ABSTRACT

Megan Elizabeth Brock. TRANSCRIPTIONAL ANALYSIS OF THE *BACTEROIDES FRAGILIS* STARCH UTILIZATION OPERON, *OSUA*. (Under the direction of Dr. C. Jeffrey Smith) Department of Microbiology and Immunology, December 2011.

The opportunistic pathogen *Bacteroides fragilis* is a symbiotic organism that inhabits the human gastrointestinal tract where it utilizes dietary and host-derived polysaccharides as carbon and energy sources. If abdominal injury occurs, this otherwise commensal organism can migrate from the anaerobic environment of the large intestine to the more aerobic peritoneum. In this new extraintestinal environment, *B. fragilis* frequently contributes to the development of intra-abdominal abscesses, and is often the most common isolate from such anaerobic infections which can lead to systemic infections and death if left untreated. The organism's ability to shift from commensalist to pathogen is inextricably linked with the complex oxidative stress response (OSR) it has evolved. The studies described in this thesis have focused on the characterization of the promoter for the oxidative starch utilization operon, *osu*, and the identification of regulatory sequences involved in transcription activation during growth in maltose or exposure to oxygen. The results of this promoter deletional analysis study have demonstrated that the *osu* promoter is indeed responsive to both maltose and oxygen, and that regulatory regions important for activation of transcription in response to both stimuli are likely found within the same 50 bp region of the promoter. Consistent with this observation was the discovery of a LacI-type binding region in this site. In addition, studies demonstrated that there is the possibility of an additional weak oxygen-responsive promoter that exists in a separate region of the *osu* promoter. The previously identified transcriptional activator, OsuR, may also play a critical role in

transcription activation of the *osu* promoter, regardless of whether the inducing agent is maltose or oxygen. The mechanism of *osu* protection during oxidative stress is not fully understood, but results of this thesis offer a more complex model of transcriptional activation of the *osu* operon than was initially theorized. More studies will be necessary to further elucidate the role of *osu* in maintaining the OSR of *B. fragilis* during oxygen exposure.

TRANSCRIPTIONAL ANALYSIS OF THE *BACTEROIDES FRAGILIS* STARCH UTILIZATION OPERON, *OSUA*

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By

Megan Elizabeth Brock

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CHAPTER ONE

INTRODUCTION

The studies described in this thesis have focused on the genetics and physiology of the anaerobic opportunistic pathogen, *Bacteroides fragilis*. The ability of this species to withstand exposure to oxygen has been suggested to contribute to the organism's pathogenicity. In particular, the ability of *B. fragilis* to survive periods of oxidative stress is critical for its shift from a commensalist to an opportunistic pathogen. The premise of this research project was based on a previously identified four-gene operon which was found to be polycistronic, involved in both starch utilization and oxidative stress. Previous work suggested that expression of this operon may be important for survival during oxygen exposure. This operon, termed *osu*: oxidative starch utilization, has been evaluated for its role in the oxidative stress response of *B. fragilis* in this research. The objectives of this project were to further characterize this locus, its role in the oxidative stress response, and the regulation of *osu*. This thesis will provide an overview of the *Bacteroides* species and the specific niche associated with this anaerobic organism. In addition, general physiology and metabolism of *B. fragilis* will be discussed, particularly as it pertains to polysaccharide utilization and oxidative stress. Finally, the virulence factors and mechanisms that contribute to cell protection and the pathogenicity of the organism will be presented. An overview of other characterized gene regulators that contribute to the extraintestinal survival of *B. fragilis* will also be discussed.

1.1 The *Bacteroides*

Within the phylum *Bacteroidetes*, there are at least four phylogenetic groups that have been delineated, including the class of bacteria known as *Bacterioidia*. This class contains five families within the order *Bacteroidales*, including the family *Bacteroidaceae.* The human gastrointestinal tract contains anaerobic bacteria, with the predominant portion belonging to a genus in this family known as *Bacteroides* (17) Although the taxonomy of *Bacteroides* has undergone major revisions since their initial characterization, several criteria have emerged to categorize this pleomorphic group of bacteria. The *Bacteroides* species are a genus of Gram-negative, obligate anaerobes that are non-spore forming, non-motile rods (44). *Bacteroides* typically comprise a large portion of the normal microflora of the human colon, although some species can be isolated in sites that include the oral cavity and other regions of the gastrointestinal tract (35). The colon has the largest population of bacteria found in the human body, with approximately 10^{11} organisms per gram (wet weight) of feces. The commensal *Bacteroides* account for greater than 30% of the bacterial species found in this habitat, and play an integral role in the body's normal intestinal function (44). Such beneficial roles include the digestion and breakdown of complex carbohydrates, as well as the development of the immune system. Like many intestinal bacteria, *Bacteroides* are saccharolytic and are capable of breaking the glycosidic bonds found in polysaccharides. The products of this metabolism are then absorbed by the host in the large intestine. Thus, in addition to producing the energy necessary for their own growth, *Bacteroides* can also provide nutrients to the host from otherwise undigestable sources in a mutualistic fashion (3). Furthermore, *Bacteroides* help limit the colonization of potential pathogenic bacteria in the gastrointestinal tract (43). Recent studies have found that some metabolic products generated by *Bacteroides* species during carbohydrate

breakdown prevent proliferation of transient bacteria like *Clostridium difficile* by creating an inhospitable environment (13).

1.2 *B. fragilis* as an opportunistic pathogen

Despite these beneficial functions, *Bacteroides* species also exhibit potential as opportunistic pathogens. Although they are not invasive or deliberate pathogens, injury to the bowel can release bacteria into the peritoneum. After injury to the colon, the microflora located there can migrate to the peritoneal cavity, where some are able to colonize this new environment. Creating a new niche in the extra-intestinal space, these organisms frequently cause intra-abdominal infections. Generally, anaerobic infections in humans are characterized by the formation of extra-intestinal abscesses resulting from trauma to the abdomen, post-operative wound infections, appendicitis, diverticulitis, and bacteremia (11). Intestinal mucosal disease, including peptic ulcers and ulcerative colitis may also be the underlying cause of anaerobic infections. Of all anaerobic infections in humans, *Bacteroides* make up the majority of anaerobes present, with *B. fragilis* being the most commonly isolated organism of this genus (38).

Complications associated with such intra-abdominal infections include sepsis, shock, respiratory failure, and secondary peritonitis accompanying an abscess. The formation of such abscesses can complicate treatment and seriously hinder recovery from infection. In particular, treatment of the infection can become increasingly difficult, eventually requiring surgical intervention. Current therapies for such infections often involve surgical drainage of the intra-abdominal abscess and a course of antibiotics. Still, the impact of these anaerobic infections on

human health can be profound, and it is estimated that the mortality rate ranges from 5-40%, depending on the location and severity of abscesses (38).

The formation of an intra-abdominal abscess occurs in several steps. First, the host's immune response causes tissue inflammation at the site of inoculation. During this initiation phase, the organism must adhere to a surface and survive clearing mechanisms of the host tissue. Granuloma formation can result, and persistent granulomata eventually causes the formation of an abscess (12). Despite comprising less than 1% of the microbial species found in the human intestine, *B. fragilis* is considered one of the most clinically relevant organisms, as it is isolated in approximately 50% of intra-abdominal abscesses. *B. fragilis* is an important commensal organism within the human colon; however, outside of this normal habitat, its ability to colonize a new environment can make it a life-threatening pathogen.

1.3 Virulence Factors of *B. fragilis*

The pathogenicity of *Bacteroides fragilis* has been linked to several virulence factors, the most classic of which is the organism's polysaccharide capsule. It has been shown that *B. fragilis* produces eight unique polysaccharides which can act together in any combination to form the capsule. The result is a variable cell-surface structure that is an effective tool for evasion of the immune system's defenses. In fact, the formation of extra-intestinal abscess—which are the key feature of *B. fragilis* infections—is the immune system's response to the presence of the bacteria. In order to establish an infection, *B. fragilis* must first adhere to a tissue surface, while tolerating its newly aerobic environment. After adherence, an abscess can be formed where the organisms are now exposed to a more favorable, increasingly anaerobic

environment. The capsular polysaccharide expressed by *B. fragilis* is essential to its ability to adhere to mesothelial tissue found in the peritoneal cavity (21, 8). Furthermore, *B. fragilis* is known to have other cell surface structures that play a role in adherence and colonization, including adhesins, hemagglutinins, fimbriae, and proteases (19).

Another important virulence factor is neuraminidase, an enzyme involved in the attachment of *B. fragilis* organisms to host cells. Neuraminidase participates in the degradation of the protective mucin layer in the human colon, and thus is widely considered to increase the organism's virulence (6). This enzyme has also been found to have a role in energy metabolism during infection, by promoting the acquisition of carbon sources during the resource limited abscess phase (38).

One advantage of abscess formation is that the structure offers bacteria defense against the host immune response as well as antibiotics. In the case of *B. fragilis*, the species has an additional line of defense in its ability to acquire resistance genes. Resistance to all major classes of antibiotics has been observed in *Bacteroides* species, and it has been shown that these species are involved in genetic exchange both to and from other species. The close contact of *B. fragilis* and hundreds of other indigenous and transient bacterial species in the human intestine allows the transfer of mobilizable segments of DNA. In addition, due to its ability to survive extended periods of time in more aerobic environments, *B. fragilis* is able to exchange genetic information with aerobic organisms as well. This capability allows further diversification of antibiotic resistance in the organism (38).

Some strains of *B. fragilis* have been shown to produce enterotoxin, and are associated with diarrheal disease in humans and animals. These enterotoxigenic strains of *B. fragilis* (ETBF) are found in significantly higher numbers in isolates from individuals with diarrhea.

Recently, it was discovered that the *B. fragilis* enterotoxin (BFT) is actually a malleoprotease capable of hydrolyzing actin, tropomyosin, and fibrinogen. It has been theorized that this enzymatic activity facilitates the release of *B. fragilis* from abscesses, disseminating the bacteria into the bloodstream and contributing to its pathogenicity (15, 38)

Finally, one of the most important virulence factors is aerotolerance (41). Within the normal intestinal environment, oxygen levels are roughly 0%, while oxygen levels in the peritoneal cavity—the area outside the intestines—can be as high as 6-7%. This amount of oxygen represents a serious environmental/oxidative stressor, and yet *B. fragilis* is able to survive in this environment without replicating for at least 72 hours (31). Other factors that support the bacteria's ability to cause infection include its lipopolysaccharide (LPS or endotoxin) layer, its ability to acquire iron from host cells, and its inhibition of neutrophil activity (31, 38).

1.4 *B. fragilis* and Oxidative Stress

As one of the most aerotolerant anaerobes, it has been suggested that *B. fragilis* employs a complex oxidative stress response (OSR) to protect against the lethal effects of oxygen. Previous research has identified a gene regulon which encodes for proteins that are upregulated when the bacteria are exposed to environmental stress, i.e. oxygen. This regulator (OxyR) has been shown to induce a collection of genes encoding protective functions and adaptations that provide defense against oxidative stress (31, 30). For example, OxyR is responsible for the transcription of catalase (encoded by *katB*), alkyl hydroperoxide reductase (*ahpCF*), thioredoxin peroxidase (*tpx*), and the nonspecific DNA binding protein (*dps*), all of which play a role in detoxification and protection of the bacterial cell during oxygen exposure.

Further investigation into the OSR of *B. fragilis* has revealed additional genes, independent of the OxyR regulon, which are activated during oxygen exposure. These genes encode proteins whose functions are more metabolic in nature, such as aerobic ribonucleotide reductase (*nrdAB*), aspartate decarboxylase (*asdA*), and the oxygen induced starch utilization protein, *osu* (31). Microarray data have identified additional genes which are activated by oxidative stress, in the form of exposure to air (~21% O_2), H_2O_2, or 5% O_2. These genes included both OxyR-dependent and OxyR-independent genes, with functions that ranged from detoxification and metabolism, to nucleic acid repair, protein repair, and redox balance. By using several approaches, there have been postulated to be more than 300 genes induced during oxygen exposure in the absence of the OxyR regulon. Thus, the role of OxyR is an acute reaction to the presence of oxygen, involving detoxification, in an effort to curb the damaging effects of aerobic exposure (41).

Genetic studies on the induction of the OxyR regulon are consistent with its role as the primary protective reaction to the stress (31). In one study, expression of individual OxyR regulated genes—*ahpCF* and *katB*—was found to be upregulated by exposure to oxygen. Furthermore, mutants for these genes exhibited a significant decrease in cell viability over a 48 hour period, whereas the wild-type parent strain remained more viable during the same period (29). In a related study, researchers measured the induction of these and other OxyR-dependent genes at various oxygen concentrations in order to determine the sensitivity of the regulon to environmental stress. Results showed that induction was obtained at even the lowest oxygen concentrations (0.5%), though the highest levels of induction were demonstrated at 2% oxygen. Slightly lower induction levels of these OxyR genes were seen at 5% and 10% oxygen

concentrations (31). This correlates with the average oxygen levels found in normal, healthy human tissues, which typically ranges from 3-5%.

