proteins involved in maintaining membrane integrity or have a role in regulating physical properties of the membrane. Further tests looking at membrane integrity or examining proteins in the membrane should be undertaken to see if this gain in substrate utilization has an effect on these systems.

The OI-98 deletion mutant gained the ability to use L-glutamic acid as a substrate (Figure. 3.24, results). L-glutamic acid is one of 20 amino acids (Newsholme *et al*. 2003). In mammals, such as humans, L-glutamic acid has been shown to have a role in the intestine maintaining gut integrity and promoting cell function (Reeds *et al*. 2000; Newsholme *et al*. 2003). Looking at Table 8.1 (Appendix I), OI-98 has one ORF of unknown function; gain in substrate utilization suggests that the OI-98 mutant could have a role in regulation of L-glutamic acid since the mutant gained the ability to use this substrate as a carbon source. It also suggests that because L-glutamic acid has a role in the intestine of mammals this gain in ability could affect colonization of this mutant and could potentially make it a better pathogen since it adds a carbon source source for this pathogen in the intestine. Further studies looking at colonization of this pathogen may elucidate further roles for this specific OI.

The OI-102 deletion mutant gained the ability to use D-serine and D-threonine as carbon sources but lost the ability to use sucrose as a carbon source (Figure 3.26, results). The ability of O157:H7's to use sucrose as a carbon source and not D-serine has been shown by other research groups (Moritz and Welsh 2006; Murkherjee *et al*. 2006). The OI-102 mutant has the opposite phenotype; it is sucrose negative and D-serine positive.

Looking at Table 8.1 (Appendix I), it is seen that the OI-102 mutant contains ORFs relating to prophages, ORFs of unknown function, a resolvase, a sucrose permease, a fructokinase, a sucrose hydrolase and a sucrose specific transcriptional regulator. Since this OI contains ORFs related to sucrose metabolism the loss in ability to use sucrose by this mutant is not unexpected. The ability to use sucrose as a carbon source is thought to be environment based and is an ability that has evolved in O157:H7's due to the sucrose rich diets of their hosts (Moritz and Welsh 2006). Moritz and Welsh (2006) also theorize that the ability to use serine could be under a strong state of catabolite repression. The results lend support to that theory since by losing the ability to use sucrose the mutant gained the ability to use serine which suggests that the ability to use serine is under repression by the sucrose genes in O157:H7's, in other words OI-102 appears to regulate the use of D-serine and removal of this regulation allows for utilization of this substrate by the deletion mutant. The mutant also gained the ability to use D-threonine as a carbon source, a polar amino acid similar to serine (Sawers 1998). This gain in ability could also be due to suppression of this gene by this OI. Threonine is degraded by threonine dehydratase an enzyme that has the ability to degrade threonine and serine (Sawers 1998). This enzyme is coded for by the *tdc* operon which has been shown to be under catabolite repression by glucose and pyruvate. This suggests that this OI could involved in regulating this operon and removal of this OI removes the suppression and the deletion mutant gained the ability to use threonine and serine. Further tests looking at the ORFs of unknown function could elucidate a role for these ORFs in regulation of serine and threonine metabolism.

The OI-172 deletion mutant gained the ability to use tween 20, M-tartaric acid and lost the ability to use α-ketobutyric acid and α-hydroxybutyric acid (Figure 3.28, results). Tween 20 is a surfactant that is known to be a solubilizing agent for membranes and is used in protein chemistry to stabilize proteins (Ayorinde *et al*. 2000; Yao *et al*. 2009), suggesting that this OI could have proteins involved in membrane integrity or have other roles involving the membrane. M-tartaric acid is an isomer of tartaric acid (Alfredsson *et al*. 1972). α-Keto-butyric acid is a substrate that can be converted to propionyl-CoA (Sawers 1998) and through subsequent conversions can be converted to succinyl-CoA an intermediate in the citric acid cycle (Haller *et al*. 2000). This suggests that the citric acid cycle could be affected by removing a substrate that can eventually be converted to a substrate used in this cycle. α-Hydroxy butyric acid is a derivative of butyric acid (Smith *and* Strang 1958) and butyric acid is one of the principal organic acids present in the intestine (Herold *et al*, 2009). Herold *et al*. (2009) studied the expression of *iha*, an adhesin present in O157:H7 strains and it was shown that the addition of short chain fatty acids such as butyric acid to growth medium strongly induced expression of this adhesin in O157:H7 strain EDL933. This suggests that removal of this island could impact colonization of this bacterium in the intestine. Looking at Table 8.1 (Appendix I)), the OI 172 contains many ORFs of unknown function and a few ORFs thought to be enzymes. Further tests looking at membrane integrity, at the citric acid cycle and/or growth in the intestine should be undertaken to fully determine the role of these ORFs in *E. coli* O157:H7.

The SL 72 deletion mutant gained the ability to use L-glutamine as a carbon source (Figure. 3.45, results). L-glutamine is one of the twenty amino acids and has been

shown to have a role in the intestine in mammals (Reeds *et al*. 2000; Newsholme *et al*. 2003). Interestingly this is also the same difference in substrate utilization pattern seen when comparing the two O157:H7 strains EDL933 and Sakai where EDL933 can also use L-glutamine as a carbon source (Figure. 3.10, results). This suggests that the ability to use L-glutamine could be regulated or repressed by the SL 72 in Sakai but not in the corresponding OI in EDL933 since EDL933 and each of the OI mutants can use L-glutamine as a carbon source. Looking at Table 8.1 (Appendix I), it is seen that the SL 72 has many ORFs of unknown function; e.g., ORFs relating to transposons, tellurium resistance, membrane proteins, urease proteins, adhesive proteins and a transcriptional regulator to name a few. These unknown ORFs, membrane proteins or the transcriptional regulator could possibly explain the gain in ability to use L-glutamine when the SL is removed from Sakai. Further tests would need to be undertaken to fully characterize all of the roles SL 72 has in Sakai.

The PM system has been used successfully to study mutant phenotypes. Erol *et al*. (2006) created an H-NS mutant of *E. coli* O157:H7; H-NS is a protein that plays a role in condensation of chromosomal DNA and therefore can influence the expression of many genes. It is an important regulator of virulence in several pathogens. They used PM plates and found that the H-NS mutant was not able to utilize 42 carbon and 19 nitrogen substrates that the wild type parental O157:H7 metabolized. This was a quick and straight forward way to study this mutant and showed the protein's importance. Another study by Quan *et al*. (2002) compared a *Salmonella cysB* mutant and an *E. coli cysB* mutant to their respective wild types and to each other. Despite having the same role in each species it was found that while for the most part the mutants behaved the same, there were

exceptions such as D-glucuronate where the *Salmonella CysB* mutant showed a decrease in function compared to the wild type while in the case of *E. coli* no decrease was observed between wild type and mutant. My results show the same type of results since for 5 of the mutants, we were able to find differences in substrate utilization that provide insight into the roles of these specific OIs but do not fully elucidate the complete role of the islands and SL. Tohsato *and* Mori (2008) used the PM system to analyze 45 deletion mutants with deletions in glycolysis, TCA cycle and pentose phosphate pathway and compared their mutants to the wild type parent *E. coli* K12 strain BW25113. They found that there were 3 clusters (C1, C2 and C3) of mutants surrounding the pathways. i.e., C1 was located at the beginning of glycolysis, while C2 and C3 represented mutants with up and down regulation in respiration due to their mutation. Overall they found that their study did not elucidate roles for genes of uncertain function and further studies were needed. This is also similar to the results of my thesis data since further studies are needed to fully elucidate the roles of the OIs and the SL studied because only 5 mutants had gains and losses of function and the role of these OIs and SL were not completely clear despite the changes in PM profile.

3.4.4 Comparison of substrate utilization patterns in *E. coli* O157:H7 strains EDL933 and Sakai with their respective OI and SL deletion mutants for significant differences in level of substrate utilization

All of the mutants except for OI-135 had significant differences in the level of substrate utilization observed compared to the parental O157:H7 strains. Most of the mutants had differences in the level of utilization for substrates that are known to have an important role in the bacterium or that had differences common only to a few of the mutants (Table 3.19). The OI-39, 55, 102, 172 and 176 and the SL 72 deletion

Table 3.19. Comparison of the O157:H7 parental strain EDL933 and its OI deletion mutants and O157:H7 parental and its SL mutant for significant differences in level of substrate utilization for substrates that have an important role in the bacterium.

	***E. coli* OI and SL deletion mutants**											
Substrates	OI-39	OI-51	OI-55	OI-59	OI-87	OI-89	OI-98	OI-102	OI-108	OI-172	OI-176	SL 72
α-D-glucose	–	–	–	–	–	–	–	–	–	↑	–	↓
Glucose-6-phosphate	–	–	–	–	–	–	–	–	–	↑	–	↓
Fructose-6-phosphate	↑	–	↑	–	–	–	–	–	–	↑	–	–
Pyruvic acid	↑	–	–	–	–	–	–	↓	–	↑	↑	–
L-arabinose	–	↑	–	–	–	↑	–	↑	–	↑	–	↓
D-galactose	–	–	–	–	–	–	–	–	–	↑	–	–
D-ribose	–	↓	–	–	↓	↓	–	–	–	↑	–	–
Thymidine	–	↓	↓	↑	↓	–	–	–	–	–	–	–
D-xylose	–	–	–	–	–	–	–	↑	↓	↑	↑	–
succinnic acid	–	–	–	–	–	–	–	–	↓	↑	–	–
L-rhamnose	–	–	–	–	–	–	↑	–	–	–	–	–

Note: – depicts substrates for which that particular mutant and the parental strain EDL933 (OI) or Sakai (SL 72) do not have a significant difference in level of substrate utilization.

↑ depicts substrates for which EDL933 or Sakai has a higher level of substrate utilization compared to its deletion mutant.

↓ depicts substrates for which EDL933 or Sakai has a lower level of substrate utilization compared to its deletion mutant.

Note: The OI deletion mutant OI-135 was not included in this table because it was shown to not have any significant differences in level of substrate utilization in comparison to EDL933

mutants have significant differences in level of substrate utilization for substrates involved in glycolysis. Glycolysis is a pathway known to occur in bacteria (Lodish *et al*. 2000) and is thought to have a role in growth of *E. coli* in the intestine of animals such as mice (Miranda *et al*. 2004). Miranda *et al.* (2004) studied colonization of *E. coli* O157:H7 strain EDL933 and *E. coli* K-12 strain MG1655 in mice and found that when EDL933 was the sole bacterium to colonize the mice it utilized glycolytic substrates for growth and survival. When in the presence of MG1655 in the intestine, EDL933 used glycolytic substrates for rapid growth for one day suggesting that it either out-competed MG1655 for the glycolytic substrates or that MG1655 used alternate substrates during that time period. The results from this study show that there are differences in level of substrate utilization for substrates that are part of the glycolytic pathway. Two of the mutants OI-102 and the SL 72 had a higher utilization rate compared to EDL933 and Sakai suggesting that they may give the bacteria an advantage in colonization of the mouse intestine. The remaining mutants OI-39, 55, 172 and 176 had a reduced rate of utilization compared to EDL933 suggesting they may not be as effective in colonizing the mouse intestine.

The OI-51, 87, 89, 102 and 172 and the SL 72 have differences in level of substrate utilization for one or more of the following, L-arabinose, D-galactose and D-ribose. These sugars have also been shown to have a role in growth of O157:H7 strain EDL933 in the intestine (Fabich *et al*, 2008). Two of the above mentioned sugars D-galactose and D-ribose have been shown by Fabich *et al*. (2008) to be used exclusively by EDL933 compared to *E. coli* K-12 MG1655. The OI-51, 89, 102 and 172 deletion mutants have a reduced level of utilization for one or more of these sugars, suggesting

that they may not be as effective in colonizing or maintaining growth in the intestine compared to EDL933. The OI-51, 87, 89 and the SL72 deletion mutants have an increased level of utilization for these sugars suggesting that they could have an advantage in colonization or maintenance of growth levels in the intestine compared to EDL933 (OI deletion mutants) or Sakai (SL deletion mutant). Two of the mutants OI-51 and 59 have increases and decreases in substrate utilization depending on the sugar suggesting that these mutants may have defects affecting only colonization or maintenance of growth levels in the intestine.

Four of the mutants OI-51, 55, 59 and 87 had a different level of substrate utilization for thymidine in comparison to EDL933. Thymidine is a DNA nucleoside and is needed/used in DNA replication (Moran *et al*. 1997). The OI-59 mutant had an increased level of utilization while the other 3 mutants exhibited a decrease in level of utilization. The change in substrate utilization level for thymidine could have an impact on processes such as DNA replication.

The OI-102, 108, 172 and 176 deletion mutants exhibited differences in level of substrate utilization for D-xylose. D-xylose is a sugar that can be used by *E. coli* as a carbon source (Hernandez-Montalvo *et al*. 2001; Watanabe *et al*. 2008). Hernandez-Montalvo *et al*. (2001) studied sugar consumption of glucose, arabinose and xylose for a mutant strain of *E. coli* and compared it to the parental strain. Overall they found that the parental strain's metabolism of arabinose and xylose was repressed by the presence of glucose and that in a mixed sugar medium glucose was metabolized first, followed by arabinose and then xylose. Three of the deletion mutants OI-102, 172 and 176 showed a decrease in xylose utilization compared to EDL933 suggesting that xylose metabolism

could be upregulated by these OIs. The OI-108 mutant exhibited higher utilization for D-xylose which could suggest a possible loss of repression for this substrate by removal of this OI. Studies of sugar mixtures with these mutants should be undertaken to see if repression of D-xylose is affected by glucose for these islands; since the Biolog system only tests one substrate at a time, we cannot determine if repression by glucose is affected in these mutants.

The OI-108 and 172 deletion mutants also saw effects on the level of substrate utilization for succinic acid. Succinic acid is a substrate that has a role in the citric acid cycle (Lodish *et al.* 2000). The OI-108 exhibited increased utilization for this substrate while the OI-172 mutant exhibited decreased utilization for succinic acid. The decrease in utilization in OI-172 could be due to the loss of function of α-keto butyric acid, which has been shown to have a role in the citric acid cycle. Further tests looking at the citric acid cycle for these mutants could elucidate roles for these OIs that could show a role in regulation of this process. The OI-98 mutant only exhibited one difference in substrate utilization in comparison to EDL933. The OI-98 mutant had a decrease in utilization for L-rhamnose. L-Rhamnose is a sugar that can be metabolized into dihydroxyacetone phosphate which can be metabolized in glycolysis (Wegerer *et al*. 2008). This suggests that under specific conditions the glycolysis pathway could be affected by the decrease in substrate utilization by this OI.

The PM method has previously been shown to be able to find differences in how substrates are utilized between mutants and parental strains. Koo *et al*. (2004) compared a mutant *E. coli* K12 strain BM101 to the parental K12 strain MG1655 and found that there were no differences in substrate utilization between the two strains. However, they found

that the mutant showed faster color development than the parental strain. This supports my results since the majority of the OI deletion mutants did not show any differences in substrate utilization but did exhibit differences in level of utilization for substrates that are important in this bacterium. All of the mutants except for the OI-135 deletion mutant exhibited differences in level of substrate utilization which is similar to the outcome seen in the study done by Koo *et al*. (2004).

3.4.5 Comparison of substrate utilization patterns in *E. coli* O157:H7 deletion mutants with the *E. coli* K-12 reference strain DH5α for significant differences in level of substrate utilization

All of the deletion mutants showed similarities and differences in level of substrate utilization relative to the *E. coli* K-12 reference strain DH5α (Table 3.20). However, the OI-39, 108 and 135 deletion mutants did not show a significant difference in substrate utilization relative to DH5α (compared to EDL933 and DH5α) for substrates involved in glycolysis, a pathway thought to have a role in colonization of the mouse intestine (Miranda *et al*. 2004). Glycolysis has been shown to have a crucial role in colonization of the mouse intestine in *E. coli* K-12 MG1655 (Chang *et al*. 2004).

The OI-51, 55, 59, 87, 89, 172 and 176 deletion mutants did not show a significant difference in substrate utilization for substrates involved in glycolysis and other sugars shown to be important in colonization of the mouse intestine (Miranda *et al*. 2004; Fabich *et al*. 2008). The OI-172 mutant and the SL 72 mutant also behaved like DH5α for succinic acid (OI-172) and α-keto glutaric acid (SL 72), substrates with roles in the citric acid cycle (Lodish *et al.* 2000). These mutants appeared to behave like the *E. coli* K-12 reference strain DH5α for these substrates.

Table 3.20. Comparison of the *E. coli* K-12 DH5α and the OI and SL deletion mutants for significant differences in level of substrate utilization for substrates that have an important role in the bacterium.

	***E. coli* OI and SL deletion mutants**												
Substrates	OI-39	OI-51	OI-55	OI-59	OI-87	OI-89	OI-98	OI-102	OI-108	OI-135	OI-172	OI-176	SL 72
α-D-glucose	–	*	–	*	–	*	–	–	–	*	*	*	–
Glucose-6-phosphate	*	–	–	–	–	–	–	–	–	–	*	*	–
Fructose-6-phosphate	*	*	*	*	*	*	*	*	*	*	*	*	–
L-arabinose	–	*	*	*	*	–	–	–	–	–	–	–	–
D-galactose	–	–	–	–	–	*	–	–	–	–	*	*	–
succinnic acid	–	–	–	–	–	–	*	–	–	–	*	–	–
L-glutamic acid	–	–	–	–	–	–	*	–	–	–	–	–	–
D-serine	–	–	–	–	–	–	–	*	–	–	–	–	–
Sucrose	–	–	–	–	–	–	–	*	–	–	–	–	–
α-keto-glutaric acid	–	–	–	–	–	–	–	–	–	–	–	–	*
# of cases no longer significantly different	**7**	**5**	**2**	**3**	**3**	**7**	**9**	**8**	**7**	**6**	**20**	**7**	**5**

Note: – depicts substrates for which that particular mutant and the DH5α do not have a significant difference in level of substrate utilization.

* depicts a substrates for which there is no longer a significant difference in level of substrate utilization between the mutant and the DH5α strain.

Shading depicts substrates which are gains in function in the PM profile of the mutant in the EDL933 comparison that are no longer different in the DH5α comparison.

The OI-98 and 102 mutants also showed similarity to the DH5α strain for substrates involved in glycolysis and the citric acid cycle. However, these mutants also behaved like the reference strain DH5α for L-glutamic acid (OI-98) and D-serine and sucrose (OI-102). These were gains in function by the removal of the OI seen when comparing the mutant to EDL933; these gains in function model the *E. coli* K-12 reference strain DH5α.

These cases for each mutant where we no longer see differences in substrate utilization for the mutant and DH5α comparison mean that the mutant is behaving more like the reference strain for utilization of these substrates than the pathogenic strains EDL933 or Sakai. Also each mutant was similar in substrate utilization for substrates known to have an important role in processes such as glycolysis. This lends support to the theory that removal of a PAI from a pathogen can result in bacteria that behave more like a reference strain.

3.4.6 Future tests and Final conclusions

The PM method has a wide range of uses and can pick up on differences between bacteria in a quick and straight forward fashion (Potera 2006) as seen with our results. In our study we gathered valuable information on the effect of removal of specific OIs and SLs from O157:H7 but, we still lack sufficient information to make conclusive statements about the nature of genes on these blocks of DNA. More tests with different PM plates or tests targeting specific gene functions may give us insight into the roles that these OI's and SLs play in *E. coli* O157:H7. Differences in levels of substrate utilization were often for substrates such as L-arabinose or fructose-6-phosphate which have been shown to have a role in colonization of the gut by bacteria such as *E. coli* (Fabich *et al*.

2008; Miranda *et al*. 2004). Further tests looking at gut colonization of the OI and SL deletion mutants could reveal other properties of these mutants relating to pathogenesis of the bacteria.

Chapter IV

Cell Growth in *E. coli* O157:H7

4.1 Introduction

EHEC forms an important class of STEC that are intestinal pathogens that have potential to cause severe infections such as haemolytic uremic syndrome (HUS), hemorrhagic colitis (HC) and can lead to complications such as seizures and even coma (Cleary 2004; Karch *et al.* 2005). One of the most important serotypes found in EHEC is *E. coli* O157:H7, as it typically associated with exceptionally virulent strains (Cleary 2004). *E. coli* O157:H7 is involved in many outbreaks and is the predominant strain found in outbreaks in North America (Yoon and Hovde 2008). Ingestion of contaminated meat and unpasteurized milk represents one of the main ways that EHEC pathogens enter the food chain (Karch *et al.* 1999). Contamination of processed meat products such as ground beef has been responsible for various outbreaks of *E. coli* O157:H7 including an outbreak in France in 2005 (King *et al*. 2009) and in the state of Washington in the USA in 1992 and 1993 (Bell *et al.* 1994). In both cases the source was frozen hamburger patties. *E. coli* O157:H7 outbreaks have also occurred by contamination of fresh produce like lettuce, berries and bean sprouts (Sivapalasingam *et al.* 2004).

Growth of *E. coli* O157:H7 is an important factor in its survivability and pathogenecity and has been studied under different conditions (Coleman *et al*. 2003). Conner and Kotrola (1995) studied growth of *E. coli* O157:H7 under acidic conditions at different temperatures. Their preliminary studies found that as temperature was increased, growth under acidic conditions (a lower pH) also increased. They also found that the *E.*

coli O157:H7 strains were susceptible to mandelic acid and that lactic and acetic acid did not seem to affect growth of the cells. Ding *et al*. (2010) examined growth of *E. coli* O157:H7 on beef patties that were untreated, or treated with high or low levels of acidic electrolyzed oxidizing water at different temperatures. The study showed that growth on beef patties treated with acidic electrolyzed oxidizing water or with slightly acidic electrolyzed oxidizing water had lower numbers of bacteria growing on the patties than the control (untreated patty) and it took bacteria growing on the acidic patties (treated) longer to arrive at the stationary phase of the curve. Ukuku *et al*. (2009) studied growth of *E. coli* O157:H7, *Salmonella spp.*, and *L. monocytogenes* in apple cider supplemented with the anti-bacterial peptide, nisin plus EDTA at various temperatures. *E. coli* O157:H7 was found to have a shorter lag phase in non sterile apple cider which suggests that the bacteria can survive and grow better in an un-sterilized environment.

The ability to grow on various food surfaces and in varying growth conditions is most likely related to bacterial virulence and the virulence genes and factors found on PAIs in these bacteria (Ferianc *et al*. 2002). PAIs are important since they add DNA to pathogenic bacterial genomes that, is not found in commensal strains. PAIs are characterized as large chunks of DNA that contain genes relating to virulence, are usually surrounded by insertion sequences and often contain genes related to transposases and integrases found on the island (Hacker *et al*. 1997). Identification of differences in growth of bacteria containing different PAIs could help clarify the contribution of PAIs to development of pathogenesis in bacteria.

In this study, growth curves for each of three controls (*E. coli* DH5α and O157:H7 strains Sakai and EDL933) plus 13 PAI deletion mutants grown in LB broth were compared. Specific objectives of the study were to:

1) Create growth curves for each of the mutants and the three controls.

2) To compare O157:H7 strains EDL933 and Sakai to the reference *E. coli* K12 strain DH5α.

3) To compare the OI deletion mutants and an SL deletion mutant to the reference *E. coli* K12 strain DH5α and the respective parental O157:H7 strains EDL933 or Sakai

4.2 Materials and Methods

4.2.1. Bacteria and Cultures

In this experiment *E. coli* O157:H7 strains EDL933 and Sakai were compared to the reference *E. coli* K-12 strain DH5α and to their respective *E. coli* O157:H7 OI and SL deletion mutants. The deletion mutants were also compared to the reference *E. coli* K-12 strain DH5α. All bacterial strains were grown on LB agar overnight. A single colony from each of the following strains: 3 controls (EDL933, Sakai and DH5α) and the 13 deletion mutants were streaked on LB Agar and grown overnight (12-16 h) at 37 ºC. This step was repeated twice to ensure pure colonies and to ensure the bacterial strains were in the same physiological state. After the final incubation a single colony from each plate was used to inoculate a 250 ml Erlenmeyer flask containing 25 ml of pre-warmed LB broth. These flasks were then incubated for 12-13 h at 37 ºC in a shaking incubator set at 220 rpm.

4.2.2. Preparing the cultures for the Growth curve assay and sample sampling.

The cultures for the growth curve were made by removing 1 ml from the overnight cultures and adding this to a 250 ml Erlenmeyer flask containing 99 ml of pre-warmed LB broth. This flask was then incubated at 37 ºC in a shaking incubator set at 220 rpm. Samples were taken at time equals zero and every hour for the first two hours of the assay and every half an hour for the remaining 12 hours of the assay. At each time point a 1 ml sample was removed from the flask and inserted into a clean cuvette in order to obtain a spectrophotometer reading at an OD of 600 nm. As the OD reading approached the limits of the spectrophotometer (*i.e.*, an OD reading of 1.000) a 1/10

dilution of the sample was made by removing a 100 µl sample from the flask and mixing it with 900µl of LB broth. A reading for the diluted sample was taken as described above. This process was repeated three times with three different cultures.

4.2.3. Growth curve analysis

All data collected was inserted into an Excel (Microsoft Office) spreadsheet. Growth curves were generated by plotting the OD_{600} data versus time. The data was analyzed using an SPSS statistical program (SPSS Inc, IBM, Chicago). Curves generated were analyzed using a linear regression. The linear regression for the linear part of the growth curves for each OI and SL deletion mutant and the three controls (DH5α, EDL933 and Sakai) was calculated using the model: Y = Bo + BiX, where Y = OD, Bo = the intercept, Bi = the slope and X = time. The equations generated were then compared using a univariate analysis.

4.3 Results and Discussion

Bacterial growth curves, including those for *E. coli* typically are sigmoidal in shape (Coleman *et al.* 2003); each curve possesses an initial lag phase in which the inoculated culture prepares to grow, a linear portion or log phase in which the slope of the line is indicative of the growth rate for the bacteria and, a plateau or stationary phase where the culture reaches a maximum cell density allowed by available nutrients found in the growth medium. In this study, careful pre-treatment of bacterial cultures to help ensure that they were in a similar physiological state at the beginning of each experiment and exact replication of the growth conditions used during experimental trials, allowed accurate duplication of the growth curves generated with little variation from one experiment to next; e.g., standard error for the log phase slope varied from $\pm$ 0.003 to $\pm$ 0.014 in the 16 growth curves examined (Table 4.1 and Table 4.2). This approach allowed a detailed comparison of the growth curves generated for each of the bacterial strains examined in order to determine if deletion of PAIs from *E. coli* O157:H7 can affect ability of the bacteria to grow.

4.3.1. Growth of OI and SL deletion mutants relative to their O157:H7 parents EDL933 and Sakai.

All growth curves generated in the study were unique to the specific strains examined; *i.e.*, the curve for each strain fit a linear equation (Table 4.1 and Table 4.2) with a specific slope. Analysis of these curves showed that nine of the OI mutants (OI-39, 51, 59, 89, 98, 108, 135, 172 and 176) had the same rate of growth (slope) as the EDL933 parental control (Table 4.1); *i.e.*, removal of these PAIs from the parental strain EDL933 did not affect growth rate of the cells. Of the remaining OI mutants, two (OI-55

Table 4.1. Linear regression of the growth curve data for the 12 OI deletion mutants, the O157:H7 parental strain EDL933 and the *E. coli* K-12 reference strain DH5α. The regression model used was $Y = b_o + b_iX$, where Y= OD, b_o identifies the y intercept, b_i is the slope and, X is time.

Strain	N	Slope*	Standard Error for Slope
DH5 (control)	54	0.320 [b, x]	± 0.014
EDL933 (parent)	340	0.358 [a, y]	± 0.003
OI-39 mutant	54	0.349[a, x]	± 0.004
OI-51 mutant	54	0.352 [a, y]	± 0.006
OI-55 mutant	54	0.339 [b, x]	± 0.004
OI-59 mutant	54	0.356 [a, y]	± 0.005
OI-87 mutant	54	0.339 [b, x]	± 0.003
OI-89 mutant	54	0.354 [a, y]	± 0.003
OI-98 mutant	54	0.353 [a, y]	± 0.006
OI-102 mutant	54	0.379[b, y]	± 0.004
OI-108 mutant	54	0.355 [a, y]	± 0.005
OI-135 mutant	54	0.353 [a, y]	± 0.004
OI-172 mutant	54	0.345 [a, x]	± 0.005
OI-176 mutant	53	0.358 [a, y]	± 0.005

* The first letter of the superscript identifies significant differences ($P<0.05$) from the parental strain *E. coli* O157:H7 EDL933 where a = same and b = different; the second letter identifies significant differences ($P<0.05$) from the reference strain *E. coli* DH5α, where x = same and y = different. N corresponds to the number of OD readings.

Table 4.2. Linear regression of the growth curve data for the SL deletion mutant, the O157:H7 parental strain Sakai and the *E. coli* K-12 reference strain DH5α. The regression model used was $Y = b_o + b_iX$, where Y= OD, b_o is the intercept, b_i is the slope and, X is time.

Strain	N	Slope*	Standard Error for Slope
DH5α (control)	54	$0.320^{b, x}$	± 0.014
Sakai (parent)	108	$0.367^{a, y}$	± 0.006
SL 72 mutant	54	$0.345^{b, x}$	± 0.006

* The first letter of the superscript identifies significant differences ($P<0.05$) from the parental strain *E. coli* O157:H7 EDL933 where a = same and b = different; the second letter identifies significant differences ($P<0.05$) from the reference strain *E. coli* DH5α, where x = same and y = different. N corresponds to the number of OD readings.

and OI-87) had a significantly slower growth rate ($P<0.05$) than EDL933 and, one (OI-102) had a significantly higher growth rate ($P<0.05$) than the parent strain. The OI 55 contains ORFs of unknown function and ORFs that have a role in transport. The change in growth rate seen by the OI-55 deletion mutant may be related to the removal of the transport proteins which could result in a mutant that is not able to transport molecules as effectively as the wildtype parental strain which could result in the slower growth rate seen by this deletion mutant. The OI-87 contains an ORF of unknown function and an unclassified ORF, the results suggest that these ORFs contribute the growth of *E. coli* O157:H7 since the removal of this OI results in a mutant that does not grow as effectively as the wildtype parental strain in rich medium. The OI-102 contains ORFs of unknown function, as well as ORFs coding for putative prophage DNA injection proteins, an ORF coding for a putative resolvase, a sucrose permease, a D-fructokinase, a sucrose hydrolase and a sucrose specific transcriptional regulators. The OI-102 deletion mutant is also unable to use sucrose as a carbon source (Biolog results, Figure 3.26, pg.75) and this loss of ability could also affect its growth rate since the change in carbon utilization of the bacterium could change the order *E. coli* O157:H7 consumes sugars and these sugars could be easier to degrade than sucrose allowing the bacterium to replicate faster in rich medium. In *E. coli* O157:H7 strain Sakai, removal of SL 72 produced a PAI deletion mutant that grew significantly slower ($P<0.05$) than the parent (Table 4.2). The SL 72 contains ORFs coding for hypothetical proteins, ORFs related to tellurium resistance, ORFs relating to urease synthesis, as well as, ORFs coding for transposases and adhesins, ORFs related to synthesis of membrane proteins, ORFs coding for regulatory proteins and transcriptional regulators. The slower growth rate of the SL 72 deletion mutant

suggests that this SL has an important role in growth of *E. coli* O157:H7 strain Sakai. This change in growth could be due to the loss of the regulatory proteins or the transcriptional regulators that are coded for on this PAI.

Survival strategies of bacteria grown under different environmental conditions have been examined by Roszak and Colwell (1987). They observed that typical laboratory media have higher concentrations of nutrients than most natural environments. For example most nutrient media used in the laboratory have ~2 g or more of carbon per liter while the environmental conditions examined in their studies had only ~1-15 mg carbon per liter. They suggested that bacteria grown under lower nutrient conditions likely would show more variation in growth than those grown under optimal conditions. In contrast, Vebø *et al*. (2010) looked at the growth curves from bacterial strains from different origins that were grown in urine (a relatively rich medium) and did not find any significant differences in growth. This suggests that the relatively low number of differences seen between the OI mutants examined in this study and the EDL933 parent might be the result of using LB, a complete bacterial growth medium with lots of nutrients. If true, the deletion mutants examined in this study might have shown more differences in growth patterns compared to the parental strains if growth of the bacteria was compared in minimal and complete media.

4.3.2. Growth of OI and SL deletion mutants relative to the reference *E. coli* K12 strain DH5α.

Comparison of *E. coli* O157:H7 strain EDL933 and Sakai with the reference *E. coli* K12 strain DH5α showed that both pathogenic O157:H7 strains grew significantly faster ($P<0.05$) than the reference DH5α strain (Table 4.1 and Table 4.2). Similarly, eight

of the 12 OI deletion mutants (OI-51, 59, 89, 98, 102, 108, 135 and 176) grew significantly faster ($P<0.05$) than the reference DH5α strain (Table 4.1); *i.e*., these deletion mutants grew similar to the parental O157:H7 strain EDL933. However, deletion of PAIs from OI-39, 55, 87 and 172 from O157:H7 strain EDL933 produced a growth rate in these strains that was not significantly different ($P\geq0.05$) from the reference *E. coli* K12 strain DH5α. Similarly, deletion of SL 72 from *E. coli* O157:H7 strain Sakai resulted in production of a deletion mutant with a growth rate that was not significantly different ($P\geq0.05$) from the reference *E. coli* K12 strain DH5α (Table 2). It appears that removal of some PAIs from *E. coli* O157:H7 results in production of bacteria that grow in a manner more characteristic of the reference strain.

Baba *et al.* (2006) created single gene knock out mutants for all of the non-essential genes found in *E. coli* K-12 strain BW25113 and compared growth of these mutants on minimal and rich media. They were able to classify their mutants into three distinct groupings; *i.e*., mutant strains that grew well on minimal medium, mutant strains that grew well in rich medium and mutant strains that grew well in both types of media. In this study, growth rates for the PAI deletion mutants also were divided into groups (two) indicating that some PAIs are capable of increasing and decreasing the growth rate of *E. coli*. Moreover, comparison of these changes in growth rate to that of the reference *E. coli* strain DH5α suggests that removal of some PAIs from O157:H7 results in a mutant that grows more like the reference strain then the parental O157:H7 strain..

Roos *et al*. (2006) compared the growth of asymptomatic bacteriuria (ABU) *E. coli* strains to uropathogenic *E. coli* strain CFT073 for differences in adhesion and

growth. Results from their experiments showed, that differences in growth between nine ABU *E. coli* strains and the uropathogenic *E. coli* strain CFT073 existed. Two of the ABU *E. coli* strains had higher growth rates than the pathogenic CFT073 strain and the remaining seven ABU strains had lower growth rates than the pathogenic CFT073 *E. coli* strain. They showed that differences in growth rate exist among strains of the same species. This result is similar to results from my experiments where different growth rates were seen for mutant strains originating from the same O157:H7 isolate.