1.5 *B. fragilis* and the oxidative starch utilization operon, *osuABCD*

Another important aspect of the OSR exhibited by *B. fragilis* is energy generation and the upregulation of genes involved in this process during exposure to oxidative stress. The *Bacteroides* species utilize the abundant polysaccharides found in the bacteria's native environment--the gastrointestinal tract--as carbon and energy sources. In fact, the degradation of these polysaccharides is an important characteristic of the energy metabolism of most *Bacteroides* species (38). Several genes encoding metabolic enzymes have been found to be induced by aerobic exposure, including those responsible for the production of an aerobic ribonucleotide reductase, a cation efflux pump, an aspartate decarboxylase, and a starch binding membrane protein. These findings, along with those from another study indicating that glucose uptake is stimulated by oxygen, suggest that cellular metabolism in the bacteria undergoes modification during times of increased environmental/oxidative stress. In the case of starch utilization, this adaptation was shown to be mediated in *B. fragilis* by a four-gene operon termed the oxygen-induced starch utilization operon (*osuABCD*). Examination of this operon has revealed that these genes are induced by maltose, starch, and several other maltooligosaccharides when wild-type cells are grown anaerobically (39).

Interestingly, genetic homologues of the *osu* operon exist in *B. thetaiotaomicron*. In that organism, starch utilization is mediated by at least four structural genes (*susC*, *susD*, *susE*, and *susF*) which encode for outer membrane proteins that play a role in starch binding. In *B.*

thetaiotaomicron, a regulator of the *susCDEF* operon called SusR controls the transcription of the *sus* genes in response to the presence of starch or other polysaccharides (38). Further analysis into the similarities between the *sus* and *osu* operons suggests that the *susD* and *susF* genes code for the production of outer membrane proteins similar to those produced by the transcription of *osuB* and *osuC* genes in *B. fragilis*, with 34% and 24% nucleotide homology, respectively. Furthermore, the *susC* gene found in *B. thetaiotaomicron* resembles *osuA* with a 50% identity (39).

To characterize the role of the starch utilization operon (*osuABCD*) on the OSR of *B. fragilis*, similar studies tested the effects of oxygen on expression levels of these genes. When compared to anaerobically-grown cultures of *B. fragilis*, those exposed to oxygen for 1 hour showed a 20-fold increase in the expression of *osuA* in the wild-type strain. In addition, *osuA* mutants displayed a significant decrease in cell viability during the first 24 hours of oxygen exposure (39). Both of these experiments support the importance of *osu* genes for the survival of *B. fragilis* during times of oxidative stress, though further investigation is necessary to characterize the transcriptional regulation in these genes.

1.6 Conclusion

The objective of this project was to identify regulatory sequences in the promoter region of *osuA* that control induction by exposure to either a substrate or environmental stress. The specific hypothesis was that the *osuA* promoter is differentially regulated by maltose (starch) induction and environmental (oxidative) stress. Furthermore, it was anticipated that there would

be independent, cis-acting regions of the promoter that responded to these different types of activation.

CHAPTER TWO

EXPERMENTAL MATERIALS AND METHODS

2.1 Bacterial Strains and Growth Conditions

The strains utilized to characterize the *osu* promoter in response to oxidative stress or maltose induction are described in Table 2.1. *B. fragilis* IB101, the wildtype strain used in this study, is a rifampicin-resistant derivative of the clinical isolate 638R, isolated from an intestinal abscess (26). A second strain, designated IB393, is an *osuR* deletion mutant. IB393 is an isogenic *osuR* mutant strain with an in-frame deletion of the *osuR* gene (39).

Cultures were grown in Tryptone Yeast Extract media containing tryptone (10 g/L), yeast extract (10 g/L), and glucose (5 g/L) supplemented with 0.01% hemin, 0.02% $NaHCO_3$, 0.04% Salts Solution A ($CaCl_2$, $MgSO_4$, K_2HPO_4, KH_2PO_4, and NaCl), and cysteine (0.5 g/L). Solid media was obtained by adding agar (15 g/L) antibiotics appropriate for the specific strain. For selection of *B. fragilis* strains IB101 and IB393, antibiotics were added when required at the following concentrations (μg/ml): rifampicin (Rif) 20, gentamycin (Gen) 50, erythromycin (Erm) 10, ampicillin (Amp) 100, tetracycline (Tet) 10, kanamycin (Kan) 25. All cultures were incubated at 37°C in a Coy Laboratory Products Inc. anaerobic chamber with an atmosphere of 80% nitrogen, 10% carbon dioxide, and 10% hydrogen.

E. coli was used for routine DNA manipulations. The strain used was DH10β (F^- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ^-) (Invitrogen, Carlsbad, CA). All cultures were grown in Luria-Bertani (LB) medium (0.01% tryptone, 0.005% yeast extract, 0.005% NaCl) with appropriate antibiotics as described above.

2.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed using a Robocycler Gradient 40 thermal cycler (Stratagene, La Jolla, CA). An initial denaturation cycle was performed at 95°C for 3 minutes, followed by forty-five cycles of the following lengths and temperatures: 95°C melting for 30 seconds, 48-50°C annealing for 30 seconds, 72°C elongation for 1 minute and 40 seconds. A final elongation cycle of 10 minutes at 72°C was included to ensure the complete replication of all fragments. Afterwards, samples were kept at 6°C until gel electrophoresis could be performed. Each PCR reaction was performed in triplicate, with a reaction mixture as follows: 2.5 µl 10X Hi-fi PCR reaction buffer, 1 µl 10mM $MgSO_4$, 0.5 µl dNTPs, 2 µl of 10µM forward primer, 2 µl of 10µM reverse primer, 2 µl template DNA, 0.5 µl Hi-fi Taq polymerase, and up to 15 µl sterile distilled H_2O.

2.3 Cloning and Mutant Construction

The plasmid construct containing the transcriptional fusion of the *osu* promoter with the *XA* reporter gene, is designated pFD1078 (Figure 2.1). The construct was generated from the vector pFD1045 (7387 bp) with the addition of a reporter gene downstream of the *osu* promoter. The size of the resulting construct was 8546 bp. A transcriptional fusion of the *osuA* promoter and the xylosidase/arabinosidase gene, *XA* (Figure 2.2), was constructed in order to obtain a reproducible method to measure *osu* transcriptional activity in cells. The *XA* gene is a reporter gene (1159 bp) which is used to indicate the amount of transcriptional activity and expression of an associated promoter, both qualitatively and quantitatively, through the utilization of a whole cell chromogenic assay. As part of a xylan-inducible operon, the *XA* gene codes for the

xylosidase/arabinosidase enzyme which is responsible for the cleavage of the polysaccharide xylan. Because most *Bacteroides* species, including *B. fragilis*, are not capable of effectively degrading xylan, *XA* can be used as a reporter gene. The xylosidase/arabinosidase activities were assayed using p-nitrophenol derivative substrates in whole cell assays (45).

The 8546 bp pFD1078 plasmid contains ampicillin resistance (Amp^R) crucial for growth and screening in *E. coli*, as well as erythromycin resistance (Erm^R) for screening in *Bacteroides* species. The XA reporter gene is fused 170 base pairs downstream of the *osu* promoter, and both are flanked by various restriction sites corresponding to cut sites for the deletion mutagenesis procedure described later. The pFD1078 construct was mated into *B. fragilis* in the presence of the *E. coli* helper strain RK231 utilizing a tri-parental mating procedure (36). This procedure transfers recombinant plasmids from *E. coli* to a *Bacteroides* recipient via bacterial conjugation. Gel electrophoresis and restriction digests were used to confirm the structure.

2.4 Deletion Mutagenesis Strategy

A deletion mutagenesis strategy (24) was employed in order to create separate identifiable regions of the promoter that could be investigated using xylosidase/arabinosidase activity assays (Figure 2.3). First, a set of deletions was made from the 3' end and 5' end of the promoter region utilizing PCR amplification and the primers included in Table 2.2. Primers were designed using the PstI site at the 5' end, and the upstream NarI site at the 3' end. The resulting promoter fragments were ligated into a NarI/PstI double digested pFD1078 vector in *E. coli*. Recombinant clones were confirmed using restriction digests and gel electrophoresis.

Correct clones then were transferred into the *Bacteroides* strains IB101 or IB393 using the tri-parental mating protocol described previously.

2.5 Xylosidase/Arabinosidase Assays

To determine xylosidase/arabinosidase activity of the transcriptional fusions in vivo, a whole-cell xylosidase-arabinosidase assay was performed. *B. fragilis* cells containing the appropriate plasmid were grown in Tryptone Yeast Extract Glucose, Maltose or Xylose (TYG, TYM, or TYX) broth to mid-logarithmic phase (A_{550} 0.3-0.6) and then cells were harvested by centrifugation. The harvested cells were suspended in 1.35 ml of 50 mM sodium phosphate buffer (pH 6.8) and the cell suspension was divided equally into three separate 1.7 ml microcentrifuge tubes (450 µl per tube). Fifty microliters CTAB solution (0.05% hexadecyltrimethylammonium bromide + 10 mM dithiothreitol in 50 mM sodium phosphate buffer) was added to each tube, which was then gently mixed and incubated in a 37°C water bath for 15 minutes. Next, 10 µl of 100 mM p-nitrophenyl-β-D-xylopyranoside in DMSO (PNPX) was added to each reaction, and incubated at 37°C for between 15 and 60 minutes. The reaction was stopped by addition of 50 µl of 2% sodium carbonate, and the cells were removed by centrifugation. The OD of the supernatant solution was measured at A_{405} and recorded.

To quantify and normalize the results of the whole cell color-change assay, the following formula was used:

$$[A_{405} / (T \times V \times A_{550})] \times 1000,$$

where T is equal to time of assay in minutes, and V is the volume of cells used in the assay

calculated to the original culture using the formula below.

V = (volume in assay ÷ volume of suspended cells) x original culture volume

2.6 Initial Characterization of *osu*

Overnight cultures of IB101(pFD1078) were grown anaerobically in 5 ml TYG (0.5% glucose) with the appropriate antibiotics. Strains were then subcultured, grown anaerobically, and their OD at A_{550} was measured and recorded over time. Results were graphed as Absorbance versus Time on a scatter plot.

Xylosidase/arabinosidase assays were also performed on the collected samples and this data was used to create an Activity curve. Results were graphed as average Activity versus Time on a scatter plot.

2.7 Induction by Substrate

Triplicate cultures of *B. fragilis* strains were grown anaerobically overnight in TYX (0.3% xylose) with the appropriate antibiotics. The strains were then subcultured in TYX and grown to an A_{550} of ~0.30 in mid-logarithmic phase. At this point, 1 M maltose solution was added to one flask containing culture, to reach a final concentration of 10 mM, which was then marked as "+maltose" Likewise, 1 M glucose solution was added to a second flask containing culture, to reach a final concentration of 10 mM, which was then labeled as "+glucose." The third flask contained no additional substrate, and served as the xylose control. Growth of the cultures (OD at A_{550}) was measured over time and, at each timepoint, a 2.5 ml sample of each culture was removed, added to a sterile 14 ml Falcon tube, and centrifuged at 8000 rpm for 10

minutes to pellet the cells. After decanting the supernatant, the cells were frozen in order to perform xylosidase/activity assays at a later time. Results were graphed as average Activity versus Time on a scatter plot.

2.8 Induction by Environmental Stress

Two separate overnight cultures of *B. fragilis* strains were prepared in TYG with appropriate antibiotics, and were grown anaerobically at 37°C. The strains were then subcultured in the same media to an OD of approximately 0.30 (A_{550}). At this point, one flask of culture was marked "aerobic" and placed in an aerobic incubator at 37°C with constant shaking; the second flask was marked "control" and left in the anaerobic incubator at the same temperature. After exposure to air in the aerobic sample, growth was continually measured in both samples. At each timepoint, a 2.5 ml sample was removed from each culture and added to a 14 ml sterile Falcon tube. Cultures were centrifuged at 8000 rpm for 10 minutes to pellet the cells, and after decanting the supernatant, cell pellets were either assayed or frozen for later use. Results were graphed as average Activity versus Time on a scatter plot.

Figure 2.1. Functional map of pFD1078. The map was determined from the nucleotide sequence of the plasmid and shows the relevant genes and functional regions. Several restriction enzyme recognition sites used during the course of this work are indicated.