4.3.3. Influence of PAIs on bacterial cell density at stationary phase

Comparison of the maximum cell density obtained at stationary phase by cultures of *E. coli* O157:H7 strains EDL933 and Sakai with the reference *E. coli* K12 strain DH5α showed that both O157:H7 strains reached a higher cell density when grown in LB medium than the reference *E. coli* strain DH5α (Figure. 4.1 and Figure. 4.2); *i.e.*, the O157:H7 strains EDL933 and Sakai reached a maximum cell density at an OD_{600} of 4.2 and 4.6 respectively cv. 3.7 for the reference DH5α strain. This also was true for the O157:H7 PAI deletion mutants. When the maximum cell density reached during growth of the PAI deletion mutant cultures was compared to the maximum cell density reached by the reference strain DH5α, the maximum cell density reached by all of the O157:H7 PAI deletion mutants was always higher than that reached by the reference strain DH5α (Figure. 4.3 to 4.6); *i.e.*, maximum cell densities reached by the EDL933 PAI deletion mutants ranged from an OD_{660} of 4.0 seen for the OI-55 deletion mutant (Figure. 4.4) to 4.7 in the OI-172 deletion mutant (Figure. 4.4) cv. an OD_{660} of 3.7 for the reference strain DH5α. Similarly, the maximum cell density reached by the SL 72 deletion mutant (an

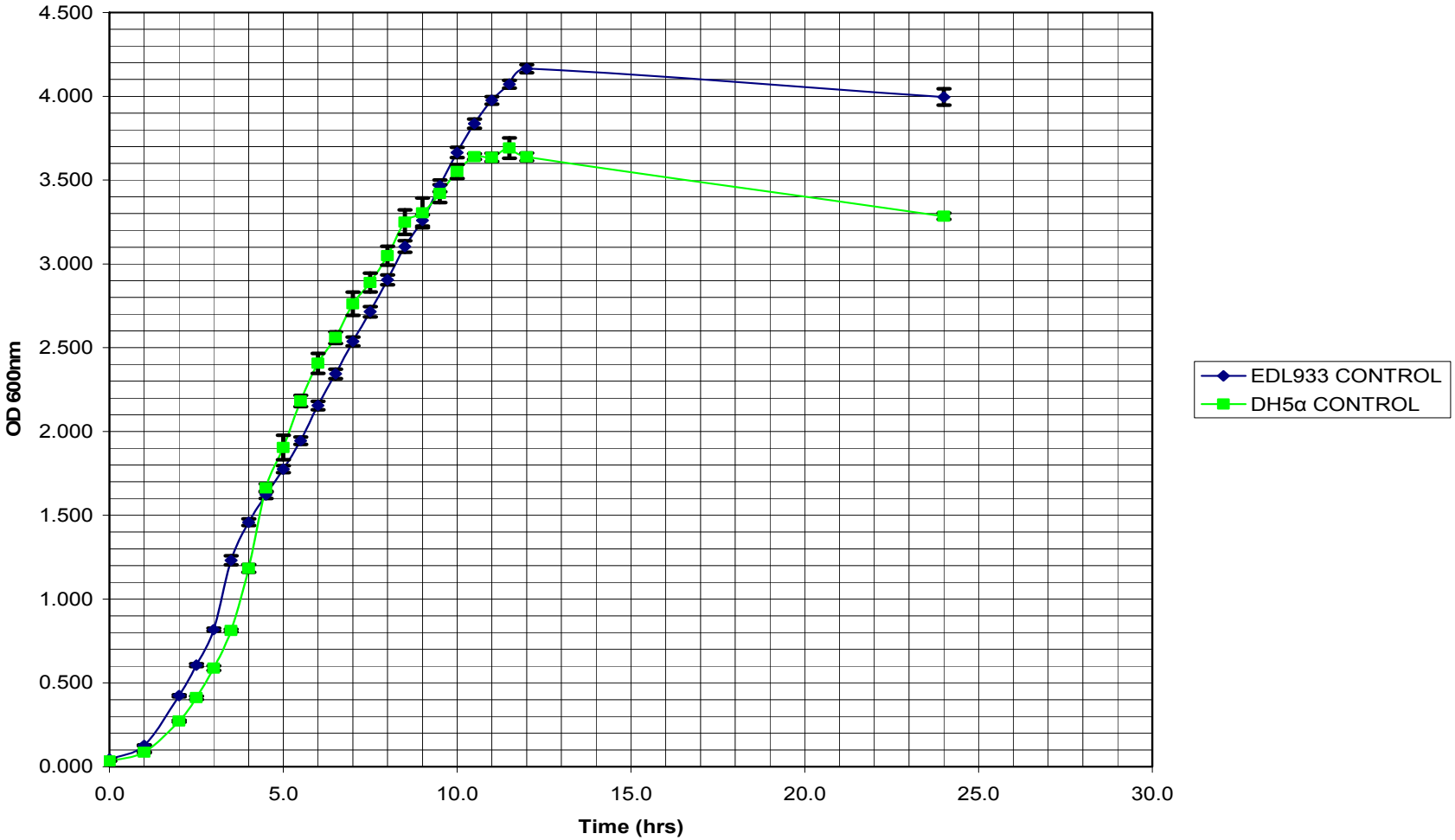


Figure. 4.1. Comparison of the reference K-12 strain DH5α growth curve with the parental strain O157:H7 EDL933. The linear part of the curves are different and appear to have a different growth rate between them. The O157:H7 pathogenic EDL933 parental strain diverges from the reference strain DH5α and they do not have the same growth pattern.

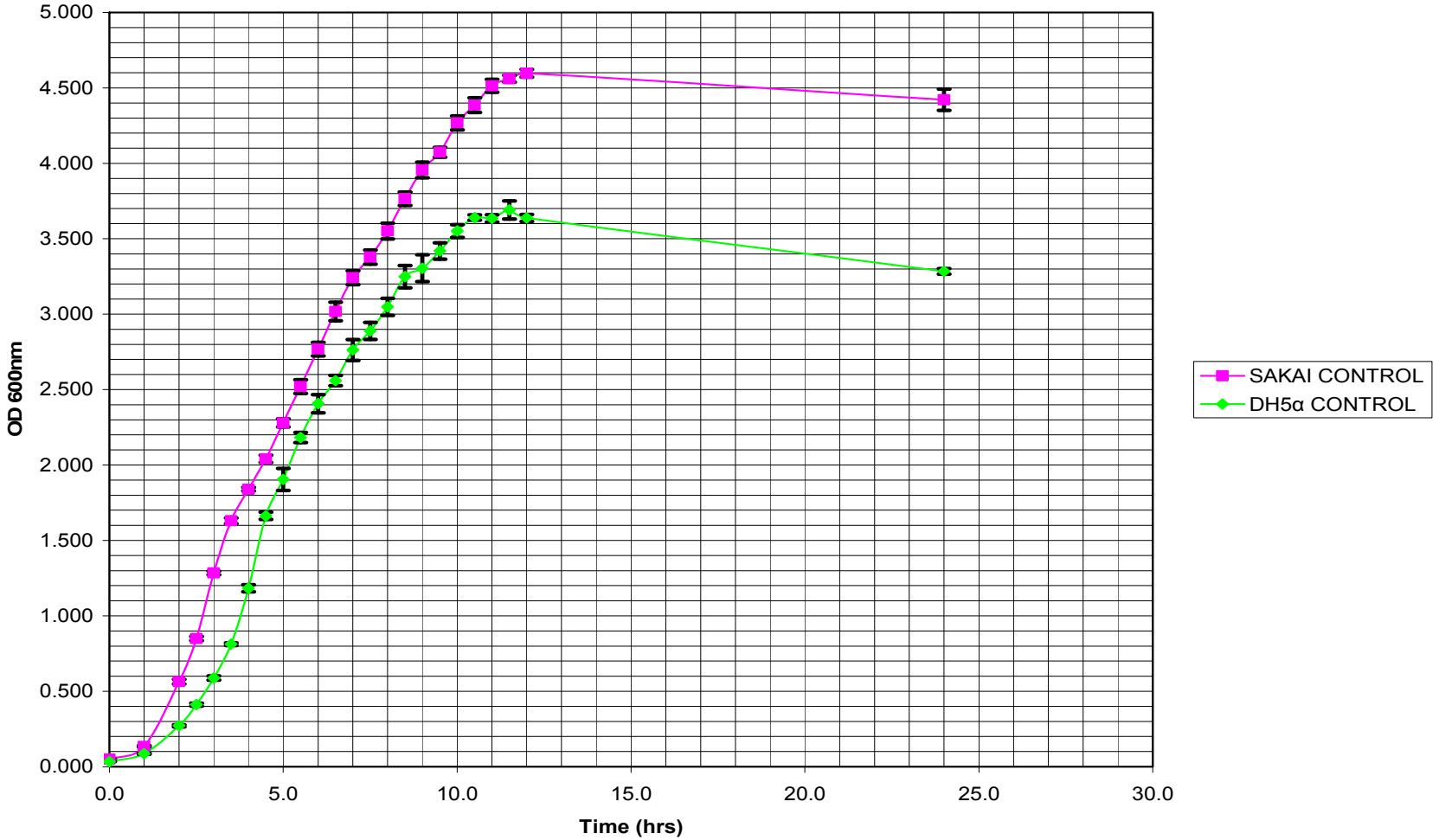


Figure. 4.2.Comparison of the reference K-12 strain DH5α growth curve with the parental strain O157:H7 Sakai. The linear part of the curves are different and appear to have a different growth rate between them. The O157:H7 pathogenic Sakai parental strain diverges from the reference strain DH5α and they do not have the same growth pattern.

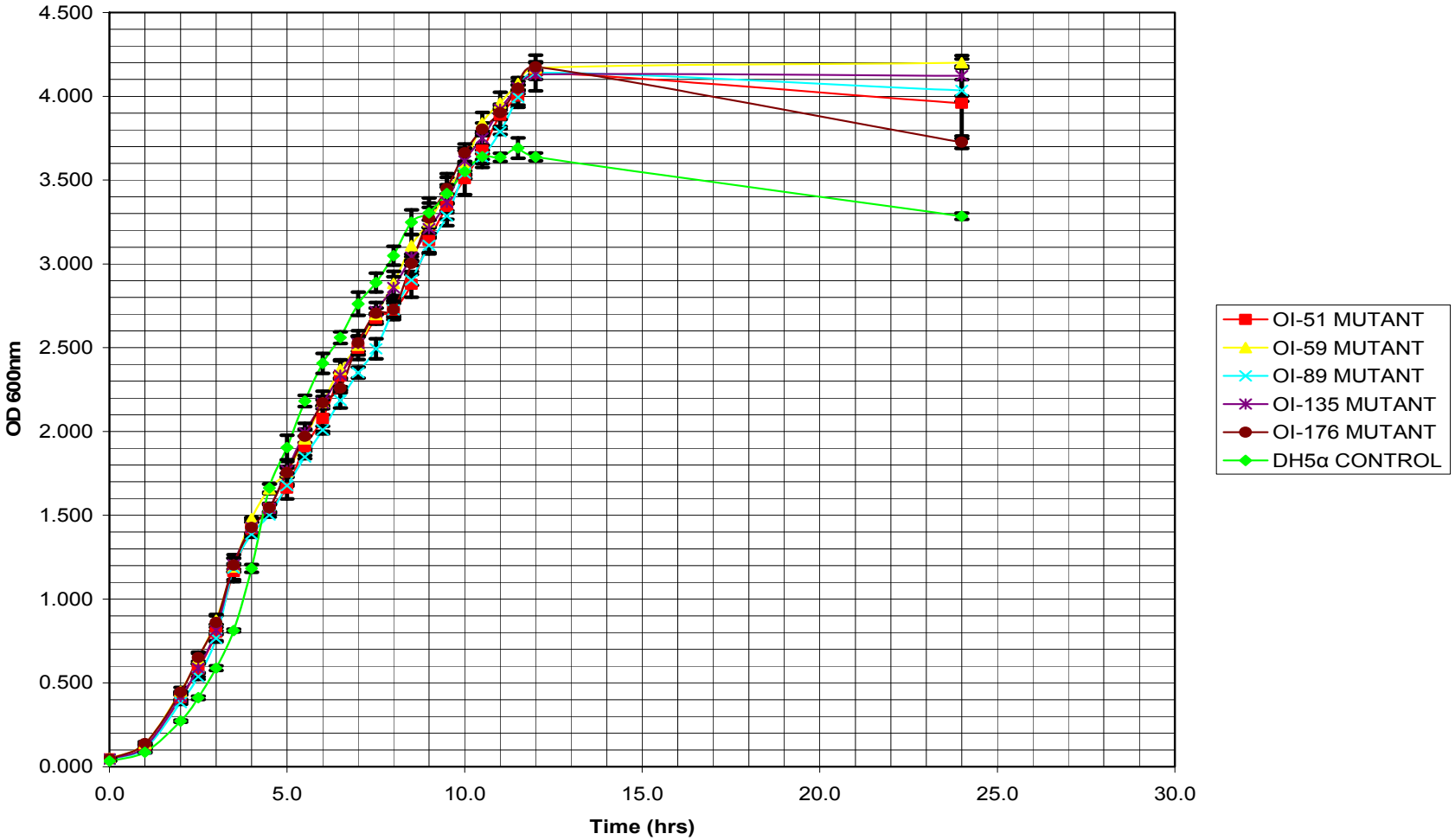


Figure. 4.3. Comparison of OI 51, 59, 89, 135 and 176 deletion mutant growth curves with the reference K-12 strain DH5α. The linear part of the curves are different and appear to have a different growth rate between them. The O157:H7 deletion mutants diverged from the reference strain DH5α and they did not have the same growth pattern.

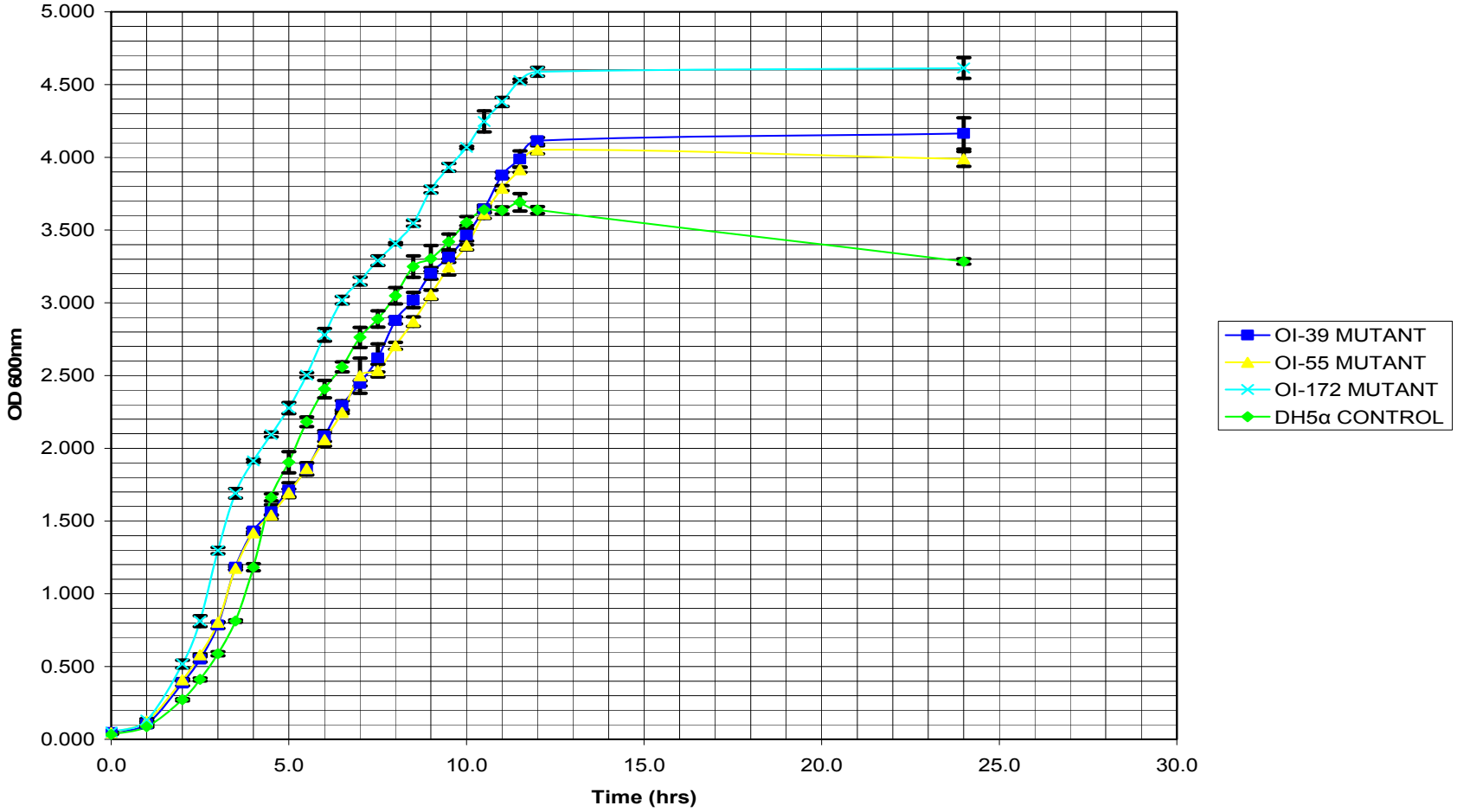


Figure. 4.4. Comparison of OI 39, 55 and 172 deletion mutant growth curves with the reference K-12 strain DH5α. The linear part of the curves appear to have a similar growth rate despite having different intercepts. The O157:H7 deletion mutants did not diverge from the reference strain DH5α and they did have the same growth pattern.