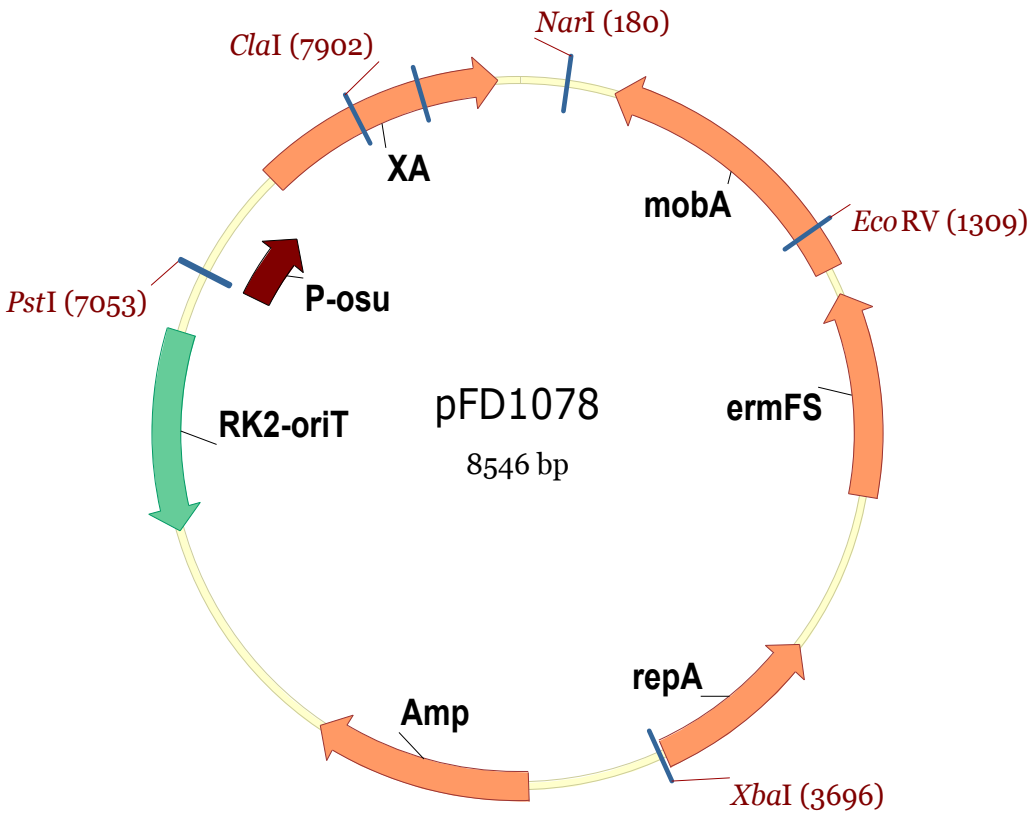
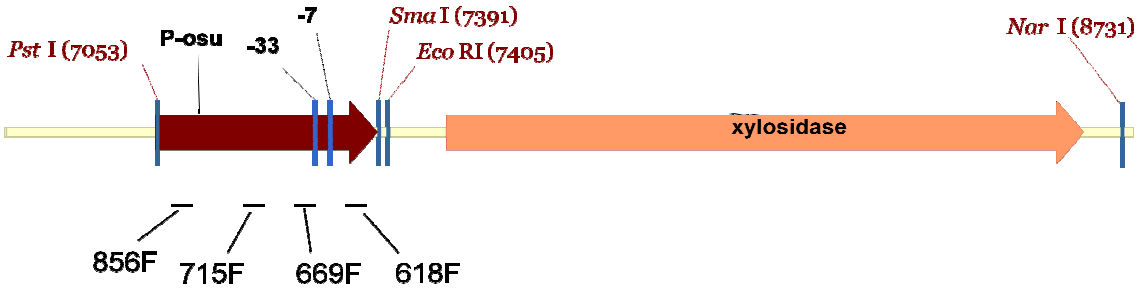

ClaI (7902)
NarI (180)
XA
mobA
EcoRV (1309)
PstI (7053)
P-osu
pFD1078
8546 bp
RK2-oriT
ermFS
repA
Amp
XbaI (3696)

Figure 2.2. Transcriptional fusion of *osu* promoter and *XA* gene. The xylosidase gene is shown by the orange arrow and the *osu* promoter region, P-osu, is indicated by the red arrow. The putative -7 and -33 motifs of the promoter are also indicated.



Promoter Region pFD1078

osuA : XA fusion

Figure 2.3. Deletion strategy for course mapping of the *osuA* promoter region. Triangles represent the putative OsuR binding site and black rectangles are the promoter -7 and -33 regions plus the transcriptional start site. The arrowheads represent the fusion boundary. Numbers correspond to deletion mutants.

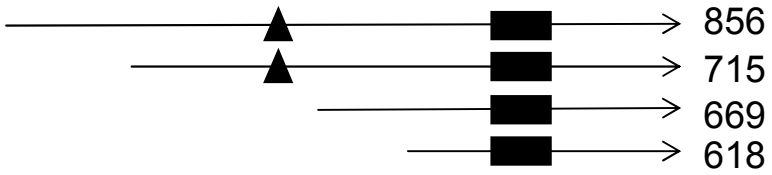

856
715
669
618

Table 2.1. *Bacteroides* strains used in this study.

Strain	Phenotype	Source
B. fragilis		
IB101	Wildtype derived from clinical isolate 638R, Rif^R	(26)
IB393	ADB77 derived from 638R, Δ*osuR*, $thyA^+$, Rif^R	(39)
E.coli		
DH10B	F^- end A1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ^-	Invitrogen
Plasmids		
pFD1078	8.5 kb, *osuA* promoter plasmid containing P-osu and the xylosidase reporter gene, Erm^R (Amp^R)	This study
pMEB856	Full length *osuA* promoter cloned into NarI/PstI restriction sites (Erm^R), derived from pFD1078	This study
pMEB757	99 bp deletion of upstream *osuA* promoter, double-digested with NarI/PstI and cloned into NarI/PstI restriction sites (Amp^R, Erm^R)	This study
pMEB715	141 bp deletion of upstream *osuA* promoter, double-digested with NarI/PstI and cloned into NarI/PstI restriction sites (Amp^R, Erm^R)	This study
pMEB669	187 bp deletion of upstream *osuA* promoter, double-digested with NarI/PstI and cloned into NarI/PstI restriction sites (Amp^R, Erm^R)	This study
pMEB618	238 bp deletion of upstream *osuA* promoter, double-digested with NarI/PstI and cloned into NarI/PstI restriction sites (Amp^R, Erm^R)	This study

Table 2.2. Primers used in current study for cloning of deletion mutants into pFD1078.

Primer	Sequence[a]	Insert Length
856F (full-length)	agctctgcagACGCCAAGCTTGCATGCCTG	1678 bp
799F	agctctgcagCCGGATCGATGCTGTAACTTCC	1621 bp
757F	agctctgcagTGTAAAGGAGACTGAAAAGTTCAG	1579 bp
715F	agctctgcagCCGCTTTTATGATGCAATCG	1537 bp
669F	agctctgcagTTTGAATTTAAATAAGCGACTG	1491 bp
618F	agctctgcagTCAAGAATCGGGATGAAGTC	1440 bp
539F	agctctgcagATGCATGAAGCAAGTTGGA	1361 bp
1078Nar (reverse)	ATACCGCACAGATGCGTAAGGA	-------

[a] Primers contained 5' leader with PstI site shown in lowercase letters. The 1078Nar primer was used for all cloning and matched a region of pFD1078 just downstream of the NarI site.

CHAPTER THREE

RESULTS

3.1 Optimization of Xylosidase/Arabinosidase Assay

In order to establish the linearity of this assay, it was necessary to optimize the procedure for the strains used in this study. First, the amount of cells used in each assay was optimized by inoculating fresh media with overnight cultures of IB101(pFD1078) and cells were harvested after 4 hours of incubation. Xylosidase endpoint assays were then performed for 60 minutes and the absorbance (A_{405}) was measured for either 2.5 ml or 5 ml samples. The results of this study are contained in Figure 3.1. When 5 ml samples of culture were used in the assay, absorbance values were significantly higher overall for all samples. As a result of the large cell number, absorbance values were out of the linear range and hence were difficult to compare (Figure 3.1). In contrast, decreasing the cell volume added to the assay led to significantly lower absorbance values, and therefore increased ability to compare samples and their activity values. In this way, experimentation based on changes in media or environmental stress yielded data that were more easily observed in subsequent studies with this organism.

Similarly, the length of time in assay was varied in order to decrease background and increase the amount of measurable difference in activity between samples. Media was inoculated with overnight culture containing IB101(pFD1078), which were then grown for 4 hours. Endpoint xylosidase assays were performed on 5 ml samples removed from each culture, and an assay length of 15 or 30 minutes was carried out. Absorbance values (A_{405}) at the end of each assay showed that decreased time in assay resulted in absorbance values in the linear range for all samples (Figure 3.2A). The effect of assay time on the units of activity is shown in Figure

3.2B. Samples treated in assay for 15 minutes displayed higher activity values for all strains, as compared to the 30 minute samples (Figure 3.2B). The assay was thus optimized for the high-yield growth found with pFD1078 in liquid media so that 2.5 ml cells in mid-log phase were harvested for each sample, and the second incubation at 37°C was performed for the minimum 15 minutes after the addition of the PNPX substrate.

3.2 Effect of Growth Phase on *osu*

Within an abscess, *B. fragilis* will develop through a complete growth cycle ending in stationary phase. Upon entry into stationary phase, the organism encounters a shift in nutrient availability; where nutrients were once plentiful, they are now in short supply. At any stage, the organism can undergo a variety of different stresses. To determine the growth effect of strains containing pFD1078 with *osuA* under normal, anaerobic conditions, initial studies were performed to characterize growth. Strain IB101(pFD1078) was grown anaerobically in both glucose and maltose-based media, during which time the optical density of these cultures was measured (Figure 3.3).

For these experiments, growth in glucose and maltose media showed very similar growth curves, with growth in maltose exhibiting slightly higher absorbance values over time. Maltose is a disaccharide and is phosphorylated upon being transported into the cell. Thus, maltose may be more energy efficient and lead to higher growth yields.

Xylosidase activity was also determined at several of the growth timepoints using the optimized assay described above (Figure 3.4). The average activity values during growth phase induction of strain IB101(pFD1078) indicate that the promoter for the *osu* operon is more active

when grown in maltose media, and it is active earlier in the growth cycle. Thus, the presence of maltose appears to be inductive under normal, anaerobic conditions.

Figure 3.1. Optimization of xylosidase/arabinosidase assay cell volume. Overnight cultures of IB101(pFD1078) were grown in TYG and used to inoculate fresh media after anaerobic growth at 37°C. Cells were harvested after 4 hours of incubation and endpoint 60 min xylosidase assays were performed using 2.5 ml or 5 ml of culture. Absorbance (OD A_{405}) was measured for assayed samples.

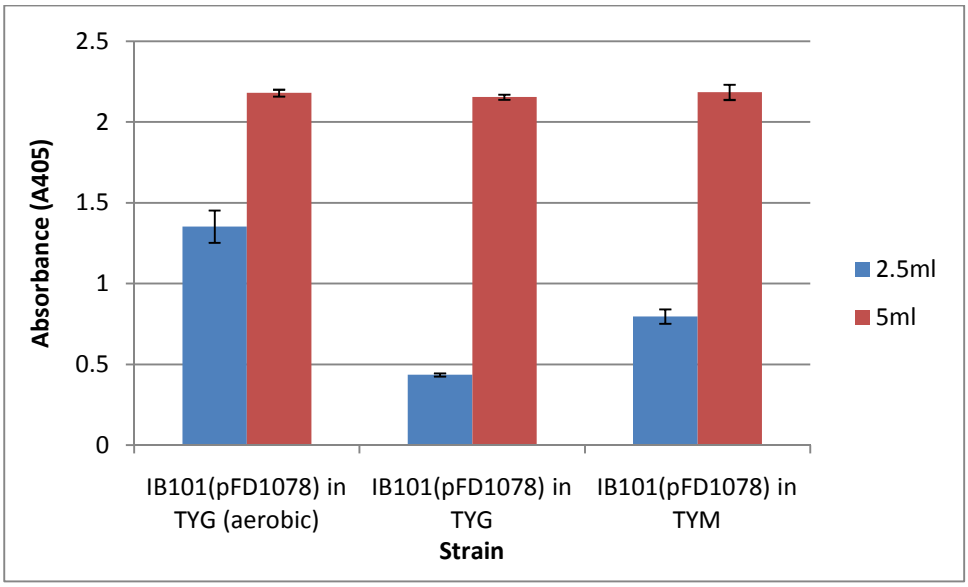
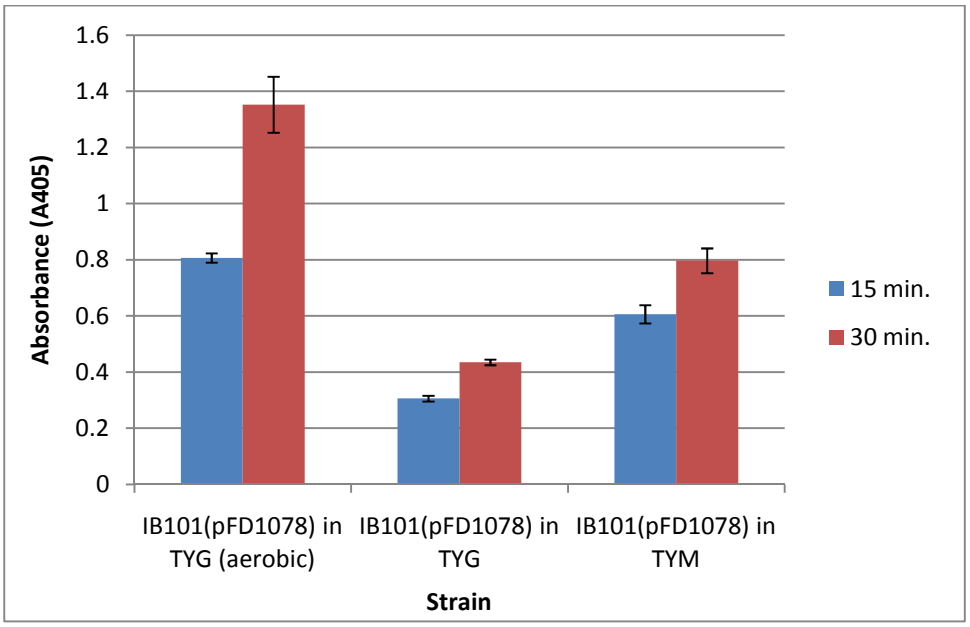

2.5
2
1.5
1
0.5
0
Absorbance (A405)
2.5ml
5ml
IB101(pFD1078) in TYG (aerobic)
IB101(pFD1078) in TYG
IB101(pFD1078) in TYM
Strain

Figure 3.2. Optimization of xylosidase/arabinosidase assay length. Overnight cultures of IB101(pFD1078) were grown in TYG and used to inoculate fresh media after anaerobic growth at 37°C. Cells were harvested after 4 hours of incubation and endpoint xylosidase assays of either 15 min or 30 min were performed using 2.5 ml of culture. Absorbance (OD A_{405}) was measured for assayed samples (A) and activity values were calculated for these samples (B).

A.


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance (A405)
15 min.
30 min.
IB101(pFD1078) in TYG (aerobic)
IB101(pFD1078) in TYG
IB101(pFD1078) in TYM
Strain

B.

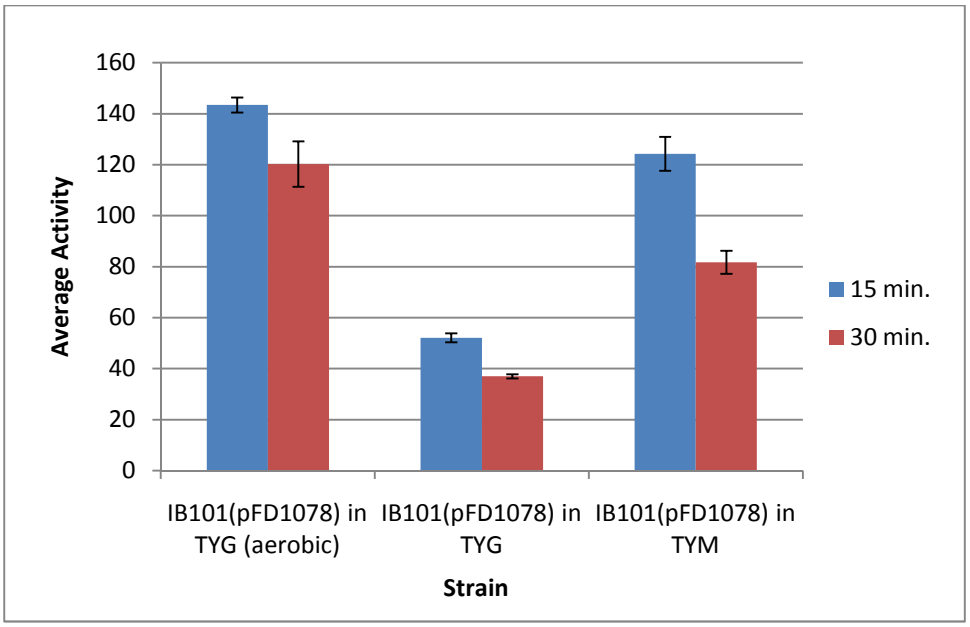

160
140
120
100
80
60
40
20
0
Average Activity
15 min.
30 min.
IB101(pFD1078) in TYG (aerobic)
IB101(pFD1078) in TYG
IB101(pFD1078) in TYM
Strain

Figure 3.3. Growth of IB101(pFD1078) in TYG or TYM. Overnight cultures of IB101(pFD1078) were grown anaerobically in 5 ml TYG (0.5% glucose) with 10 µg/ml erythromycin and the culture was used to inoculate separate 125 ml Erlenmeyer flasks containing 100 ml fresh TYG (0.5% glucose) or TYM (0.5% maltose). At the indicated timepoints, samples were removed and their OD at A_{550} was measured and recorded. Data are represented as the mean of three independent experiments.

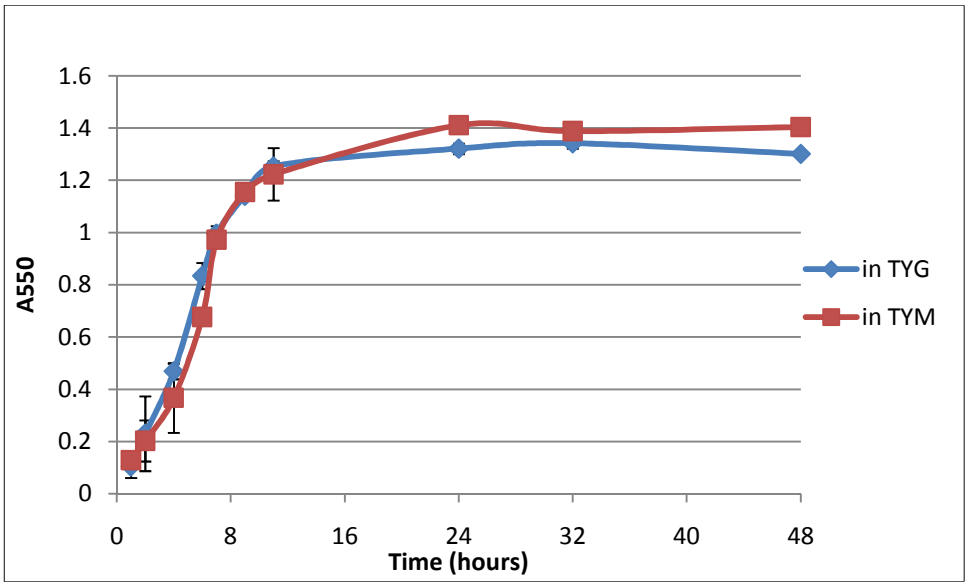
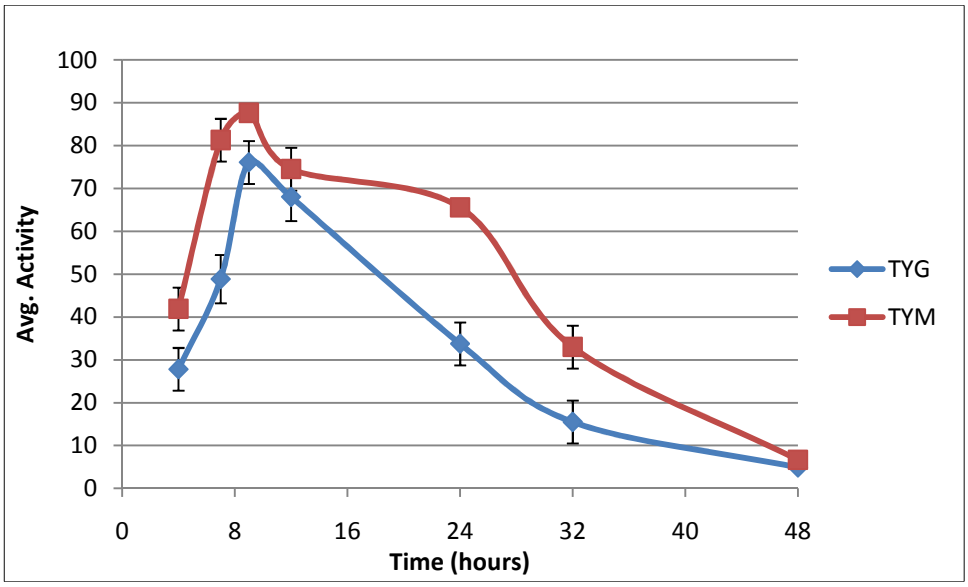

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A550
0
8
16
24
32
40
48
Time (hours)
in TYG
in TYM

Figure 3.4. P-osu activity during anaerobic growth. Overnight cultures of IB101(pFD1078) were grown anaerobically in 5 ml TYG (0.5% glucose) with 10 μg/ml erythromycin and the culture was used to inoculate separate 125 ml Erlenmeyer flasks containing 100 ml fresh TYG (0.5% glucose) or TYM (0.5% maltose). At different stages of growth, samples were removed and used for xylosidase/arabinosidase transcriptional activity assays. Data are represented as the mean of three independent experiments.


100
90
80
70
60
50
40
30
20
10
0
Avg. Activity
0
8
16
24
32
40
48
Time (hours)
TYG
TYM

3.3 Induction of *osuA* by Maltose

In a separate study designed to characterize the induction of *osuA* by substrate, the potential effects of catabolite repression and induction by maltose were studied. Catabolite repression is understood to be part of a global control mechanism utilized by various microorganisms, in which bacteria are able to quickly adapt to a preferred carbon and energy source. In order to measure the presence of this induction, xylosidase activity of mid-log phase anaerobic cultures grown in either 0.3% glucose, 0.3% maltose, or 0.3% xylose, was compared.

Using the xylosidase activity data from this experiment, the kinetics of maltose induction were able to be determined (Figure 3.5). As compared to the addition of glucose, cultures exposed to maltose showed faster and higher *osu* transcriptional activity in the first two hours after addition of the sugars.

In order to determine whether the regulatory protein OsuR may affect catabolite repression and/or maltose induction, xylosidase activity was measured in the *osuR* mutant grown to mid-log phase on xylose media, and then induced with maltose. A mutant lacking *osuR* expression would be expected to exhibit decreased *osuA* induction and thus, decreased xylosidase activity compared to the wildtype. The appropriate strain was constructed by conjugal transfer of pFD1078 into the *osuR* mutant, IB393. After successful mating of pFD1078 into IB393, maltose induction experiments were carried out, just as in the previous experiments (Figure 3.6).

Figure 3.5. P-osu activity of IB101(pFD1078) following addition of maltose or glucose to mid-log phase xylose grown cells. Triplicate cultures of *B. fragilis* strains were grown anaerobically overnight in 5 ml TYX (0.3% xylose) with the appropriate antibiotics. Strains were subcultured in 50 ml TYX (no resazurin, no antibiotics) and grown to an OD ~0.3 (A_{550}). Cultures then were supplemented with either 0.5 ml 1 M maltose solution, 0.5 ml 1 M glucose solution, or no supplement. Samples then were taken at: 10 min, 20 min, 40 min, 60 min (T_1), 2 h, 3 h, and 4 h. Data are represented as the mean of three independent experiments.