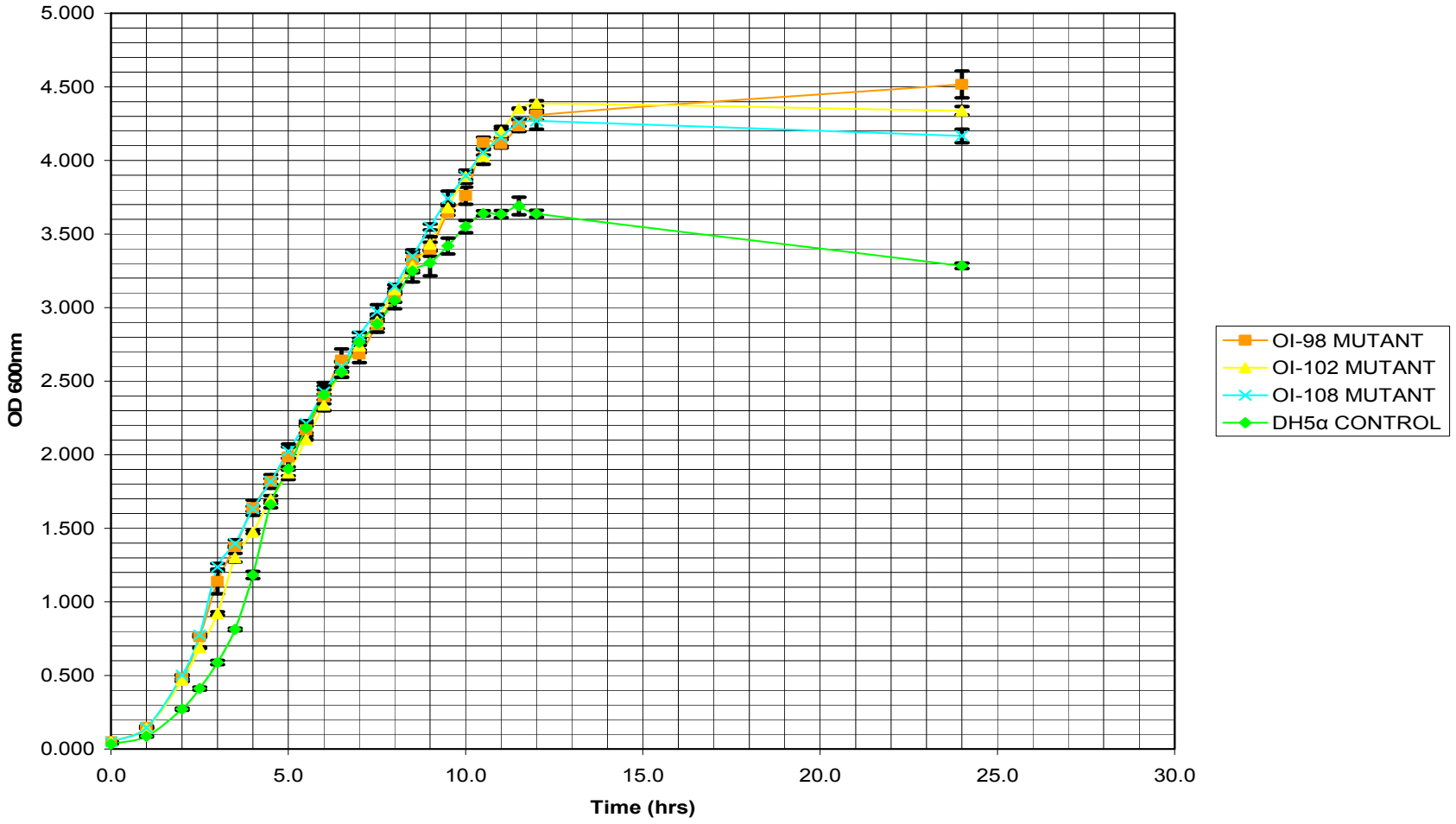


Figure. 4.5. Comparison of OI 98, 102 and 108 deletion mutant growth curves with the reference K-12 strain DH5α. The linear part of the curves appears to have a different growth rate but a similar intercept. The O157:H7 deletion mutants did diverge from the reference strain DH5α in growth rate but not for the intercept.

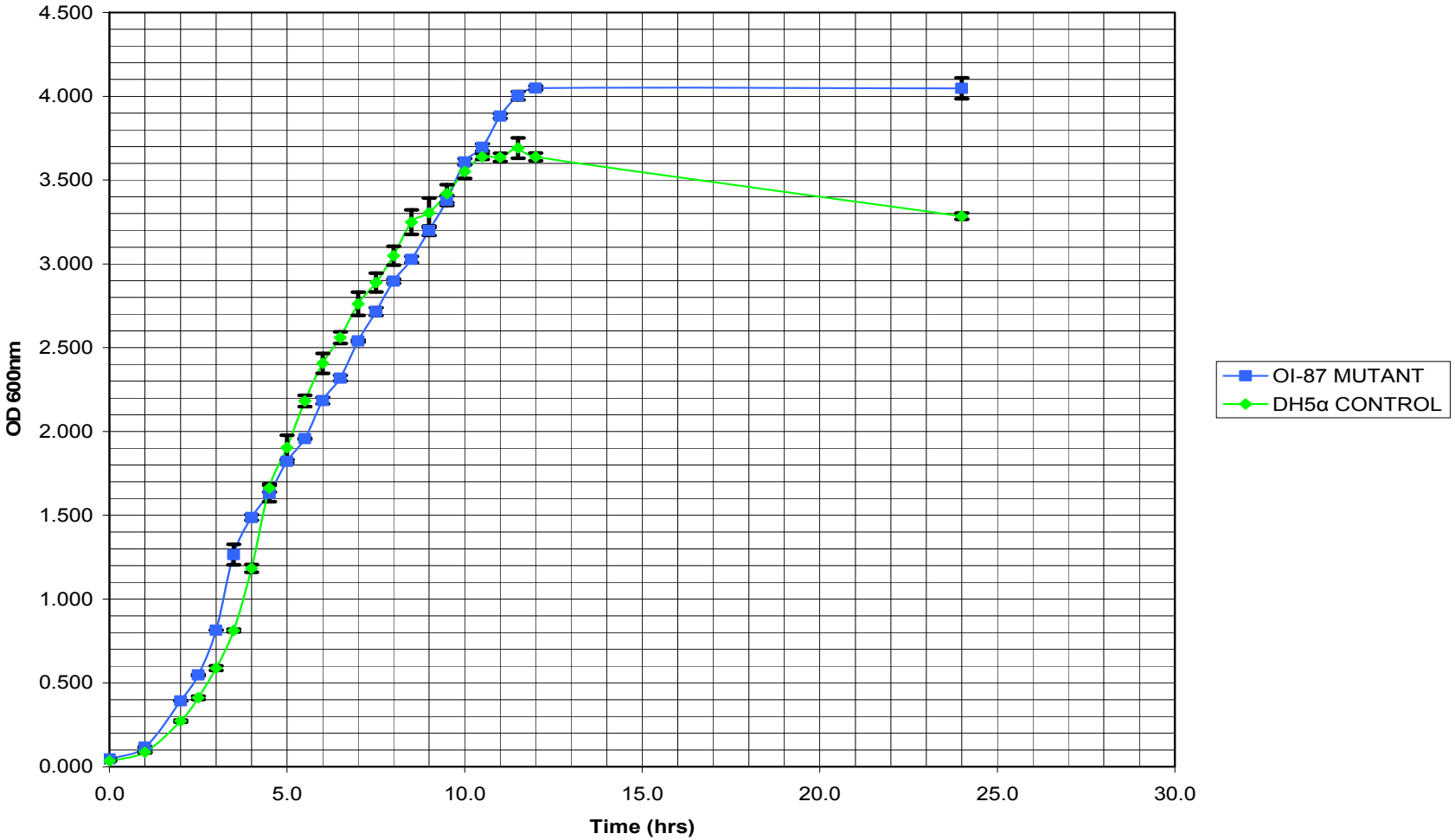


Figure. 4.6. Comparison of OI 87 deletion mutant growth curve with the reference K-12 strain DH5α. The linear part of the curves appear to have a similar growth rate and intercept. The O157:H7 deletion mutant did not diverge from the reference strain DH5α in either growth rate or intercept.

OD_{660} of 4.3) was greater than that reached by the reference strain DH5α (an OD_{660} of 3.7) (Figure. 4.7).

Many of the EDL933 PAI deletion mutants reached a maximum cell density similar to that of the O157:H7 parent strain EDL933 (Figure. 4.8 to 4.11); *i.e.*, the maximum cell density in six of the 12 OI mutants (OI-39, 51, 59, 89, 135, 176 - Figure. 4.8) was the same as that seen in the O157:H7 parental EDL933 strain (*i.e.*, an OD_{660} of 4.2). However, some PAI deletion mutants reached a cell density less than the O157:H7 parental strain (*i.e.*, an OD_{660} of 4.1 in the OI-55 deletion mutant – Figure. 4.9, and an OD_{660} of 4.0 in the OI-87 mutant – Figure. 4.11 cv. an OD_{660} of 4.2 for the parental strain) while others reached a cell density higher than the O157:H7 parental strain; *i.e.*, the maximum cell density reached by the deletion mutant OI-102 was an OD_{660} of 4.4 (Figure.4.9), while the OD_{660} for OI-98, 108 and 172 was 4.3, 4.3 and 4.6 respectively cv. an OD_{660} of 4.2 for the O157:H7 parent (Figure. 4.10). The SL 72 deletion mutant (Figure. 4.12) also reached a lower cell density (OD_{660} of 4.3) than the O157:H7 parent strain Sakai (OD_{660} of 4.6).

The data suggest that some PAIs in *E. coli* O157:H7 may have a role in substrate utilization and may help to increase the efficiency of substrate utilization in the environment. This increase in efficiency appeared to result in production of higher numbers of bacteria (a higher OD_{660}) in cultures as compared to the reference K-12 strain, *E. coli* DH5α. This effect, together with the higher growth rates observed in the O157:H7 cultures, suggests that these bacteria may be better adapted for survival in some

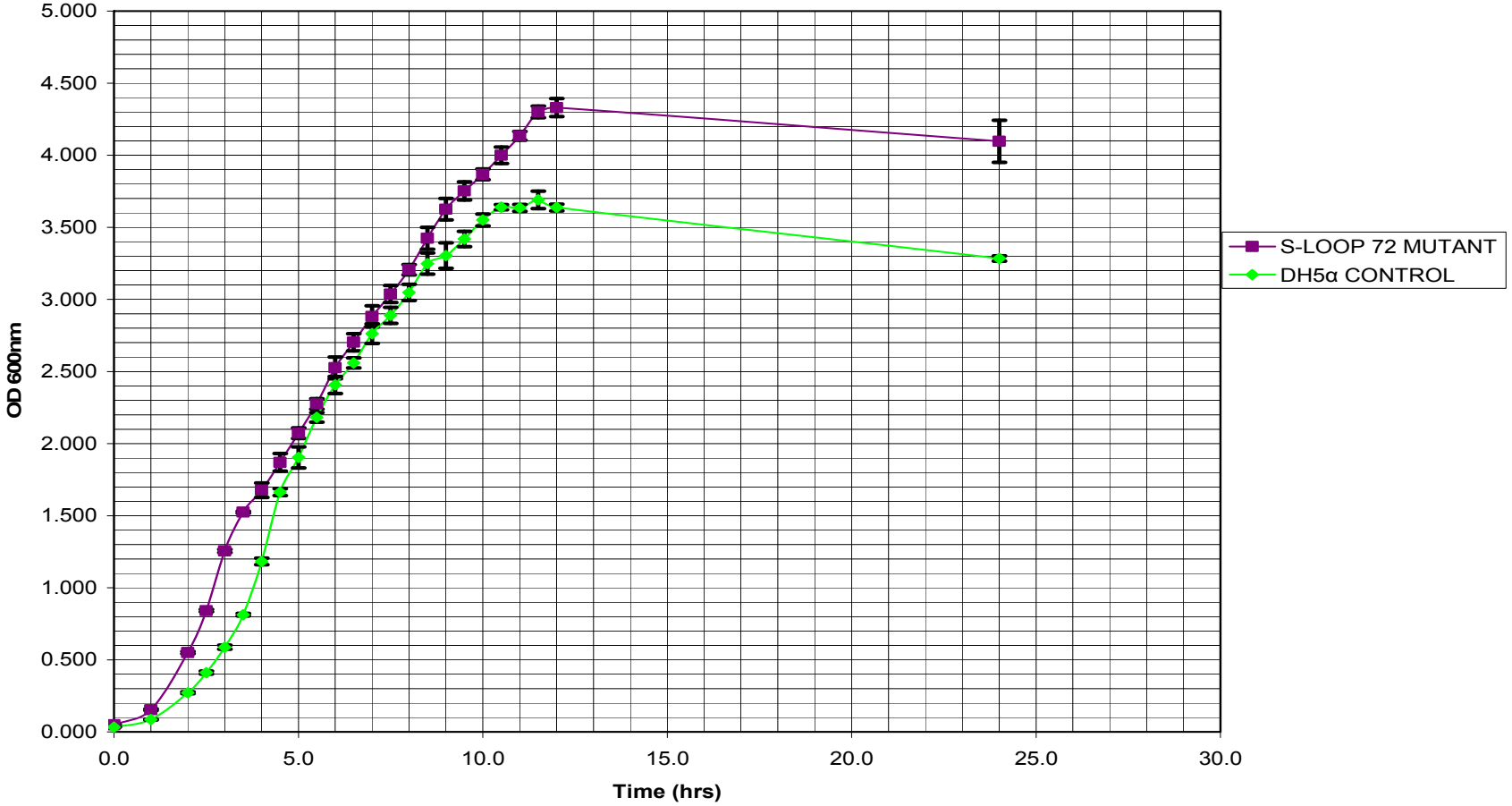


Figure. 4.7. Comparison of SL 72 deletion mutant growth curve with the reference K-12 strain DH5α. The linear part of the curves appear to have a similar growth rate and intercept. The O157:H7 deletion mutant did not diverge from the reference strain DH5α in either growth rate or intercept.

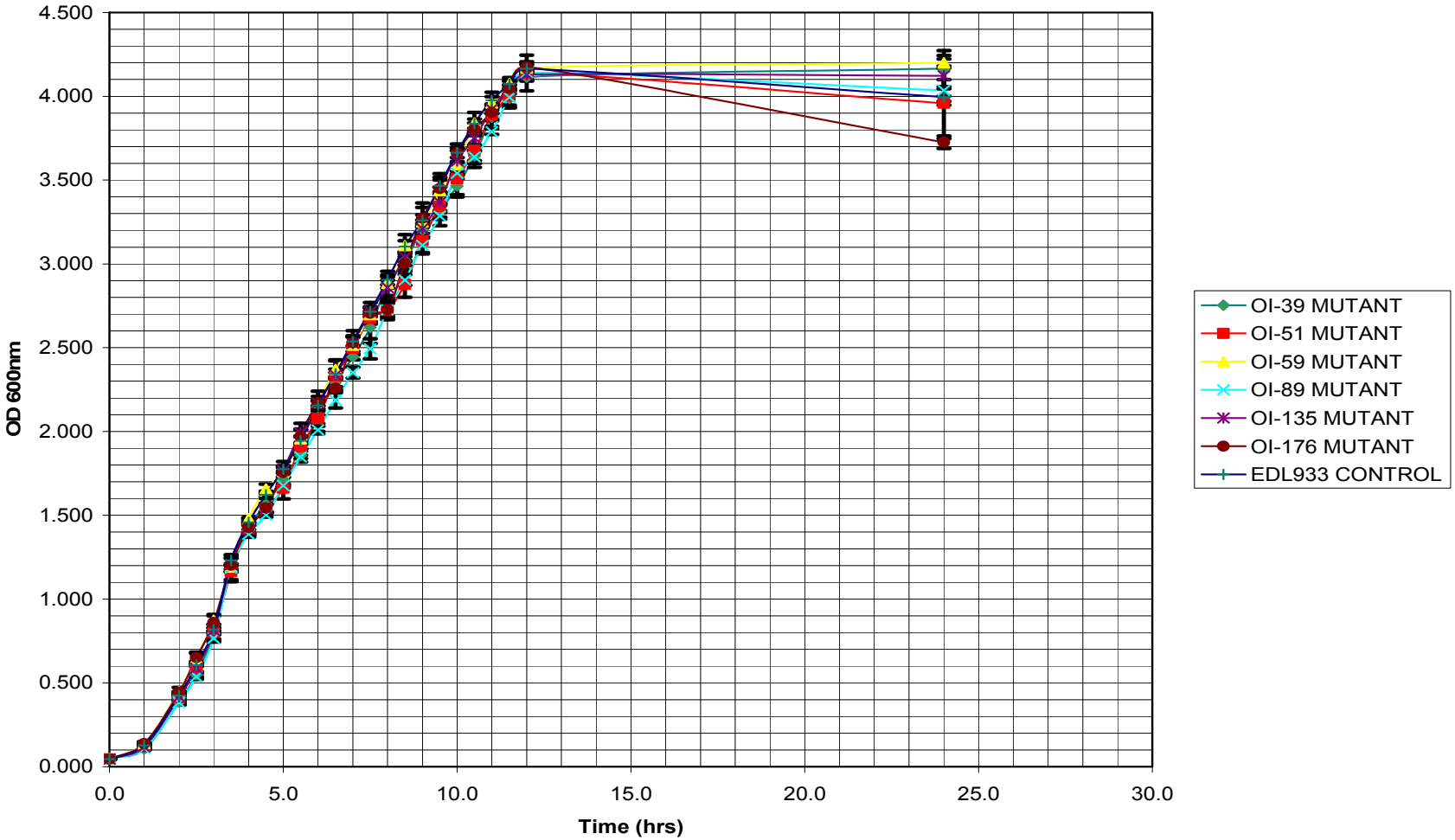


Figure. 4.8. Comparison of OI 39, 51, 59, 89, 135, and 176 deletion mutant growth curves with the parental strain EDL933. The curves are very similar and appear to have the same growth rate among them. The deletion mutants do not diverge from the EDL933 control in this case and mimic the same growth pattern as the control.