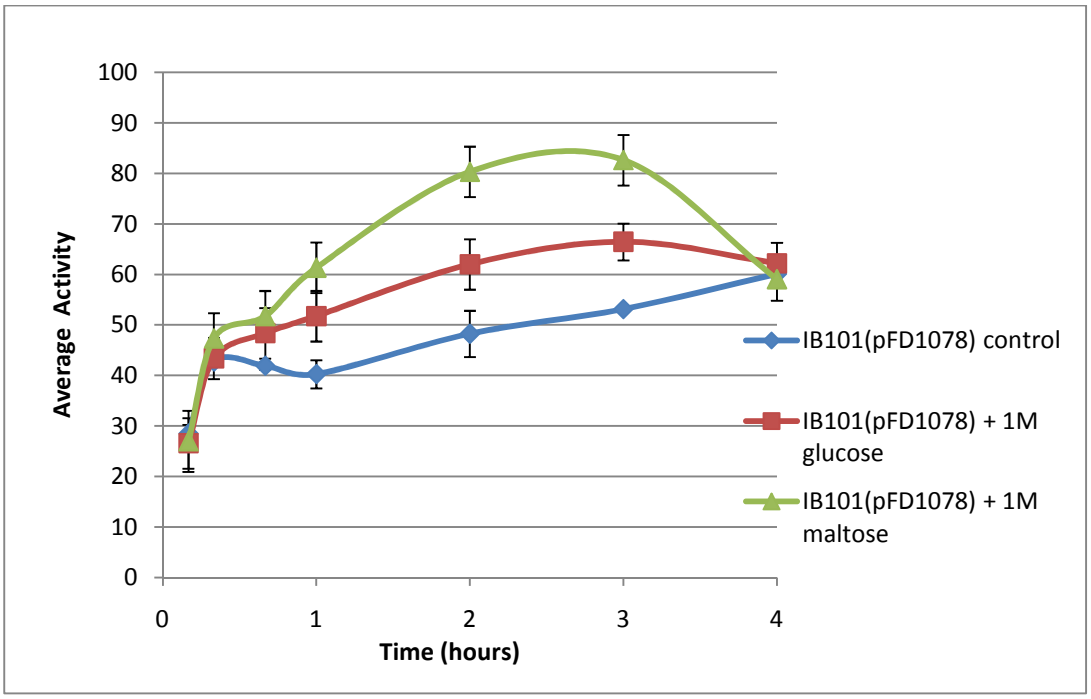
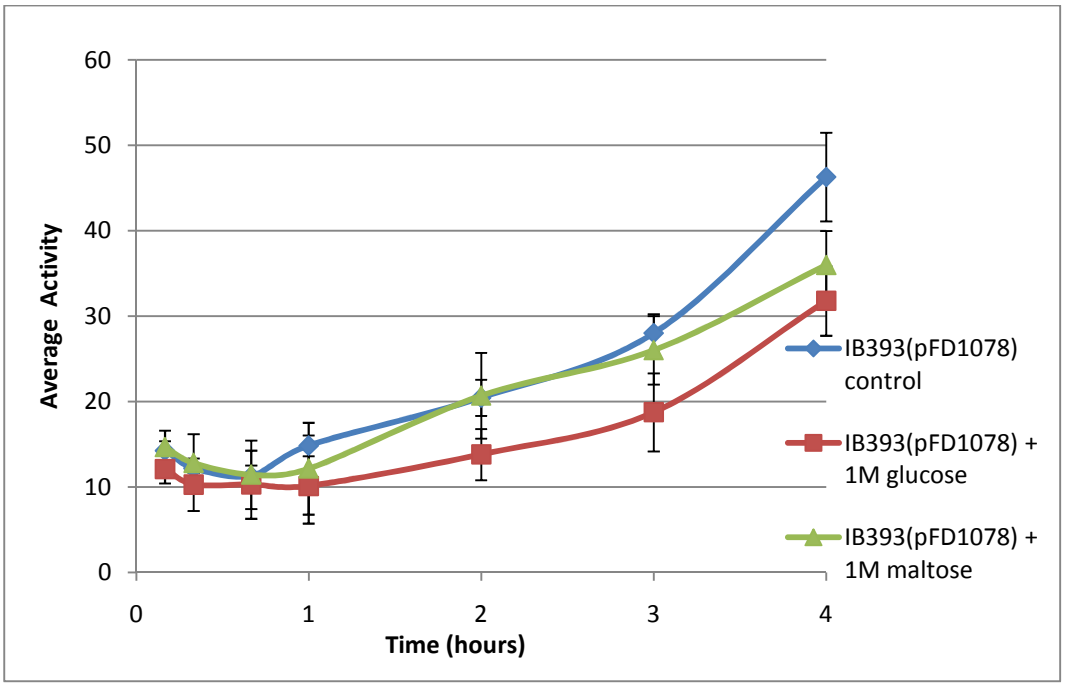

100
90
80
70
60
50
40
30
20
10
0
Average Activity
0
1
2
3
4
Time (hours)
IB101(pFD1078) control
IB101(pFD1078) + 1M glucose
IB101(pFD1078) + 1M maltose

Figure 3.6. P-osu activity of IB393(pFD1078) following addition of maltose or glucose to mid-log phase xylose grown cells. Triplicate cultures of *B. fragilis* strains were grown anaerobically overnight in 5 ml TYX (0.3% xylose) with the appropriate antibiotics. Strains were subcultured in 50 ml TYX (no resazurin, no antibiotics) and grown to an OD ~0.3 (A_{550}). Cultures then were supplemented with either 0.5 ml 1 M maltose solution, 0.5 ml 1 M glucose solution, or no supplement. Samples then were taken at: 10 min, 20 min, 40 min, 60 min (T_1), 2 h, 3 h, and 4 h. Data are represented as the mean of three independent experiments.


60
50
40
30
20
10
0
Average Activity
0
1
2
3
4
Time (hours)
IB393(pFD1078) control
IB393(pFD1078) + 1M glucose
IB393(pFD1078) + 1M maltose

These data indicate that there was no significant induction by maltose or glucose in the *osuR* mutant for the first 3 hours. As cells began to enter the stationary phase of growth at approximately 4 hours, there was some increase in activity, though this was shown to be nearly 60% less than the maximum maltose induction. This suggests that osuR is an important activator of expression in *B. fragilis*.

3.4 Induction of *osuA* by Environmental Stress

To further establish baseline information for regulation if the *osu* xylosidase fusion, induction by environmental stress was examined. A time course of xylosidase induction was determined in glucose grown cultures exposed to air. The xylosidase activity observed in IB101(pFD1078) cells shaken in an aerobic incubator for 48 hours is shown in Figure 3.7. These data demonstrate a clear relationship between activity in the *osu* promoter and oxidative stress presented by exposure to air. Though growth was hindered in the aerobic sample compared to the control, the promoter activity was significantly higher over the time course.

Oxidative induction of *osu* was measured in the *osuR* mutant IB393, to determine if this positive regulator affected the inducible response. Data for the xylosidase/activity assays performed after air induction of strain IB393(pFD1078) is given in Figure 3.8. As in the previous experiment, it was determined that aerobic induction of xylosidase does occur in the *osuR* mutant, with significant increases in activity over the anaerobic control. However, average xylosidase activity was decreased in both of these samples comapred to the wildtype.

Figure 3.7. P-osu activity of IB101(pFD1078) glucose grown cells following exposure to air. Two separate overnight cultures of *B. fragilis* strains were prepared in 5 ml TYG with appropriate antibiotics, and were grown anaerobically at 37°C. Strains then were subcultured in 100 ml TYG (no resazurin, no antibiotics) to an OD of approximately 0.3 (A_{550}). One flask of culture was added to aerobic shaker, while a control culture was grown anaerobically. Growth was continually measured in both samples at the following timepoints: 10 min, 20 min, 40 min, 60 min, 1 h (T_1), 2 h, 4 h, 12 h, 24 h, and 48 h. Data are represented as the mean of four independent experiments.

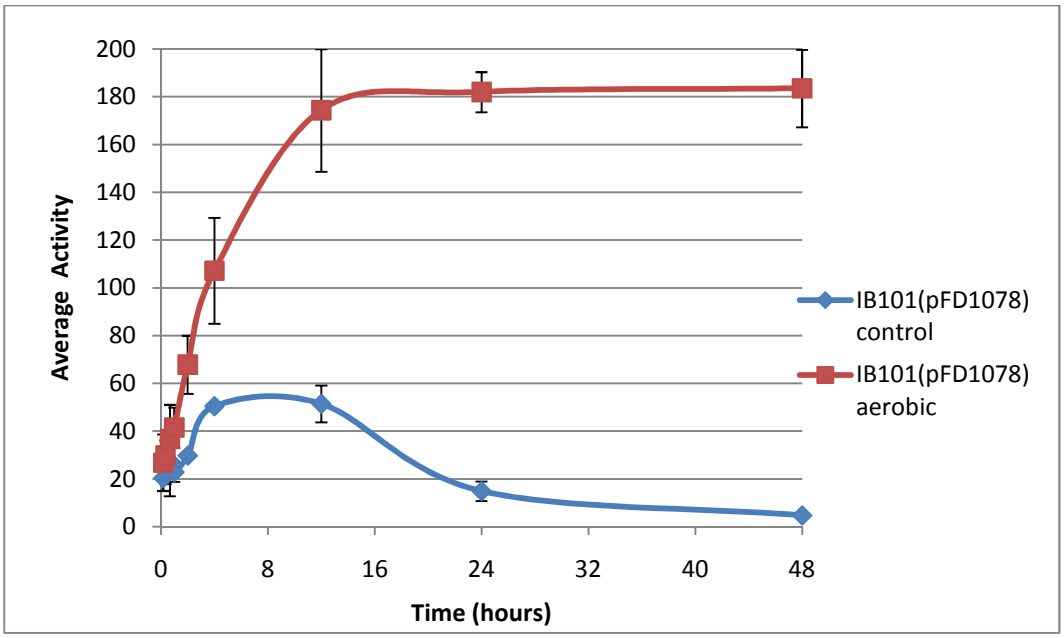
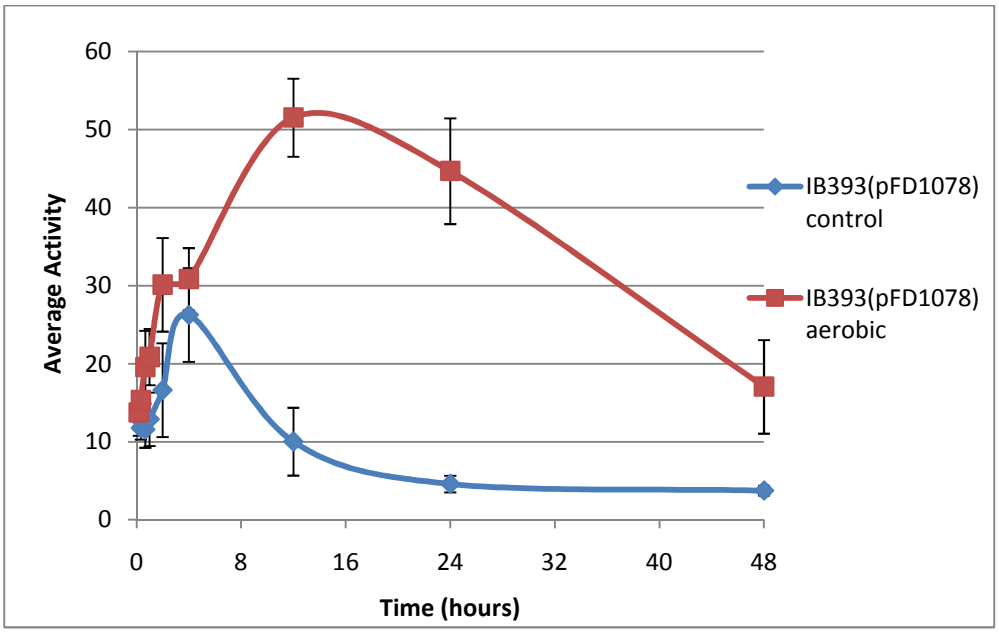

200
180
160
140
120
100
80
60
40
20
0
Average Activity
0
8
16
24
32
40
48
Time (hours)
IB101(pFD1078) control
IB101(pFD1078) aerobic

Figure 3.8. P-osu activity of IB393(pFD1078) glucose grown cells following exposure to air. Two separate overnight cultures of *B. fragilis* strains were prepared in 5 ml TYG with appropriate antibiotics, and were grown anaerobically at 37°C. Strains then were subcultured in 100 ml TYG (no resazurin, no antibiotics) to an OD of approximately 0.3 (A_{550}). One flask of culture was added to aerobic shaker, while a control culture was grown anaerobically. Growth was continually measured in both samples at the following timepoints: 10 min, 20 min, 40 min, 60 min, 1 h (T_1), 2 h, 4 h, 12 h, 24 h, and 48 h. Data are represented as the mean of three independent experiments.


60
50
40
30
20
10
0
Average Activity
0
8
16
24
32
40
48
Time (hours)
IB393(pFD1078) control
IB393(pFD1078) aerobic

3.5 Effect of P-osu Deletions on Maltose Induction

The previously described experiments demonstrated that *osu* transciption responded to two different stimuli, thus the aim of the experiment described here was to determine if specific, separable regions of the *osu* promoter are responsible for induction by air and maltose. To accomplish this goal, xylosidase activity was measured for each deletion mutant under the following conditions: 1) anaerobic cultures grown in TYG to mid-log phase; 2) aerobic exposure of TYG cultures (described in Chapter 3.6); and 3) anaerobic cultures grown in TYX followed by the addition of maltose. Activity data will be used to identify the presence of cis-acting regulatory regions in the approximately 350 bp promoter region.

As a control, the full-length promoter region was amplified by PCR from pFD1078 using the 856F and 1078Nar primers, and then was digested using PstI and NarI enzymes. After digestion, the full-length *osu* promoter fragment was ligated into a similarly digested pFD1078 plasmid. The resulting product was a plasmid of the same length as the original pFD1078 and was designated pMEB856. Maltose induction experiments were performed following the same procedure outlined previously, and as expected, these experiments yielded growth and activity curves similar to those seen in strain IB101(pFD1078) (Figure 3.9). Compared to glucose, the addition of maltose resulted in higher transcriptional activity in the first 3 hours following induction.

The next deletion examined was mutant IB101(pMEB715). Analysis of xylosidase activity data after glucose or maltose addition to culture showed activity levels similar to wildtype levels (Figure 3.10). Based on these findings, strain IB101(pMEB669) was used in subsequent experiments, as it represented the next smallest deletion mutant.

Figure 3.9. Activiy of the IB101(pMEB856) following addition of maltose or glucose to mid-log phase xylose grown cells. Triplicate cultures of *B. fragilis* strains were grown anaerobically overnight in 5 ml TYX (0.3% xylose) with the appropriate antibiotics. Strains were subcultured in 50 ml TYX (no resazurin, no antibiotics) to an OD ~0.3 (A_{550}). Cultures were supplemented with either 0.5 ml 1 M maltose solution, 0.5 ml 1 M glucose solution, or no supplement. Growth (OD at A_{550}) and xylosidase activity were measured at the following timepoints: 10 min, 20 min, 40 min, 60 min (T_1), 2 h, 3 h, and 4 h. Data are represented as the mean of three independent experiments.

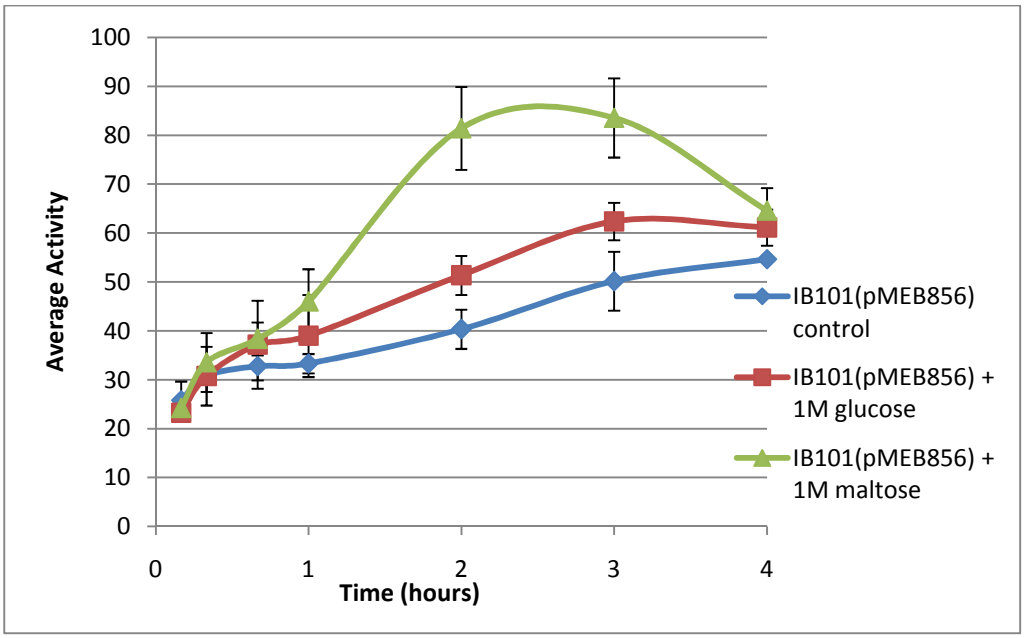
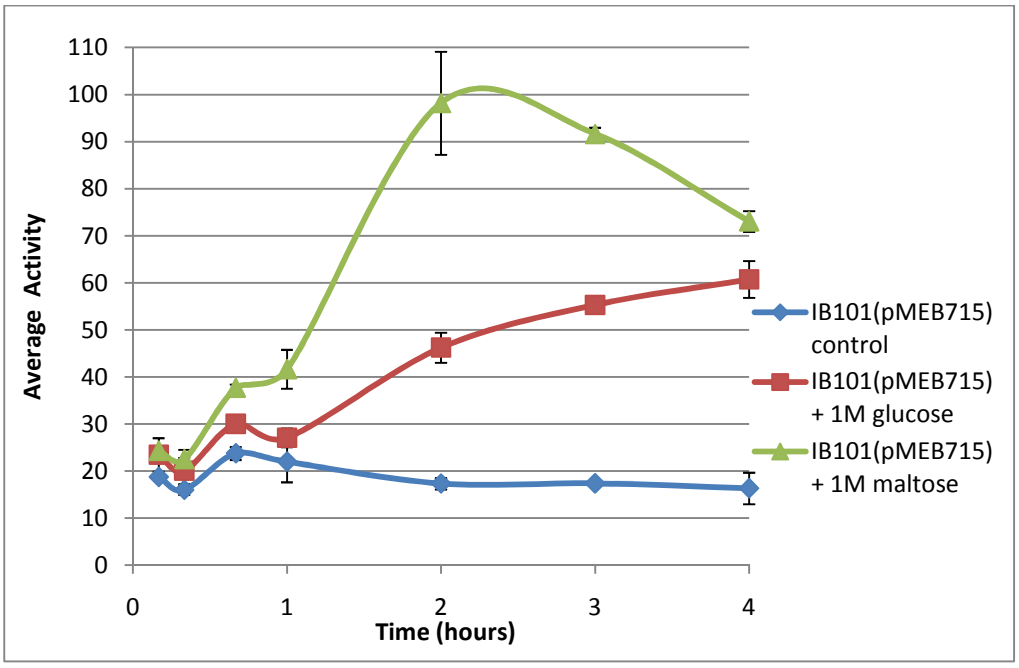

100
90
80
70
60
50
40
30
20
10
0
Average Activity
0
1
2
3
4
Time (hours)
IB101(pMEB856) control
IB101(pMEB856) + 1M glucose
IB101(pMEB856) + 1M maltose

Figure 3.10. Activity of the IB101(pMEB715) following addition of maltose or glucose to mid-log phase xylose grown cells. Triplicate cultures of *B. fragilis* strains were grown anaerobically overnight in 5 ml TYX (0.3% xylose) with the appropriate antibiotics. Strains were subcultured in 50 ml TYX (no resazurin, no antibiotics) to an OD ~0.3 (A_{550}). Cultures were supplemented with either 0.5 ml 1 M maltose solution, 0.5 ml 1 M glucose solution, or no supplement. Growth (OD at A_{550}) and xylosidase activity were measured at the following timepoints: 10 min, 20 min, 40 min, 60 min (T_1), 2 h, 3 h, and 4 h. Data are represented as the mean of three independent experiments.


110
100
90
80
70
60
50
40
30
20
10
0
Average Activity
0
1
2
3
4
Time (hours)
IB101(pMEB715) control
IB101(pMEB715) + 1M glucose
IB101(pMEB715) + 1M maltose

Results revealed that transcriptional activity of this deletion was substantially decreased for all cultures, including those where glucose or maltose was added (Figure 3.11A). Neither the addition of maltose nor glucose was sufficient to increase xylosidase activity above the control, a trend which was initially observed in the wildtype.

In order to confirm the loss of xylosidase activity in the shortened promoter, transciptional activity was measured in IB101(pMEB618), the smallest *osu* promoter fragment cloned into the wildtype. Data from these experiments demonstrate that *osu* transciptional activity in this clone showed a similar pattern to IB101(pMEB669) (Figure 3.11B). Xylosidase activity was significantly decreased for all conditions over time compared to the wildtype. Induction with maltose was not restored in this strain, a finding consistent with the previous data.

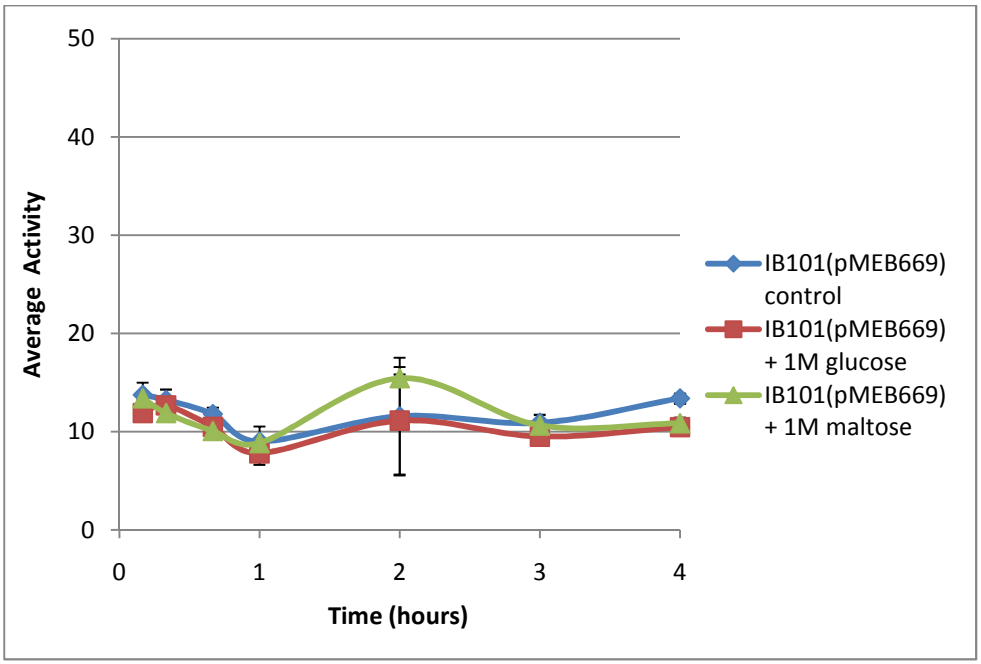
In order to determine whether these results could be due, in part, to the presence of the maltose regulator, OsuR, induction experiments in the *osuR* mutant were performed. Studies revealed that, with the full length *osuA* promoter region, induction with maltose was not observed (Figure 3.12, 39). However, there was some growth phase associated activity observed in all cultures tested. Subsequent studies analyzed the remaining deletion mutants in strain IB393. The expectation was that the results of those experiments would mirror previous data obtained with deletion mutants in the wildtype however, this was not the case. The deletion mutant plasmids in the *osuR* mutant showed a different pattern of induction, with IB393(pMEB715) and IB393(pMEB669) showing no significant activity for any of the culture conditions tested (Figure 3.13A and B, respectively).

Examination of the activity of all clones at 3 hours post-addition of maltose revealed the pattern of induction described above in a clear and organized way. Of note is the high transcriptional activity in strain IB101(pMEB856), even for the control, as well as the significant

decrease in promoter activity in strain IB101(pMEB669) and all deletion mutants in strain IB393 (Figure 3.14).

Figure 3.11. Activity of strains IB101(pMEB669) (A) and IB101(pMEB618) (B) following addition of maltose or glucose to mid-log phase xylose grown cells. Triplicate cultures of *B. fragilis* strains were grown anaerobically overnight in 5 ml TYX (0.3% xylose) with the appropriate antibiotics. Strains were subcultured in 50 ml TYX (no resazurin, no antibiotics) to an OD ~0.3 (A_{550}). Cultures were supplemented with either 0.5 ml 1 M maltose solution, 0.5 ml 1 M glucose solution, or no supplement. Growth (OD at A_{550}) and xylosidase activity were measured at the following timepoints: 10 min, 20 min, 40 min, 60 min (T_1), 2 h, 3 h, and 4 h. Data are represented as the mean of three independent experiments.

A.


50
40
30
20
10
0
Average Activity
0
1
2
3
4
Time (hours)
IB101(pMEB669) control
IB101(pMEB669) + 1M glucose
IB101(pMEB669) + 1M maltose

B.