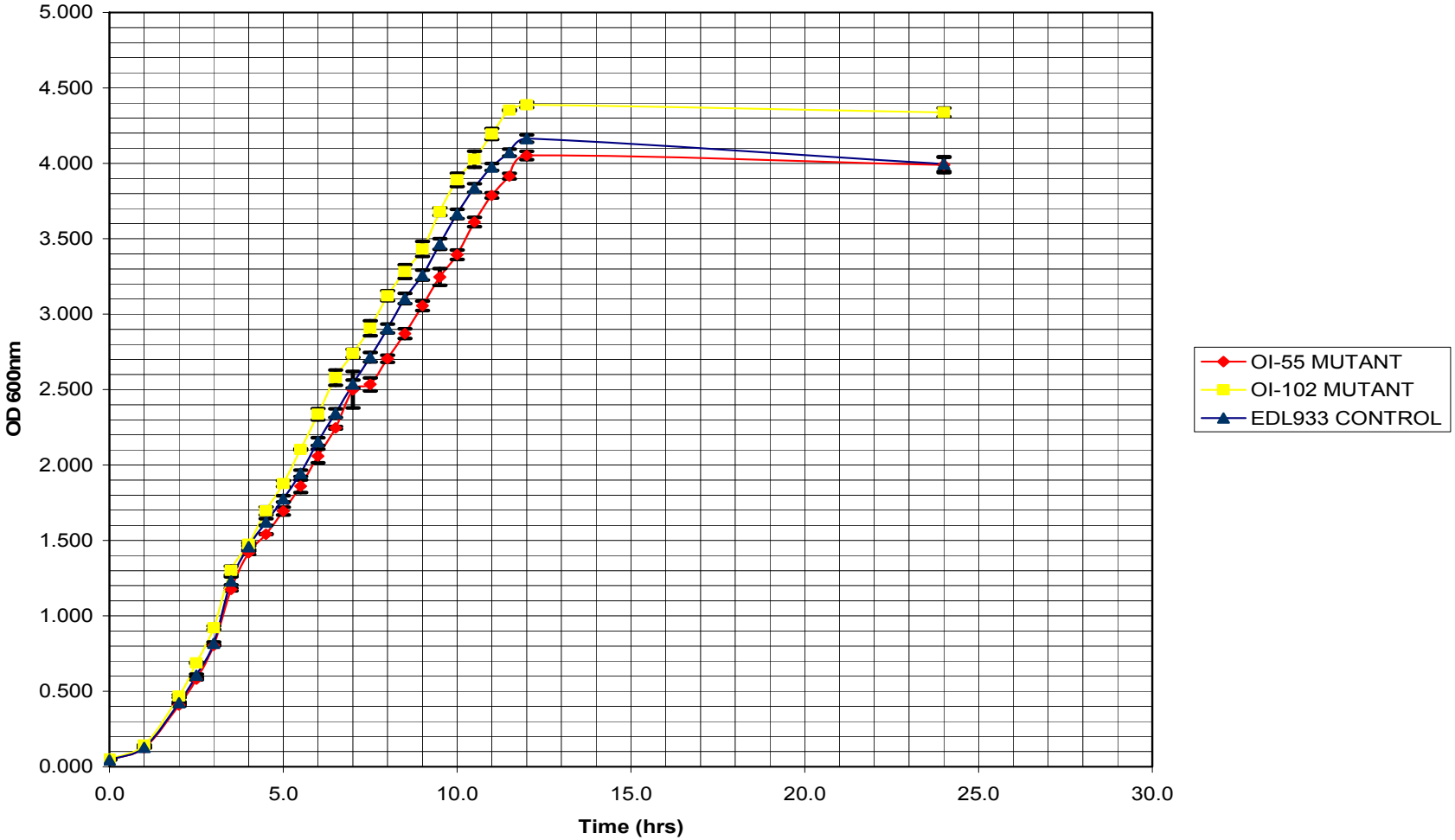


Figure. 4.9. Comparison of OI 55 and 102 deletion mutant growth curves with the parental strain EDL933. The curves are different and have different rates of growth. The OI-55 mutant has a slower growth rate than the EDL933 control. The OI-102 mutant has a faster growth rate than the EDL933 control.

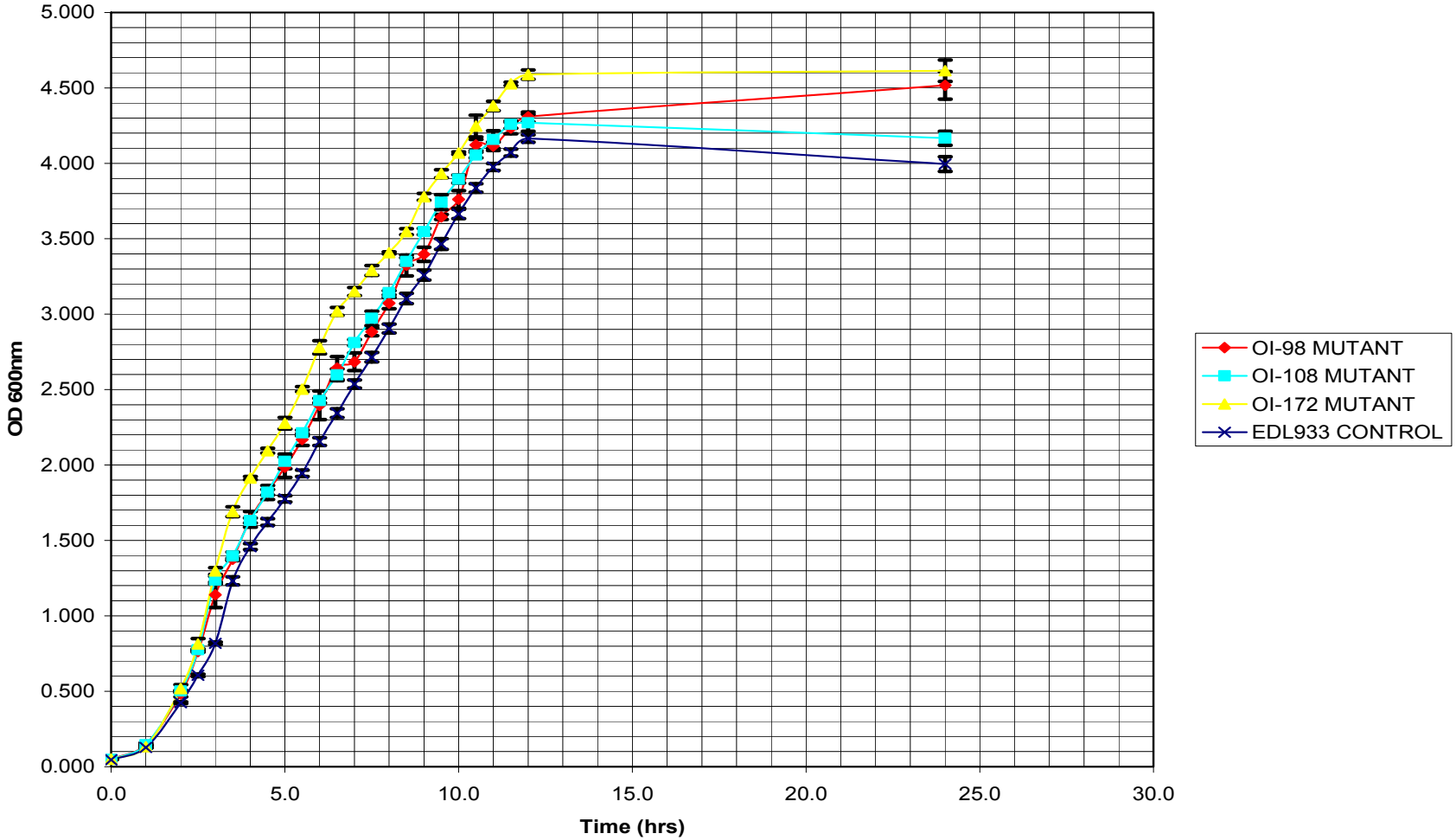


Figure. 4.10. Comparison of OI 98, 108 and 172 deletion mutant growth curves with the parental strain EDL933. The curves have a similar growth rate.The deletion mutants do not diverge from the EDL933 control in this case and mimic the same growth pattern as the control.

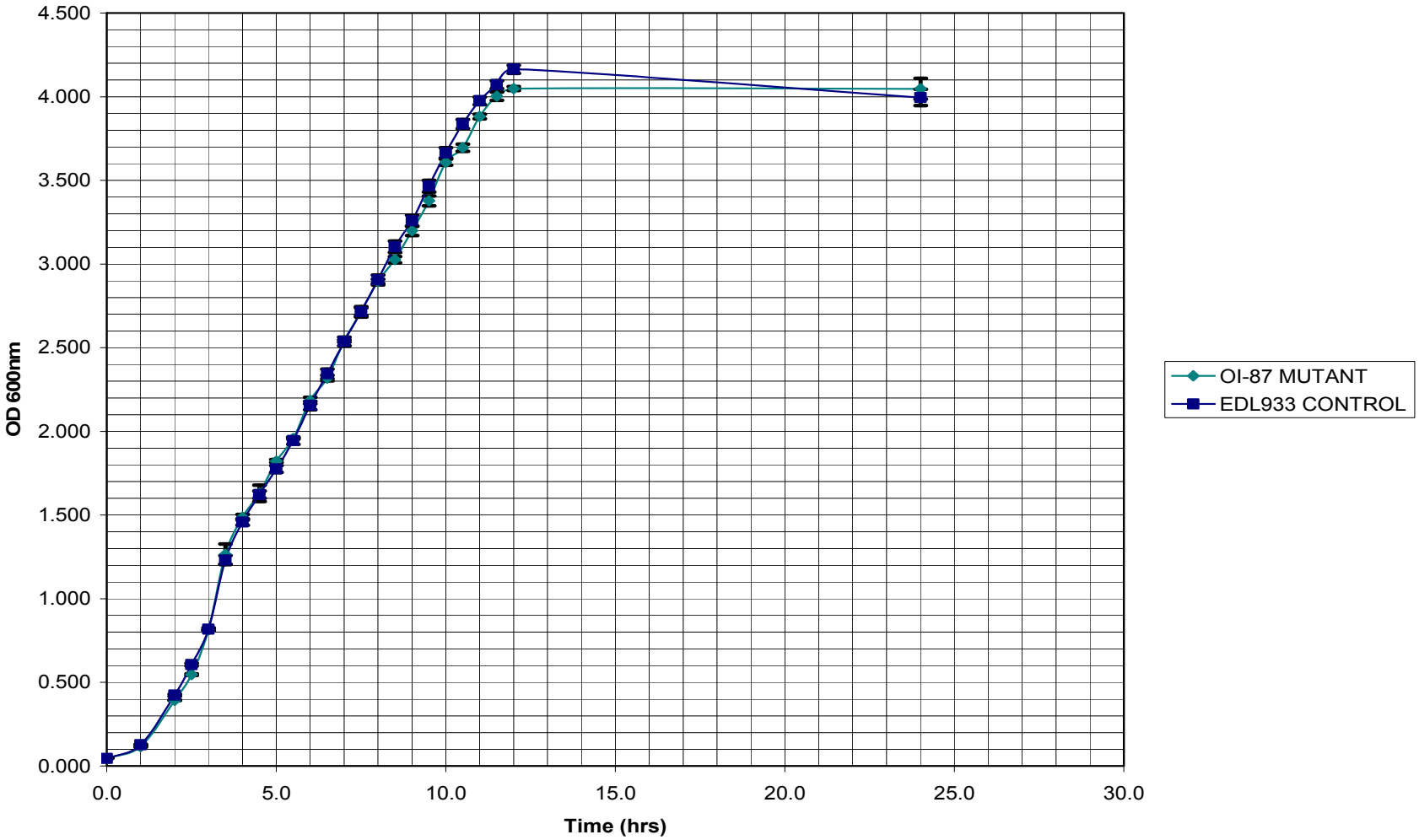


Figure. 4.11. Comparison of OI 87 deletion mutant growth curve with the parental strain EDL933. The curves have a different growth rate with the OI-87 mutant having a slower growth rate than the EDL933 control.

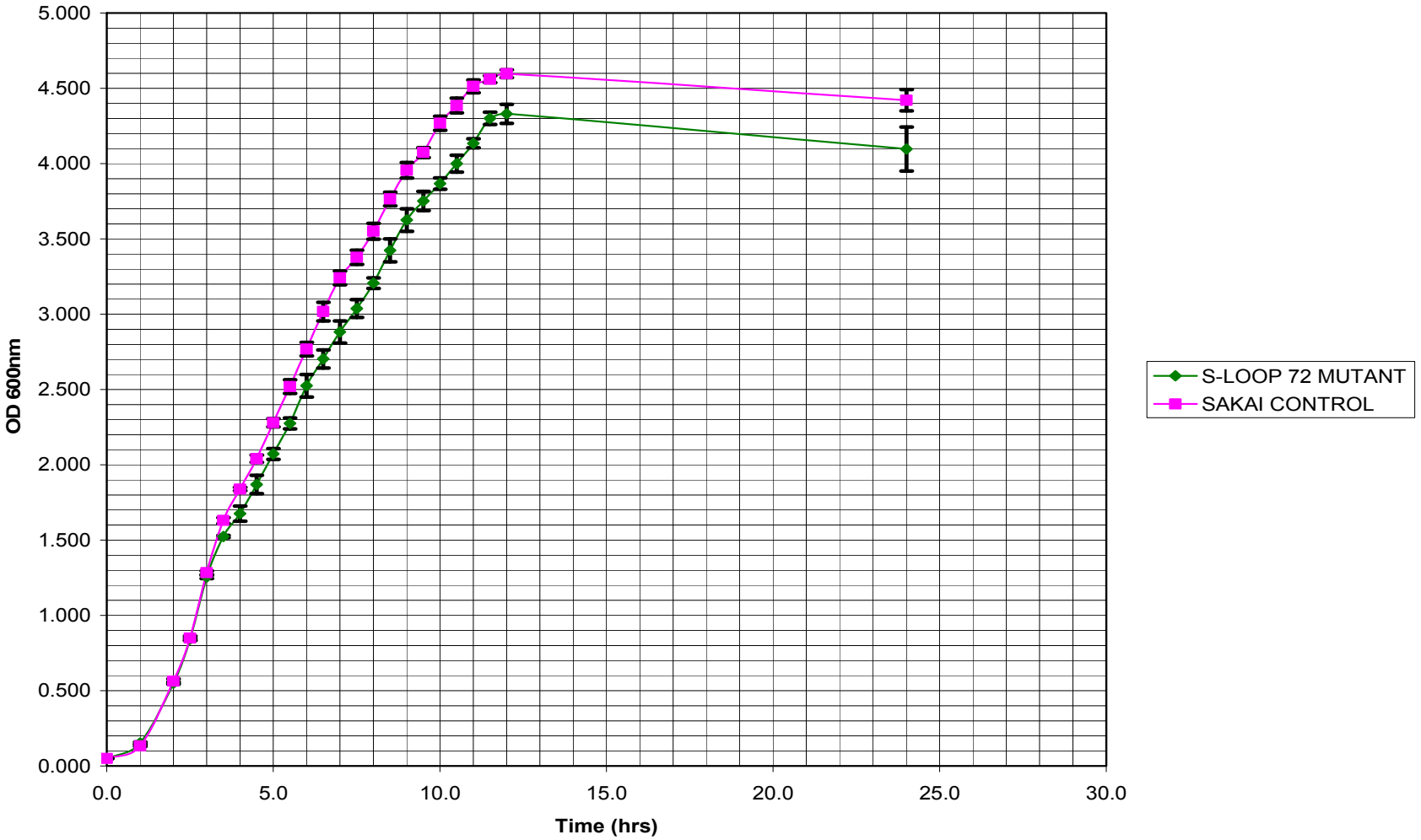


Figure. 4.12. Comparison of SL 72 deletion mutant growth curves with the parental strain Sakai. The curves were different since they had a different growth rate. The SL 72 mutant had a slower growth rate than the Sakai control.

environments than other reference *E. coli* strains. However, further studies are required in order to determine if this trend is common among other enteric bacterial pathogens.

4.3.4. Future directions

The growth of *E. coli* O157:H7 is a factor in its survivability and pathogenesis and has been studied under different growth conditions (Coleman *et al*., 2003). Future studies looking at growth of the O157:H7 deletion mutants and the controls DH5α, EDL933 and Sakai need to be undertaken to fully elucidate the roles that these PAIs have in regulation of growth of these bacteria. The data provided in this study suggests that some PAIs have a role in growth and that those roles may increase efficiency of use of nutrients in the environment. Thus for some PAIs removal of the PAI creates a strain that behaves more like a reference strain than an O157:H7 pathogen. However, these findings need to be explored in more detail.

Chapter V

Cell Viability in *E. coli* O157:H7

5.1 Introduction

E. coli O157:H7 is an intestinal pathogen that can cause a wide range of symptoms and complications from bloody diarrhea to more serious complications such as HUS (Klein *et al*. 2002; Cleary 2004). One of the main routes of *E. coli* O157:H7 infections is by ingesting contaminated food or drink (Karch *et al*., 2005). Food related outbreaks due to consumption of contaminated ground beef and other food products such as spinach have been implicated in *E. coli* outbreaks (Bopp *et al*. 2003; Grant *et al*. 2008). Some outbreaks have been waterborne due to drinking or swimming in contaminated water (Bopp *et al*. 2003; Fremaux *et al*. 2008). In a study by Rangel *et al*. (2005) which looked at outbreaks of *E. coli* O157:H7 in the Unites States from 1982-2002, 31 out of 350 outbreaks were waterborne (9.0%).