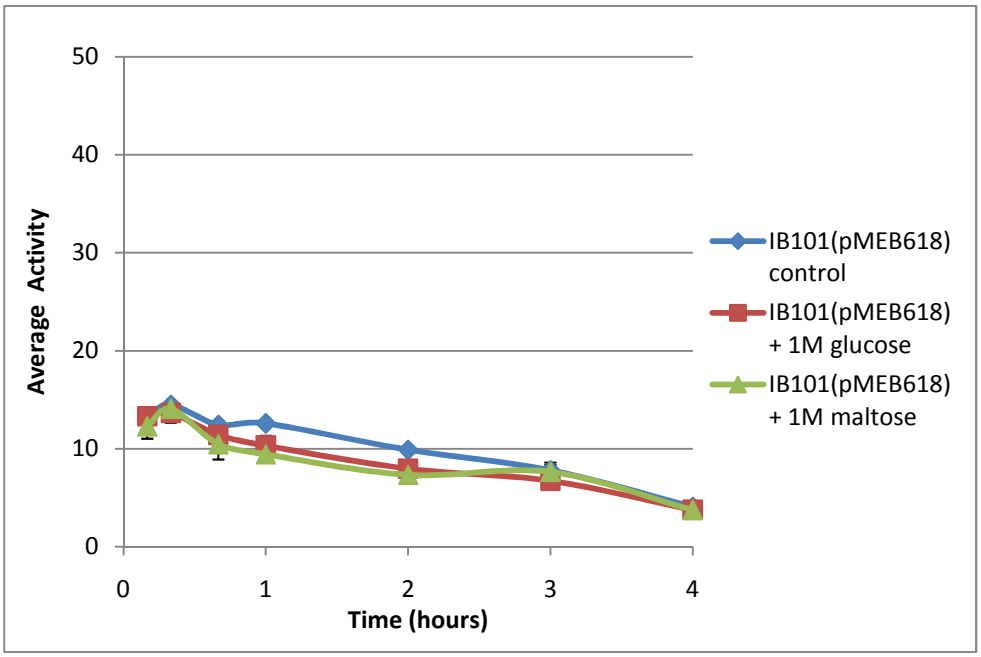

50
40
30
20
10
0
Average Activity
0
1
2
3
4
Time (hours)
IB101(pMEB618) control
IB101(pMEB618) + 1M glucose
IB101(pMEB618) + 1M maltose

Figure 3.12. Activity of the IB393(pMEB856) following addition of maltose or glucose to mid-log phase xylose grown cells. Triplicate cultures of *B. fragilis* strains were grown anaerobically overnight in 5 ml TYX (0.3% xylose) with the appropriate antibiotics. Strains were subcultured in 50 ml TYX (no resazurin, no antibiotics) to an OD ~0.3 (A_{550}). Cultures were supplemented with either 0.5 ml 1 M maltose solution, 0.5 ml 1 M glucose solution, or no supplement. Growth (OD at A_{550}) and xylosidase activity were measured at the following timepoints: 10 min, 20 min, 40 min, 60 min (T_1), 2 h, 3 h, and 4 h. Data are represented as the mean of three independent experiments.

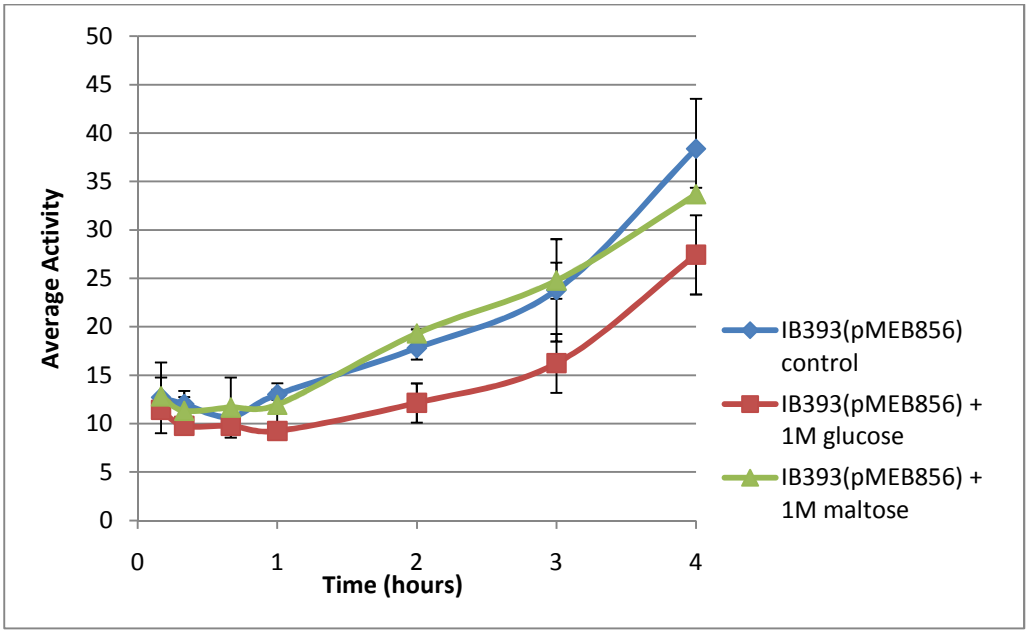
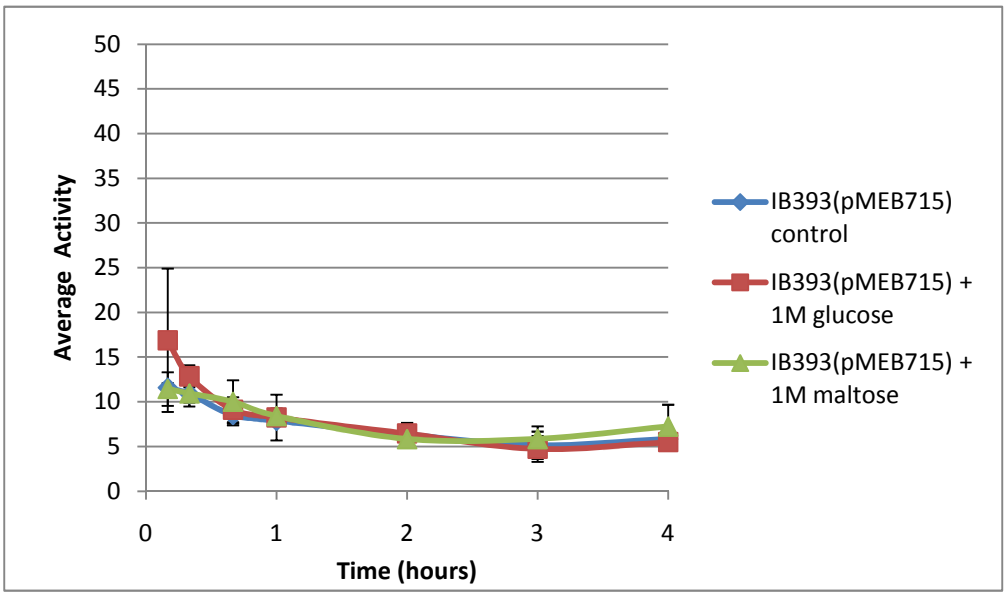

50
45
40
35
30
25
20
15
10
5
0
Average Activity
0
1
2
3
4
Time (hours)
IB393(pMEB856) control
IB393(pMEB856) + 1M glucose
IB393(pMEB856) + 1M maltose

Figure 3.13. Activity of the IB393(pMEB715) (A) and IB393(pMEB669) (B) following addition of maltose or glucose to mid-log phase xylose grown cells. Triplicate cultures of *B. fragilis* strains were grown anaerobically overnight in 5 ml TYX (0.3% xylose) with the appropriate antibiotics. Strains were subcultured in 50 ml TYX (no resazurin, no antibiotics) to an OD ~0.3 (A_{550}). Cultures were supplemented with either 0.5 ml 1 M maltose solution, 0.5 ml 1 M glucose solution, or no supplement. Growth (OD at A_{550}) and xylosidase activity were measured at the following timepoints: 10 min, 20 min, 40 min, 60 min (T_1), 2 h, 3 h, and 4 h. Data are represented as the mean of three independent experiments.

A.


50
45
40
35
30
25
20
15
10
5
0
Average Activity
0
1
2
3
4
Time (hours)
IB393(pMEB715) control
IB393(pMEB715) + 1M glucose
IB393(pMEB715) + 1M maltose

B.

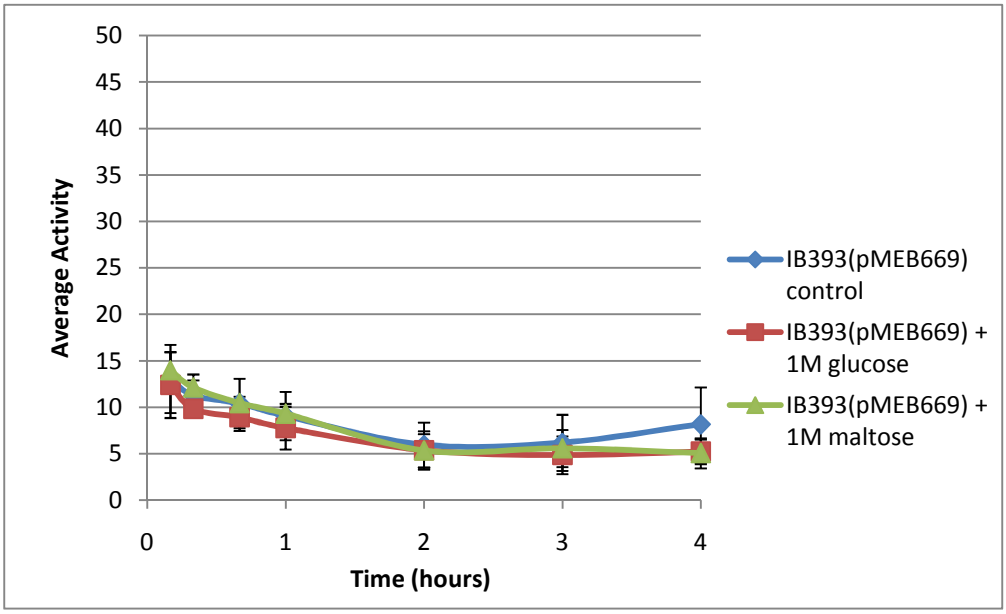
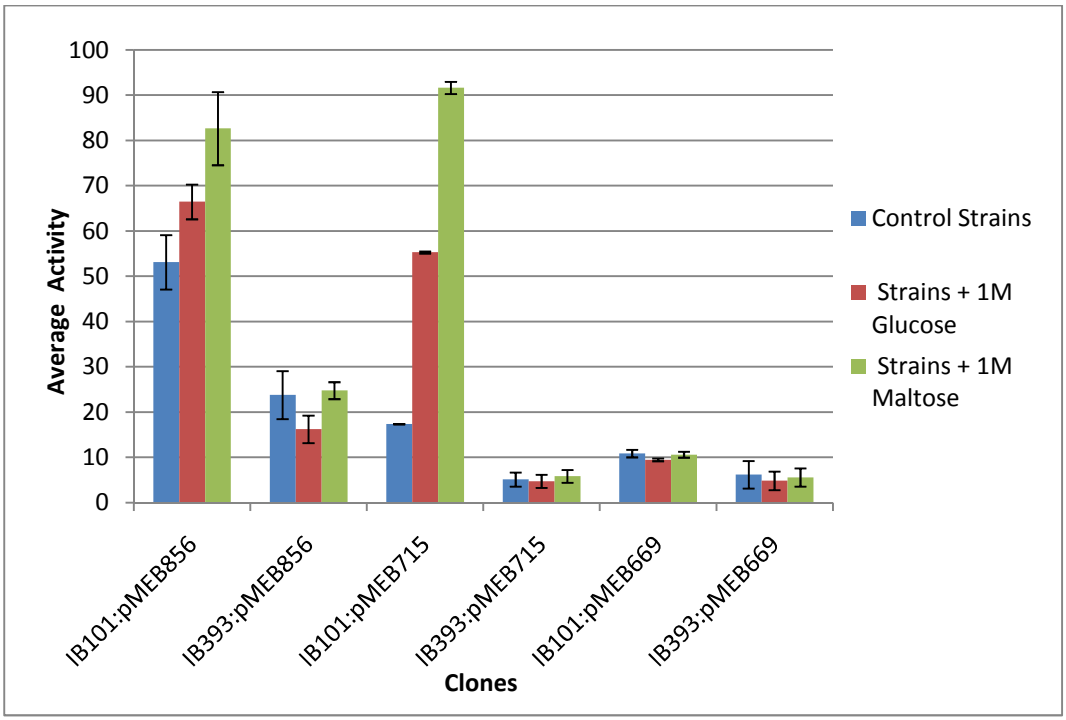

50
45
40
35
30
25
20
15
10
5
0
Average Activity
0
1
2
3
4
Time (hours)
IB393(pMEB669) control
IB393(pMEB669) + 1M glucose
IB393(pMEB669) + 1M maltose

Figure 3.14. Activity of all strains at 3 hrs of growth following addition of maltose or glucose to mid-log phase xylose grown cells. Summary data was compiled after maltose induction experiments were performed, as described previously. Data are represented as the mean of three independent experiments.