Waterborne outbreaks have the potential to infect many people (Rangel *et al*. 2005) and are important because pathogenic bacteria such as *E. coli* O157:H7 can be spread over a large geographic area and to a wide range of people in a short period of time (Olsen *et al.* 2002). A well known Canadian outbreak is the Walkerton incident. In 2000 a waterborne outbreak implicating *E. coli* O157:H7 and *Campylobacter jejuni* occurred in Walkerton Ontario (Holme 2003). Over 2000 people were infected and 7 people died. Outbreaks have also occurred in the United States. One outbreak occurred at the New York State Fair in 1999 where 775 people were infected with *E. coli* O157:H7 by a contaminated well water supply (Bopp *et al*. 2003). Another *E. coli* O157:H7

outbreak occurred in Missouri where 243 people were infected and 4 people died after drinking contaminated water from a rural township's un-chlorinated water supply (Swerdlow *et al*. 1992). The ability of *E. coli* O157:H7 to cause outbreaks from drinking contaminated water may be related to its ability to survive for long periods of time in water (Flint 1987).

Studies looking at survival of *E. coli* O157:H7 in different water environments have been undertaken. Cook *and* Bolster (2007) compared survival of *E. coli* O157:H7 and *Campylobacter jejuni* in ground water and found that *E. coli* O157:H7 could survive just as well in nutrient free water as in water containing carbon or nitrogen while *C. jejuni* died off 2.5 to 13 times quicker than *E. coli* in groundwater**.** Rozen and Belkin (2001) looked at survivability of enteric bacteria including *E. coli* in seawater. Overall they found that *E. coli* was able to survive for a long period of time in seawater, but lost the ability to form colonies and entered into a viable but non culturable state (VBNC) that allowed them to survive in the marine environment for extended periods. Smith *et al*. (1994) exposed *E. coli* to a polar marine environment for 54 days and at the conclusion of their experiments found that the cells were no longer able to grow on selective medium, although direct viable counts indicated that the cells were still present but in a VBNC state. *E. coli* can enter a VBNC state when exposed to stresses such as changes in pH, available nutrients and/or temperature or when subjected to starvation conditions (Liu *et al.*, 2008).

The ability to enter a VBNC state (Liu *et al*. 2008) as well as the ability to survive for long periods of time in water (Mori 2001) is possibly encoded on or influenced by pathogenicity islands (PAIs) found in these cells. PAIs are sections of the bacterial

genome that are found in pathogens such as *E. coli* O157:H7 that encode virulence factors or proteins that have a role in disease (Ferianc *et al.* 2002). These islands are not found in commensal strains of *E. coli* (Ferianc *et al*. 2002).

One method that can be used to study survival of *E. coli* in water is to use vital stains to identify living and dead cells after transfer of the bacteria to water. Liu *et al*. (2008) used a Live/Dead kit (Invitrogen, Carlsbad, California) to count the number of *E. coli* O157:H7 cells present in water during a starvation experiment designed to force *E. coli* O157:H7 into a VBNC state for subsequent tests. After 13 weeks cells were no longer culturable and after 16 weeks were found to be still viable as determined by Live/Dead staining.

The ability of the *E. coli* K-12 reference strain DH5α and the pathogenic *E. coli* O157:H7 strains Sakai and EDL933 plus 13 O157:H7 deletion mutants produced from these bacteria to survive in water was examined by creating survivability curves and examining them over a period of 10 days using a Live/Dead viability test. Specific objectives of this study were to:

1) Create survivability curves for each of the mutants and the three controls.

2) To compare O157:H7 strains EDL933 and Sakai to the reference strain DH5α.

3) To compare the OI deletion mutants and the SL deletion mutant to the reference control DH5α and their respective parental strains EDL933 and Sakai**.**

5.2 Materials and Methods

5.2.1. Bacterial cultures

Single colonies from each of *E. coli* O157:H7 strains EDL933 and Sakai, *E. coli* K-12 strain DH5α and the 13 OI and SL deletion mutants were streaked on LB Agar and grown overnight (12-16 h) at 37 ºC. This step was repeated twice to ensure that individual pure colonies of each strain were used and to ensure that the bacteria were in the same physiological state. After the final incubation a single colony from each plate was used to inoculate a 15 ml Falcon tube containing 10 mL of pre-warmed LB broth. These tubes were then incubated for 12-13 hr at 37 ºC in a shaking incubator set at 220 rpm.

5.2.2. Sample preparation and staining to test for cell survivability

Overnight cultures of cells (10 mL) were centrifuged at 2800 x g in an eppendorf A-4-44 rotor (Eppendorf Centrifuge, Model 5810R, Needham, Massachusetts) for 15 min. The supernatant was removed and the cells washed twice in 5 mL of sterile MilliQ water (Millipore) as described above. The supernatant was removed and the cells re-suspended in 1 mL of sterile MilliQ water. This mixture of cells and water was added to a test tube containing 9 mL of sterile MilliQ water. The tubes were then incubated at 25 ºC in a shaking incubator set at 220 rpm.

Preliminary testing indicated that taking samples every 24 h was not necessary since there was very little difference in cell survival between time points sampled closer than 48 h apart (data not shown). Consequently, bacterial samples were taken at time zero

(immediately after preparation), at day 2 (48 h), day 4 (96 h), day 6 (144 h), day 8 (192 h), and day 10 (240 h). All cells were stained using the *Bac*Light™ Live/Dead Bacterial Viability Kit (kit #L7007, Molecular Probes, Eugene, Oregon) following the manufacturer's directions. (The dye mixture was made by mixing equal amounts of compound A and compound B from the kit as per the manufacturer's instructions.) A 200 µl sample of cells was removed from each test sample, placed in a 1 mL microcentrifuge tube and then returned to the incubator until the next sample time. Tubes were spun in a microcentrifuge (Beckman Microcentrifuge 11, Palo Alto, California) for 5 min at 10,000 rpm. The supernatant was removed and the cells re-suspended in 200 µl of 0.85 % NaCl. To this mixture 1 µl of the LIVE/DEAD *Baclight* dye mixture was added to the microcentrifuge tube and mixed thoroughly by inversion. The tubes were then incubated in the dark at room temperature for 15 min.

5.2.3. Slide preparation and analysis

After incubation of the cells, 5 µl of the stained bacterial suspension was placed on a microscope slide and a cover slip added. The slides were then viewed and counted using a fluorescent microscope. Cells were viewed using a Zeiss AxioScope equipped with a standard fluorescein longpass filter set. For each strain ~1000 bacteria were counted and recorded as being alive (green) or dead (red) (Figure 5.1). These numbers were used to generate a ratio of live cells to dead cells and then used to compare the strains graphically to each other. The ratios were compared statistically using a 2-tailed T-test assuming equal variance with a P value of 0.05. The ratios were further analyzed using a statistical program (SPSS) to perform a linear regression and compare the results

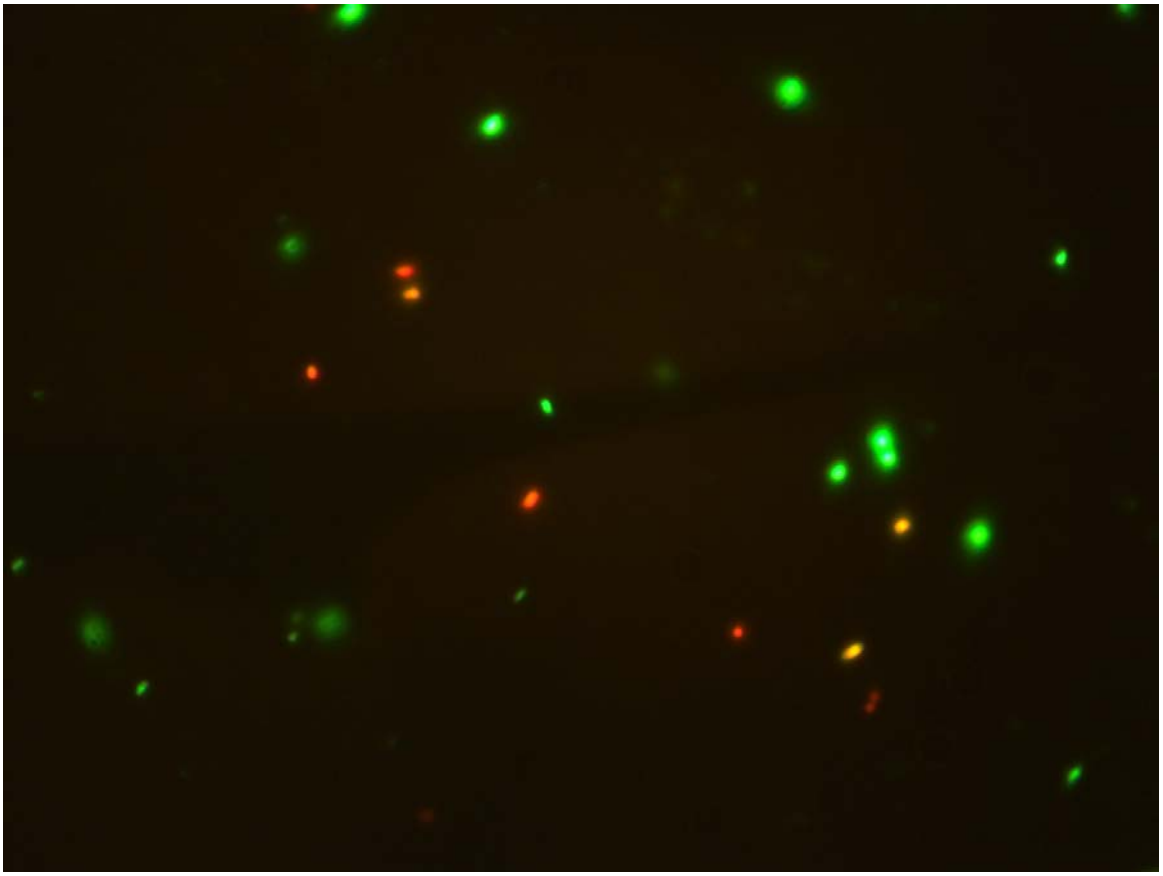


Figure. 5.1. *E. coli* O157:H7 strain EDL933 after treatment with the LIVE/DEAD *Baclight* dye. The green dots are live bacterial cells and the red dots are dead bacterial cells. Cells were viewed using a Zeiss AxioScope equipped with a standard fluorescein longpass filter set and a 100 X magnification lens using immersion oil.

using a univariate analysis. A linear regression for each of the OI and SL deletion mutant survivability curves and the parental controls (O157:H7 strain EDL933 and Sakai) was calculated using the model Y = Bo + BiX, where Y = OD, Bo = the intercept, Bi = the slope and X = time. This process was repeated three times with three different cultures.

5.3 Results and Discussion

E. coli O157:H7 strains EDL933 and Sakai were placed in sterile distilled water and their survival over the next 10 days compared to that of *E. coli* K-12 reference strain DH5α as well as to their respective *E. coli* O157:H7 OI (OI) and SL deletion mutants. The reference *E. coli* K-12 strain DH5α did not survive as long as the O157:H7 parental controls EDL933 and Sakai (Figure. 5.2); *i.e.*, *E. coli* K-12 strain DH5α showed the greatest percentage decrease in live cells (63.80%) over a 10 day period (Table 5.1). This decrease was significantly greater ($P < 0.05$) than that seen in both O157:H7 parental strains EDL933 (25.08 %) and Sakai (18.26 %).

Lui *et al.* (2008) found that *E. coli* O157:H7 appears to enter a VBNC state in response to stresses such as lack of nutrients. In contrast, in this experiments *E. coli* DH5α had low survivability in water and did not appear to enter a VBNC state in response to this type of stress. This suggests that the VBNC state could be an acquired trait by the O157:H7 pathogen, attained in order to increase survivability. Bogosian *et al*. (1996) also looked at survival of a reference *E. coli* K-12 strain W3110 in sterile artificial sea and river water, and found that plate counts of cells from each of these environments remained stable for over 50 days. However, they also found that the *E. coli* W3110 cells did not appear to enter a VBNC state. The *E. coli* DH5α strain looked at in my experiments had a limited ability to survive in sterile water as less than 5 % of the cells transferred to water were alive after 10 days, a result quite different from that of Bogosian *et al*. (1996). This difference in survival could be due to a lack of nutrients present in the sterile Milli-Q water used in my experiments while the sea and

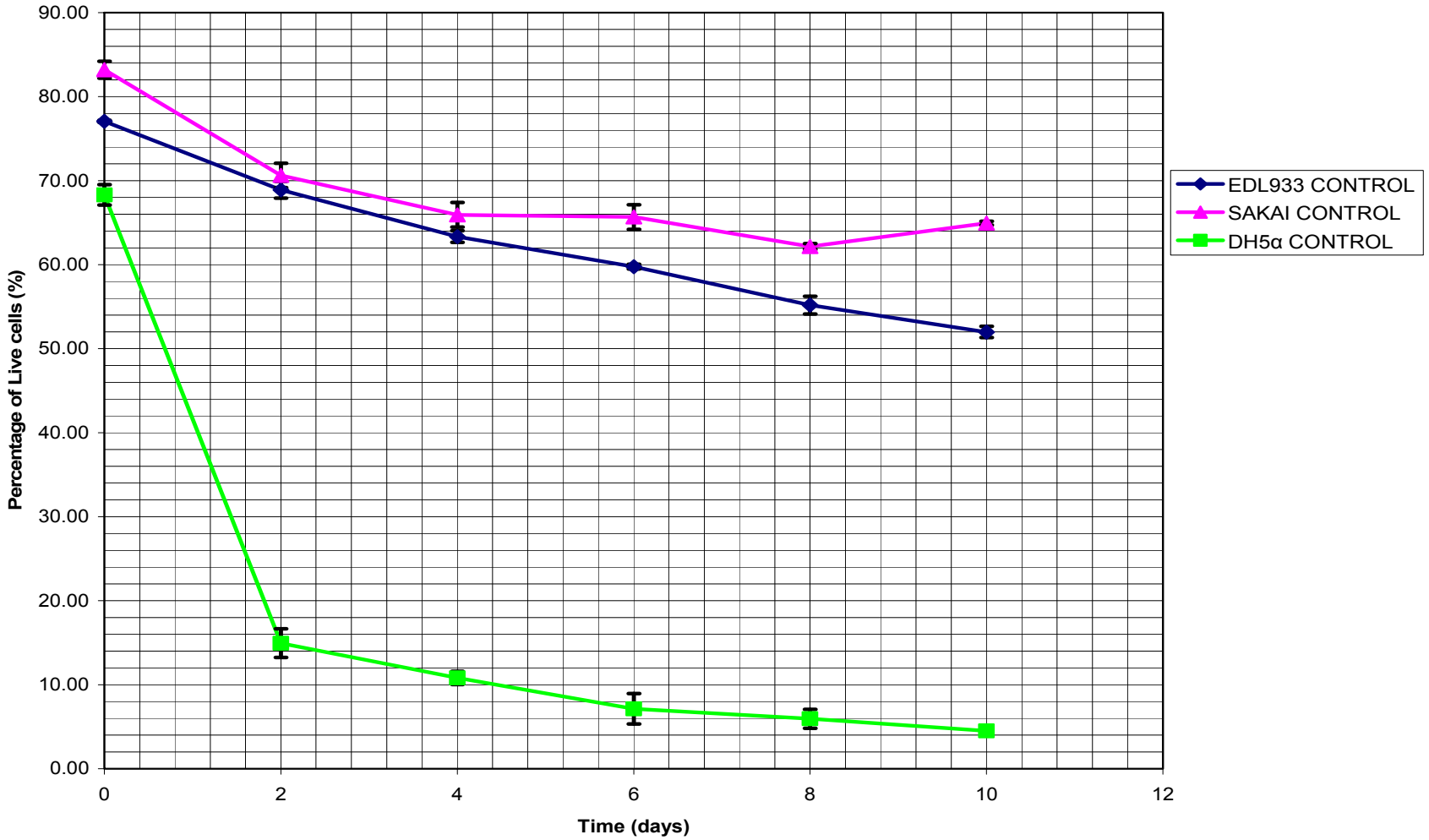


Figure. 5.2. Comparison of the *E. coli* strains EDL933 and Sakai with the reference K-12 *E. coli* strain DH5α. Survivability of *E. coli* O157:H7 strains EDL933 and Sakai relative to *E. coli* K-12 reference strain DH5α in sterile MilliQ water.

Table 5.1. Difference in percentage of live cells after 10 days in sterile MilliQ water for the *E. coli* O157:H7 strains EDL933 and Sakai relative to the reference *E. coli* K12 strain DH5α.

E. coli Strain	Percentage of live cells Day 0	Percentage of live cells Day 10	Difference over 10 days	Standard Error	Probability value (P)
O157:H7 strain EDL933	77.07	51.99	25.08	± 0.71	_[a]
O157:H7 strain SAKAI	83.20	64.94	18.26	± 1.21	_[a]
K12 strain DH5α	68.31	4.51	63.80	± 2.30	**0.000[b]***

*Significant differences in the overall percentage drop over 10 days (Comparison of O157:H7 strains EDL933 and Sakai to *E. coli* K12 strain DH5α) are bolded.

river water tested by Bogosian *et al*. (1996) may have contained some nutrients. Still, my data does support one aspect of the Bogosian *et al*. (1996) experiment that suggests that *E. coli* DH5α does not enter a VBNC state in the same way as *E. coli* K-12 strain W3110.

5.3.1 Comparison of the survivability of *E. coli* O157:H7 Strains EDL933 and the O157:H7 OI deletion mutants.

Each of the OI deletion mutants and the parental O157:H7 strain EDL933 showed a decline in the percentage of live cells over the course of 10 days (Table 5.2). While the parental O157:H7 control EDL933 showed a 25.08% drop in the number of live cells during this period (Table 5.2), the OI deletion mutants exhibited a range in the percentage decrease in live cells. The EDL933 deletion mutant OI-87 showed the largest drop in the percentage of live cells (24.84%), a value which was not significantly different ($P \leq 0.05$) from the percentage decrease in cell number compared to the EDL933. Other strains exhibiting a similar trend were the deletion mutants OI-55, 59, 89, 102 and 172. By contrast, the deletion mutant OI-51 showed a much smaller decline (12.05%); however, this value was significantly less ($P > 0.05$) than that of the O157:H7 EDL933 parent. Other deletion mutants exhibiting a decline significantly less ($P > 0.05$) than that of the O157:H7 EDL933 parent were OI-39, 98, 108, 135 and 176. In each case, these cells were able to survive longer in water lacking any nutrients than the O157:H7 EDL933 parent.

Both *E. coli* O157:H7 strain Sakai and the SL deletion mutant produced from Sakai showed an overall decline in the percentage of live cells that survived over the

Table 5.2. Difference in percentage of live cells after 10 days for each of the *E. coli* O157:H7 OI deletion mutants, the SL 72 deletion mutant and the O157:H7 parental controls EDL933 and Sakai.

***E. coli* Strain**	**Percentage of live cells Day 0**	**Percentage of live cells Day 10**	**Overall percentage difference over 10 days**	**Standard Error**	**Probability value (P)***
EDL933	77.07	51.99	25.08	± 0.71	_
OI-39	66.74	45.25	21.48	± 0.90	**0.035**
OI-51	60.32	48.28	12.05	± 2.02	**0.004**
OI-55	64.98	47.76	17.22	± 3.09	0.068
OI-59	70.74	51.45	19.29	± 6.92	0.452
OI-87	71.03	46.19	24.84	± 2.02	0.915
OI-89	70.33	47.71	22.63	± 1.80	0.275
OI-98	68.98	51.76	17.22	± 0.46	**0.001**
OI-102	71.95	50.73	21.22	± 1.34	0.064
OI-108	78.32	60.88	17.44	± 0.92	**0.003**
OI-135	75.92	60.12	15.80	± 0.49	**0.000**
OI-172	73.03	55.39	17.64	± 3.62	0.114
OI-176	73.69	56.66	17.03	± 2.74	**0.047**
SAKAI	83.20	64.94	18.26	± 1.21	-
SL 72	70.29	56.50	13.79	± 0.94	**0.044**

*Significant differences in the overall percentage drop over 10 days (Comparison of O157:H7 strain EDL933 to the OI-deletion mutants and O157:H7 strain Sakai to the SL deletion mutant) are bolded.

course of 10 days. The parental O157:H7 control Sakai showed a drop of 18.26% in the percentage of live cells over 10 days (Table 5.2) while the SL 72 mutant showed a significantly lower ($P < 0.05$) percentage drop in live cells of 13.79%.; *i.e.*, more of the SL deletion mutant cells survived in water over 10 days than the O157:H7 strain Sakai parent cells.

Waterborne outbreaks of *E. coli* O157:H7 are important because of the possibility to infect many people and to cover a wide geographical area (Olsen *et al*. 2002). *E. coli* O157:H7 has been shown to survive for long periods of time in water (Flint 1987). The results from this study show a slow decline in number of live cells over the course of 10 days and, none of the curves lead to 100% cell death (Figure. 5.3, 5.4, 5.5). In fact after 10 days, the percentage of live O157:H7 cells remaining in water was at least ~45% suggesting that all of the O157:H7 strains examined could survive in water for several weeks.

This slow decline in live cell numbers was previously shown in a study by Mori (2001). She showed that *E. coli* O157:H7 can take several weeks to completely die off in a water sample. These results are similar to those found in this study because there is still a large number of live cells in the water mixture and the slow decline in cell numbers suggest they would continue to survive for an extended period of time.. Liu *et al.* (2008) had similar results and concluded that a VBNC state was induced in *E. coli* O157:H7 under reduced nutrient conditions. In their studies *E. coli* O157:H7 were no longer culturable after 13 weeks, but were still detected as being alive (viable) after 16 weeks using a Live/Dead bacterial viability kit. Wang and Doyle (1998) also looked at *E. coli* survivability in water; they concluded that "O157:H7 is a hardy pathogen" and that it is

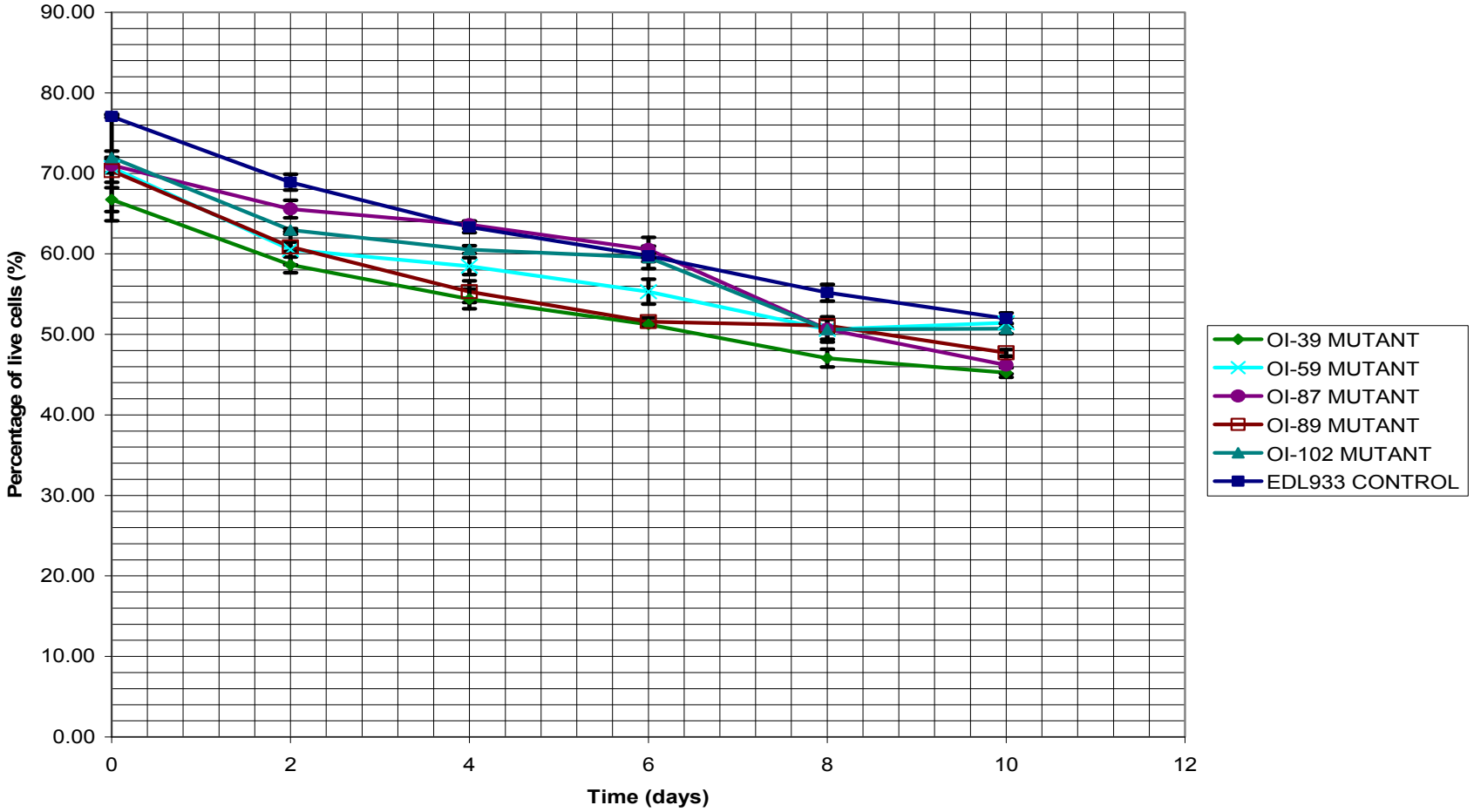


Figure. 5.3. Comparison of the O157:H7 mutants OI-39, OI-59, OI-87, OI-89 and OI-102 with the O157:H7 parental strain EDL933. The curves each show an overall slow decline with different survivability patterns over ten days with none of the OI mutants or the EDL933 control reaching 100% cell death.

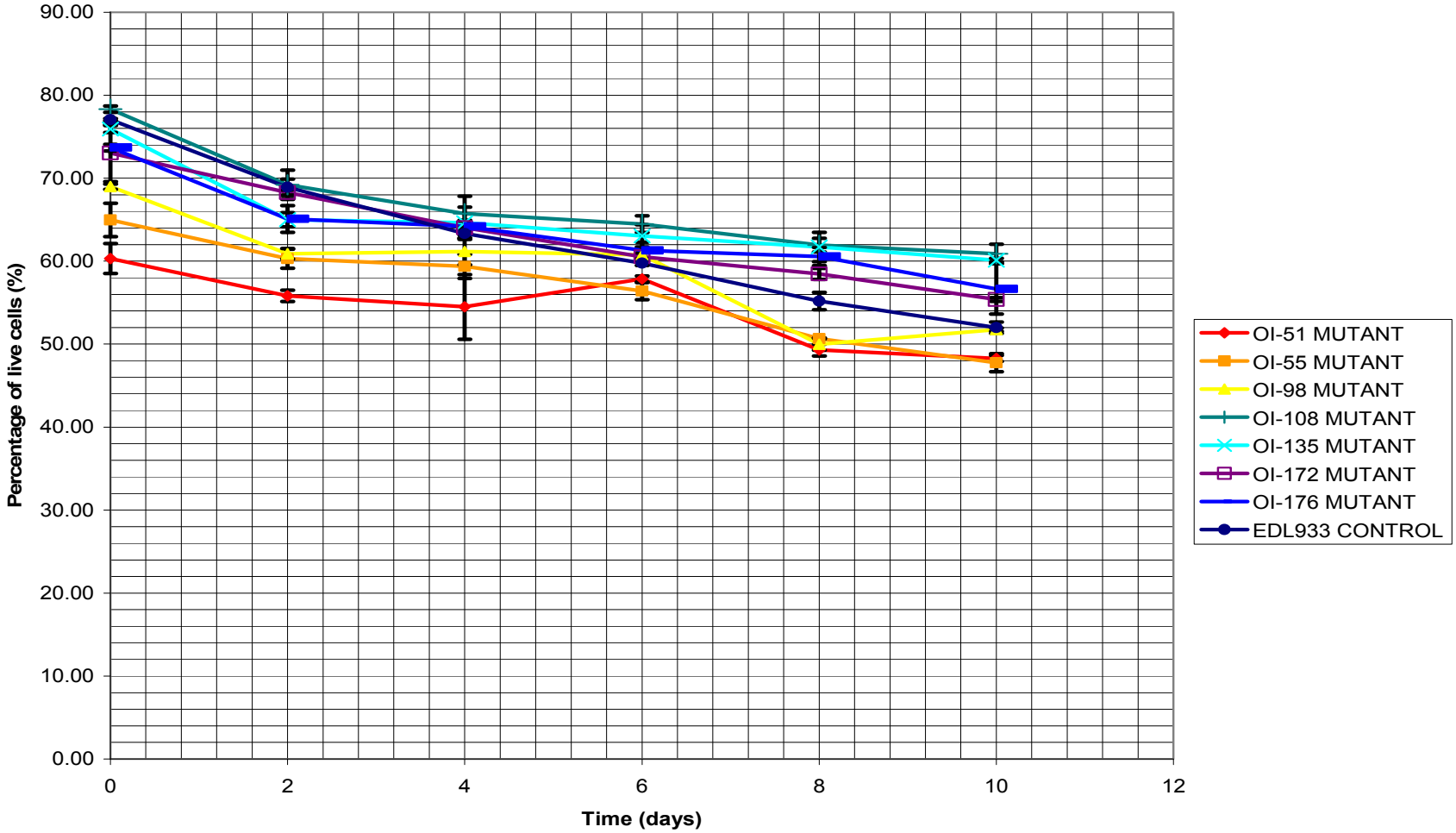


Figure. 5.4. Comparison of the O157:H7 mutants OI-51, OI-55, OI-98, OI-108, OI-135, OI-172 and OI-176 with the O157:H7 parental strain EDL933. The curves each show a slow overall decline over ten days with neither the OI mutants nor the EDL933 control reaching 100% cell death. The curves have different survivability patterns.

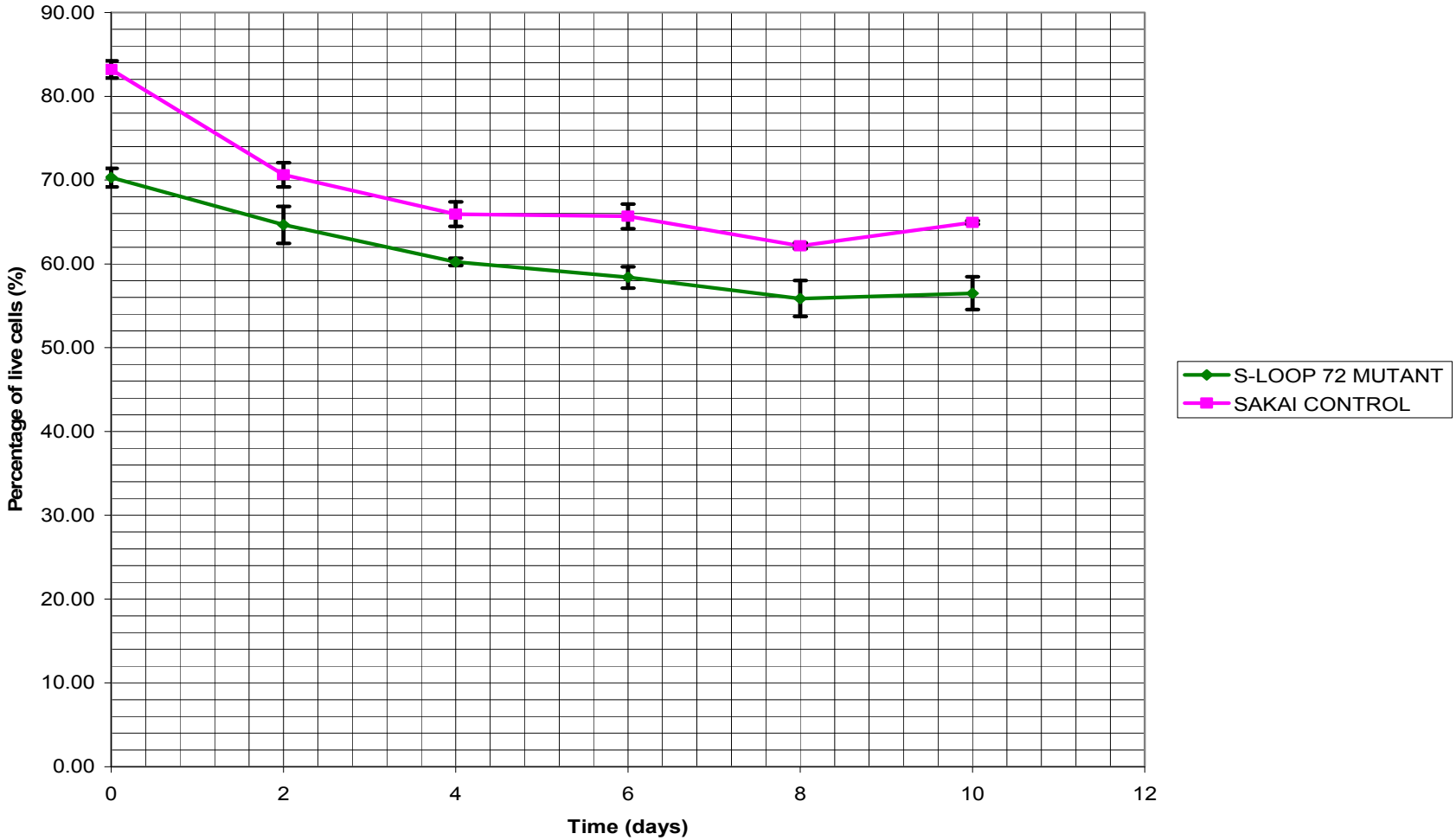


Figure. 5.5. Comparison of the SL 72 mutant with the O157:H7 parental strain Sakai. The curves each show a slow overall decline over ten days with neither the SL 72 mutant nor the Sakai control reaching 100% cell death. The curves have a similar survivability pattern

capable of surviving in water for long periods of time, especially at cold temperatures.

The rate of decline for each of the O157:H7 strains looked at in my study was examined using a best fit linear regression model. Five of the OI mutants (OI-39, 59, 87, 89 and 102) and the SL 72 mutant had statistically similar ($P \leq 0.05$) rates of decline that were not different from the parent O157:H7 strains EDL933 (OIs) and Sakai (SL) (Figure 5.3 and 5.4) (Table 5.3). An effect of removal of specific PAIs was not seen in these mutants. The remaining seven OI mutants (OI-51, 55, 98, 108, 135, 172 and 176) did show a significant difference ($P > 0.05$) in the rate of decline in cell number (slope) within cultures compared to O157:H7 strain EDL933 (Figure. 5.4) (Table 5.3). This suggests that survivability of these bacteria in water was affected by removal of a PAI from the parental O157:H7 strain. It appears that survivability in sterile water is an acquired trait by the pathogen (O157:H7) since the reference strain (DH5α) was unable to survive well in sterile water. Traits of pathogens such as *E. coli* O157:H7 that cause disease are found on PAIs and these PAIs are not present in commensal strains of *E. coli* (Ferianc *et al*., 2002). The results also suggests that more than one PAI can affect survivability of the bacterium since 7 mutants each with a different PAI removed showed a significant difference in rate of decline. Each of these removed OI has different ORFs found within them. The OI 51 contains ORFs from the prophage CP-933C; most of these ORFs from this prophage code for unknown proteins. The OI 55 contains ORFs of unknown function and ORFs that have a transport role. The OI 98 contains an ORF of unknown function. The OI 108 has unknown ORFs that are phage or prophage related; *i.e*., ORFs that code for proteins that are phage or prophage related and ORFs similar to

Table 5.3. Linear regression of the survivability curve data for 13 OI and SL deletion mutants and the O157:H7 parent strains EDL933 and Sakai. The regression model used was $Y = b_o + b_iX$, where Y= OD, b_o is the intercept, b_i is the slope and, X is time.

E. coli Strain	N	Intercept	Standard Error for Intercept	Slope	Standard Error for Slope
EDL933 control	18	7.49E+01	± 0.766	-2.430	± 0.126
OI-39 mutant	18	6.43E+01	± 0.942	-2.076	± 0.155
OI-51 mutant	18	5.98E+01	± 1.496	-1.093	± 0.247
OI-55 mutant	18	6.50E+01	± 0.913	-1.685	± 0.151
OI-59 mutant	18	6.71E+01	± 2.155	-1.848	± 0.356
OI-87 mutant	18	7.19E+01	± 1.072	-2.458	± 0.177
OI-89 mutant	18	6.66E+01	± 1.243	-2.088	± 0.205
OI-98 mutant	18	6.74E+01	± 1.274	-1.704	± 0.210
OI-102 mutant	18	6.97E+01	± 1.058	-2.059	± 0.175
OI-108 mutant	18	7.46E+01	± 1.295	-1.576	± 0.214
OI-135 mutant	18	7.15E+01	± 1.397	-1.292	± 0.231
OI-172 mutant	18	7.19E+01	± 1.002	-1.731	± 0.165
OI-176 mutant	18	7.08E+01	± 1.264	-1.452	± 0.209
Sakai control	18	7.71E+01	± 1.875	-1.671	± 0.310
SL 72 mutant	18	6.79E+01	± 1.296	-1.388	± 0.214

N is the number of data points collected for each strain.

transposases. The OI 135 contains two ORFs of unknown function. The OI 172 contains ORFs unknown function, ORFs coding for two putative intergrases, a transposase, a putative resolvase, a putative ATP-dependent helicase and two putative helicases; ***i.e.,*** genes likely involved in bacteriophage propagation and DNA synthesis, as well as regulation of cell growth and cell division. The OI 176 contains an ORF of unknown function. Each of the PAIs removed have specific role/roles in this bacteria and one role of each of the seven PAIs (OI 51, 55, 98, 108, 135, 172 and 176) is in survivability of *E. coli* O157:H7 in water. This suggests that traits that are important to the survival of the bacteria are found on many different PAIs.

5.4 Summary

E. coli O157:H7 has been shown to survive for over a 100 days in water (Mori, 2001; Liu *et al.*, 2008), an ability that appears to far exceed that of reference *E. coli* K12 strain DH5α(in sterile water). This ability appears to be due, at least in part, to the presence of one or more PAIs in the bacteria. Presence of these islands seems to increase the efficiency with which these bacteria are able to use reserve nutrients in the cell and/or metabolized cellular components, allowing them to survive over extended periods of time. This observation is consistent with current approaches to culture of environmental isolates of *E. coli* O157:H7 which requires resuscitation of the bacteria into supplemented nutrient broth before using standard culture techniques for maintaining the bacteria in a laboratory setting. This property of the bacteria to survive in water for long periods of time may allow them to survive long enough to be able to be ingested and infect the intestines of animals, giving them a selective advantage over other bacteria.

Chapter VI

Summary of General Conclusions

6.1 Phenotypic Microarray Conclusions

6.1.1 Comparison of the PM profiles of the OI and SL deletion mutants to the parental controls Sakai and EDL933

The PM method showed various differences in substrate utilization when comparing the deletion mutants to the parental controls. Most of the changes were differences in level of substrate utilization while only 5 mutants showed true differences in substrate utilization (positive or negative for a substrate and the opposite result was seen for the parental strain). These changes helped elucidate or clarify possible roles for these OIs and the SL deletion mutants. As mentioned the bulk of the OI deletion mutants (OI-39, 51,55,59,89, 108, 135 and 176) did not have any differences in PM profile with the EDL933 parental control for substrate utilization. The remaining 4 OI deletion mutants (OI-87, 98, 102 and 172) and the SL 72 deletion mutant each had differences in substrate utilization in comparison to the EDL933 (OI) or Sakai (SL) parental control. The OI-87 mutant gained the ability to use tween 20 as a carbon source, the OI-98 mutant gained the ability to use L-glutamic acid, the OI-102 mutant gained the ability to use D-serine and D-threonine as carbon sources but lost the ability to use sucrose, the OI-172 deletion mutant gained the ability to use tween 20, M-tartaric acid and loss the ability to use α-ketobutyric acid and α-hydroxybutyric acid and the SL 72 mutant gained the ability to use L-glutamine as a carbon source. These differences were seen when comparing the PM profiles of the mutants and its respective parental control. Each of these gains or loss

of functions could potentially have an impact on adaptability, survivability and pathogenesis of *E. coli* O157:H7.

All of the deletion mutants except the OI-135 deletion mutant had differences in level of substrate utilization in comparison to the parental controls, EDL933 and Sakai. There were similarities seen across the mutants for differences in level of substrate utilization, where many of the same substrates showed differences in level of utilization in comparison to the parental controls. This suggests that processes or substrates that have an important role in *E. coli* O157:H7 are regulated by more than one PAI and that differences in level of substrate utilization could result in a less efficient pathogen and further tests need to be undertaken to see if this is the case.

6.1.2 Comparison of the PM profiles of the OI and SL deletion mutants to the *E. coli* K-12 reference strain DH5α

In each of the comparisons of the *E. coli* O157:H7 deletion mutants and the reference *E. coli* K-12 DH5α there were substrates that showed similarities in level of substrate utilization that were originally differences in level of substrate utilization in the DH5α and the O157:H7 EDL933 and Sakai parental strain comparisons. This suggests that the mutants are behaving more like the reference strain for these substrates than the pathogenic parental supporting the hypothesis that by removing a PAI from a pathogen results in a mutant that is less pathogenic than the parent. There was also a pattern seen where these similarities in substrate utilization between the mutants and the DH5α control were for many of the same substrates.

6.2 Cell Growth Conclusions

6.2.1 Comparison of the OI and SL deletion mutants to the parental controls Sakai and EDL933 growth curves

The growth curves generated were unique to each strain tested and allowed for comparison between the mutants and the controls which showed the differences and similarities between the growth rates of the mutants and the 3 controls EDL933, Sakai and DH5α. Analysis of the mutant and parental curves showed that nine of the OI mutants (OI-39, 51, 59, 89, 98, 108, 135, 172 and 176) had the same rate of growth (slope) as the EDL933 parental, meaning the removal of these OIs did not affect the growth of mutants compared to EDL933 in LB broth. Three of the mutants OI-55, OI-87 and the SL 72 mutant had a slower rate of growth compared to EDL933 (OI) and Sakai (SL) and the OI-102 mutant had a higher rate of growth compared to EDL933. This shows that removal of these 3 OIs and the SL 72 had an impact on the growth of the bacterium in LB broth suggesting these PAIs have a role in growth of *E. coli* O157:H7.

6.2.2 Comparison of the OI and SL deletion mutants to the *E. coli* K-12 reference strain DH5α growth curves

Comparison of *E. coli* O157:H7 mutants with the reference *E. coli* K12 strain DH5α showed that some of the strains had a similar growth patterns while others had a different pattern. Eight of the 12 OI deletion mutants (OI-51, 59, 89, 98, 102, 108, 135 and 176) grew faster than the reference DH5α strain same as the EDL933 parental strain. The remaining four OI deletion mutants, OI-39, 55, 87, 172 and the SL 72 deletion mutant had a growth rate that was similar to the reference strain DH5α which is different than the parental strains EDL933 (OI) and Sakai (SL). This suggests that the mutants are behaving or have a growth pattern that is more similar to the DH5α strain in this

comparison, suggesting that the removal of some PAIs from *E. coli* O157:H7 results in a mutant that grows in a manner more characteristic of a reference strain in a rich medium.

6.3 Cell Viability Conclusions

6.3.1 Comparison of cell viability of the OI and SL deletion mutants to the parental controls Sakai and EDL933

The survivability curves generated in sterile water for each of the mutants and the parental controls EDL933 and Sakai were unique to each strain and also allowed for comparison to show similarities and differences between the mutants and their respective parental controls. The comparison showed that 6 of the deletion mutants (OI-39, 59, 87, 89, 102 and the SL 72) showed no difference in rate of decline (survival) in comparison to the parental strains EDL933 (OI) and Sakai (SL). The removal of these above mentioned PAIs appear to not have an effect on survivability of the pathogen in sterile water. The remaining 7 OI deletion mutants (51, 55, 98, 108, 135, 172 and 176) did show a difference in rate of decline in comparison to EDL933, suggesting that these PAIs have a role in survivability of the bacterium in sterile water.

6.4. Future Directions

Further tests need to be undertaken with these mutants to fully elucidate the roles of these OI and SL in *E. coli* O157:H7 strains EDL933 and Sakai. Each method used to study the deletion mutants did open further area's of research. The PM method could be expanded to include more types of PM plates such as, nitrogen utilization or phosphorus utilization which could elucidate roles for the ORFs of unknown function. The growth curves could be undertaken under different growth conditions to see if growth is affected if the deletion mutants are grown in minimal media or at a different temperature. The survivability curves could be undertaken for a longer period of time. *E. coli* O157:H7

had been shown to survive for over 100 days in water (Mori, 2001; Liu et al, 2008). The removal of specific PAI could affect long term survivability of the mutants in water.

Other future experiments could be to do partial deletions of the islands and SL removed to narrow down the ORF causing the differences in substrate utilization, growth and/or survivability. Also the removed islands could be transferred to *E. coli* K-12 to see if the addition of the PAI would cause the *E. coli* K-12 reference strain DH5α to behave like the pathogen *E. coli* O157:H7 with the addition of the new DNA. Also a few of the OI deletion mutants contain genes that are prophage related, 51, 102, 108 and 172. It has been shown that these phage and prophage play an important role in *E. coli* O157:H7 pathogenesis (Hallewell, 2008) and the removal of these islands with ORFs related to phages may change the virulence of this pathogen. Studies looking at phage and phage production should be undertaken to see if removal of the island affects the pathogenicity of *E. coli* O157:H7. These types of experiments described above would help clarify the roles these specific PAIs have in E. *coli* O157:H7.

6.5 Overall conclusions

The three tests used to compare the O157:H7 deletion mutants with the O157:H7 controls EDL933 and Sakai and the *E. coli* K-12 reference strain DH5α have elucidated possible roles for these specific PAIs or elucidated further areas of study to determine the specific roles of these PAIs. Each of the tests found similarities and/or differences between the deletion mutants and the three controls giving valuable information about the role the specific PAI has in the bacterium. In some cases such as with the OI-39 deletion mutant the role of the OI was not specific but suggested areas for further study in order to

determine the specific role of this island. In other cases such as the OI-102 deletion mutant it was found to have a role in sucrose metabolism indicating a specific role for this island.

The results suggest that removal of a PAI has an effect on the resulting bacterium. The effects on the mutant can range from differences in substrate utilization, differences in how substrates are utilized, to differences in growth or survivability. In six cases the deletion mutants showed differences with the O157:H7 parental control for more than one test. The OI 87, 102 and the SL 72 deletion mutants each had significant differences in substrate utilization and growth compared to the O157:H7 parental strains EDL933 (OI) and Sakai (SL). The OI 98 and 172 deletion mutants had significant differences in substrate utilization and survivability compared to the EDL933 O157:H7 parental. The OI-55 deletion mutant had significant differences in growth and survivability compared to EDL933. This demonstrates that PAIs can have more than one role in *E. coli* O157:H7 strains EDL933 and Sakai and these roles can affect various systems in this bacterium. The removal of a PAI also suggest that the resulting mutant behaves more like a reference strain depending on the tests suggesting the resultant mutant is becoming less pathogenic. Overall it can be concluded that PAIs have an important role in *E. coli* O157:H7 and the removal of these PAIs affect the bacterium and may likely affect the pathogenesis or pathogenicity of the bacterium.

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Appendix I

Genes and ORFs of each OI from *E. coli* O157:H7 strain EDL933 and SL from *E. coli* O157:H7 strain Sakai deleted.

OI and SL ORFs and genes		
PAI Type	**#**	**ORFs and Genes**
OI	39	ORF; Unknown function
OI	39	ORF; Unknown function
OI	51	putative integrase of prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	putative single stranded DNA-binding protein of prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	putative capsid protein of prophage CP-933C
OI	51	putative head maturation protease of prophage CP-933C
OI	51	putative portal protein of prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	putative holin protein of prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	putative terminase of prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	51	unknown protein encoded by prophage CP-933C
OI	55	putative molybdenum transport protein
OI	55	ORF; Unknown function
OI	55	putative iron compound ABC transporter, ATP-binding protein
OI	55	putative iron compound ABC transporter, permease protein
OI	55	ORF; Unknown function
OI	55	ORF; Unknown function
OI	59	putative regulator; Not classified

OI	59	ORF; Unknown function
OI	87	ORF; Unknown function
OI	87	ORF; Not classified
OI	89	ORF; Unknown function
OI	89	ORF; Unknown function
OI	89	ORF; Unknown function
OI	89	ORF; Unknown function
OI	98	ORF; Unknown function
OI	102	putative prophage DNA injection protein
OI	102	putative prophage DNA injection protein
OI	102	ORF; Unknown function
OI	102	ORF; Unknown function
OI	102	ORF; Unknown function
OI	102	ORF; Unknown function
OI	102	ORF; Unknown function
OI	102	ORF; Unknown function
OI	102	putative resolvase
OI	102	sucrose permease
OI	102	D-fructokinase
OI	102	sucrose hydrolase
OI	102	sucrose specific transcriptional regulator
OI	108	ORF; Other or unknown (Phage or Prophage Related)
OI	108	ORF, hypothetical protein
OI	108	putative chaperone protein
OI	108	ORF; Other or unknown (Phage or Prophage Related)
OI	108	ORF; Other or unknown (Phage or Prophage Related)
OI	108	ORF; Other or unknown (Phage or Prophage Related)
OI	108	putative transposase
OI	108	ORF; Unknown function (Insertion Sequence Associated)
OI	108	partial putative transposase
OI	108	partial putative transposase
OI	108	unknown protein encoded by prophage CP-933Y
OI	108	unknown protein encoded by prophage CP-933Y
OI	108	unknown protein encoded by prophage CP-933Y
OI	108	unknown protein encoded by prophage CP-933Y
OI	108	putative antiterminator of prophage CP-933Y
OI	108	unknown protein encoded by prophage CP-933Y

OI	108	unknown protein encoded by prophage CP-933Y
OI	108	unknown protein encoded by prophage CP-933Y
OI	108	IS30 transposase
OI	108	ORF; Other or unknown (Phage or Prophage Related)
OI	108	ORF; Other or unknown (Phage or Prophage Related)
OI	108	ORF; Other or unknown (Phage or Prophage Related)
OI	108	ORF; Other or unknown (Phage or Prophage Related)
OI	108	ORF; Other or unknown (Phage or Prophage Related)
OI	108	ORF; Other or unknown (Phage or Prophage Related)
OI	108	putative enzyme; Integration, recombination (Phage or Prophage Related)
OI	108	putative enzyme; Integration, recombination (Phage or Prophage Related)
OI	108	putative DNA binding protein
OI	108	ORF; Other or unknown (Phage or Prophage Related)
OI	135	ORF; hypothetical protein
OI	135	ORF; hypothetical protein
OI	172	putative integrase
OI	172	ORF; hypothetical protein in IS
OI	172	putative transposase
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	putative resolvase
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	partial putative integrase
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	172	putative ATP-dependent helicase
OI	172	ORF; Unknown function
OI	172	putative helicase
OI	172	putative helicase

OI	172	ORF; Unknown function
OI	172	ORF; Unknown function
OI	176	ORF; Unknown function
SL	72	putative integrase
SL	72	putative membrane protein
SL	72	transposase
SL	72	putative regulatory protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	IS30 transposase
SL	72	putative transposase
SL	72	putative complement resistance protein precursor
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	putative diacylglycerol kinase
SL	72	putative outer membrane protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	urease-associated protein UreD
SL	72	urease gamma subunit
SL	72	urease beta subunit
SL	72	urease alpha subunit
SL	72	urease accessory protein UreE
SL	72	urease accessory protein UreF
SL	72	urease accessory protein UreG
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	ribosomal protein L31-like protein
SL	72	hypothetical protein
SL	72	putative colicin immunity protein
SL	72	putative membrane protein
SL	72	hypothetical protein
SL	72	hypothetical protein

SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	TerW protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	putative tellurium resistance protein TerZ
SL	72	putative tellurium resistance protein TerA
SL	72	putative tellurium resistance protein TerB
SL	72	putative tellurium resistance protein TerC
SL	72	putative tellurium resistance protein TerD
SL	72	putative tellurium resistance protein TerE
SL	72	hypothetical protein
SL	72	putative tellurium resistance protein TerF
SL	72	hypothetical protein
SL	72	Iha adhesin
SL	72	hypothetical protein
SL	72	hypotheical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	putative glucosyl-transferase
SL	72	ferric enterochelin esterase
SL	72	transposase
SL	72	InsA protein of insertion sequence IS1
SL	72	putative membrane protein
SL	72	hypothetical protein
SL	72	hypothetical protein

SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	putative transposase ORFB protein of insertion sequence IS629
SL	72	putative transposase ORFA protein of insertion sequence IS629
SL	72	HecB-like protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	putative transcriptional regulator
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	BfpM-like protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	AidA-I adhesin-like protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	putative DNA repair protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein
SL	72	hypothetical protein

* The OI data is taken from the sequenced O157:H7 strain EDL933
** The SL data is taken from the sequenced O157:H7 strain Sakai