100
90
80
70
60
50
40
30
20
10
0
Average Activity
Control Strains
Strains + 1M Glucose
Strains + 1M Maltose
IB101:pMEB856
IB393:pMEB856
IB101:pMEB715
IB393:pMEB715
IB101:pMEB669
IB393:pMEB669
Clones

3.6 Effect of P-osu Deletions on Environmental Stress Induction

The *osuA* gene was initially identified during an experiment to screen for genes upregulated by oxidative stress, suggesting that it was oxygen responsive (37). We therefore set out to further elucidate the potential role of this operon in the oxidative stress response of *B. fragilis* by identifying regulatory region(s) of this promoter that are oxygen inducible.

The full-length promoter region control plasmid, pMEB856, was tested for oxygen induction following the procedure outlined previously. Transcriptional activity data from those experiments showed data consistent with strain IB101(pFD1078), as expected (Figure 3.15). The next deletion mutant to be evaluated was strain IB101(pMEB715). After exposure to environmental stress, this strain continued to show high amounts of *osu* transciptional activity in the aerobic sample when compared to the control (Figure 3.16). The data also resembled strain IB101(pMEB856) in xylosidase activity over time. As a result of these findings, the next deletion mutant to be tested was strain IB101(pMEB669). Studies revealed that, in this mutant, *osu* activity was substantially decreased (Figure 3.17A). Despite exposure to oxygen, the aerobic sample of this strain did not overtake the anaerobic control in terms of transciptional activity. In fact, the activity of the anaerobic control was lower overall than in strain IB101(pFD1078) or IB101(pMEB715). To confirm the loss of this activity, the smallest deletion mutant in the wildtype strain, IB101(pMEB618), was investigated under the oxygen induction protocol.

Figure 3.15. Activity of IB101(pMEB856) glucose grown cells following exposure to air. Two separate overnight cultures of *B. fragilis* strains were prepared in 5 ml TYG with appropriate antibiotics, and were grown anaerobically at 37°C. Strains then were subcultured in 100 ml TYG (no resazurin, no antibiotics) to an OD of approximately 0.3 (A_{550}). One flask of culture was added to aerobic shaker, while a control culture was grown anaerobically. Growth was continually measured in both samples at the following timepoints: 10 min, 20 min, 40 min, 60 min, 1 h (T_1), 2 h, 4 h, 12 h, 24 h, and 48 h. Data are represented as the mean of three independent experiments.

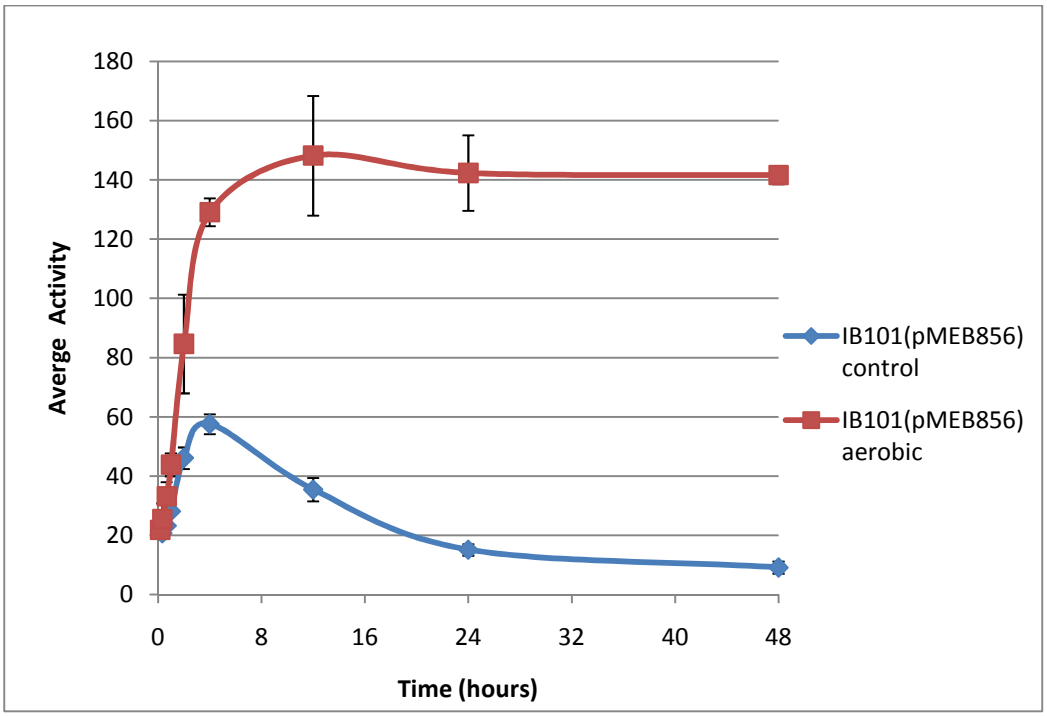

180
160
140
120
100
80
60
40
20
0
Averge Activity
0
8
16
24
32
40
48
Time (hours)
IB101(pMEB856) control
IB101(pMEB856) aerobic

Figure 3.16. Activity of IB101(pMEB715) glucose grown cells following exposure to air. Two separate overnight cultures of *B. fragilis* strains were prepared in 5 ml TYG with appropriate antibiotics, and were grown anaerobically at 37°C. Strains then were subcultured in 100 ml TYG (no resazurin, no antibiotics) to an OD of approximately 0.3 (A_{550}). One flask of culture was added to aerobic shaker, while a control culture was grown anaerobically. Growth was continually measured in both samples at the following timepoints: 10 min, 20 min, 40 min, 60 min, 1 h (T_1), 2 h, 4 h, 12 h, 24 h, and 48 h. Data are represented as the mean of three independent experiments.

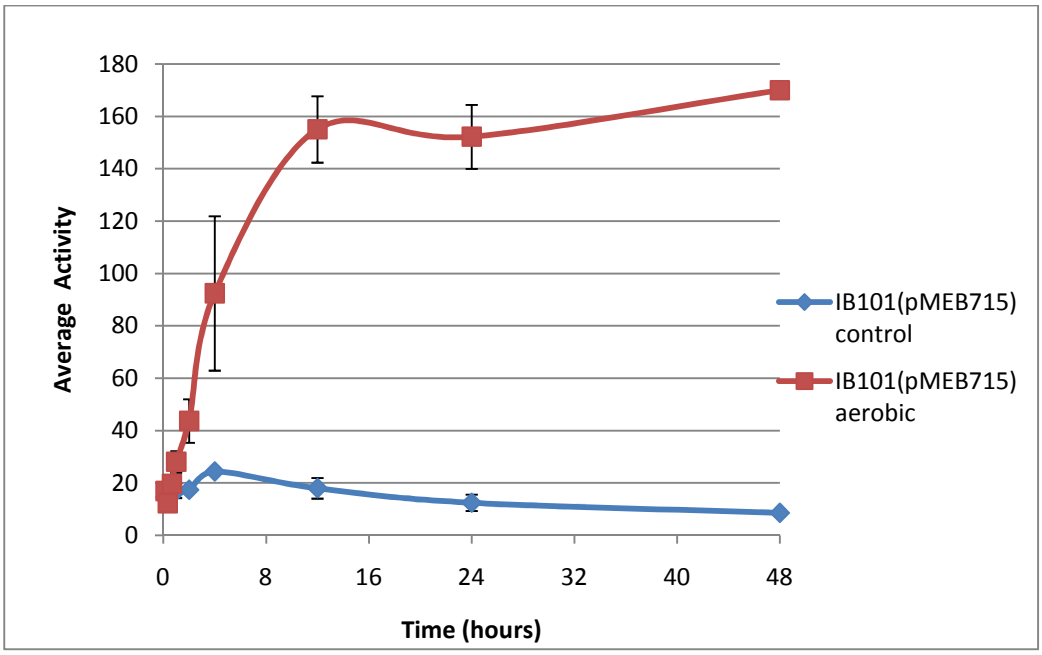
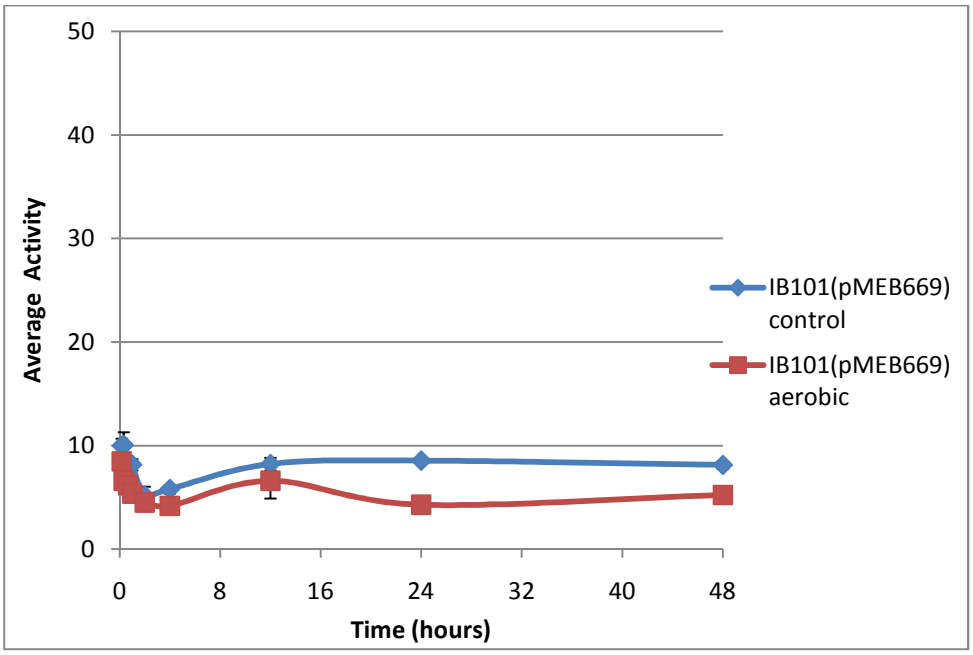
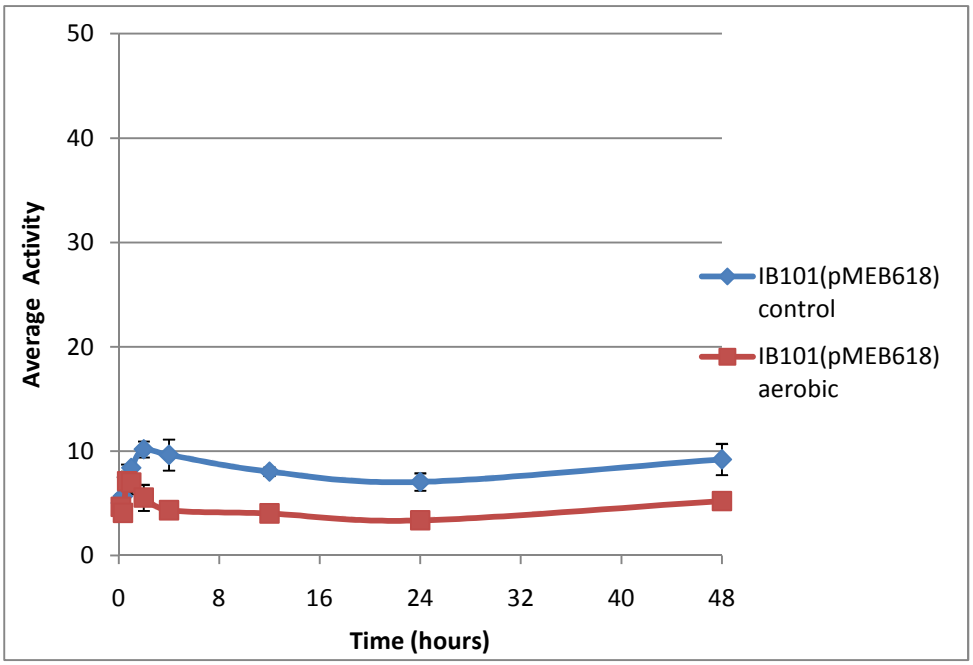

180
160
140
120
100
80
60
40
20
0
Average Activity
0
8
16
24
32
40
48
Time (hours)
IB101(pMEB715) control
IB101(pMEB715) aerobic

Figure 3.17. Activity of strains IB101(pMEB669) (A) and IB101(pMEB618) (B) glucose grown cells following exposure to air. Two separate overnight cultures of *B. fragilis* strains were prepared in 5 ml TYG with appropriate antibiotics, and were grown anaerobically at 37°C. Strains then were subcultured in 100 ml TYG (no resazurin, no antibiotics) to an OD of approximately 0.3 (A_{550}). One flask of culture was added to aerobic shaker, while a control culture was grown anaerobically. Growth was continually measured in both samples at the following timepoints: 10 min, 20 min, 40 min, 60 min, 1 h (T_1), 2 h, 4 h, 12 h, 24 h, and 48 h. Data are represented as the mean of three independent experiments.

A.


50
40
30
20
10
0
Average Activity
0
8
16
24
32
40
48
Time (hours)
IB101(pMEB669) control
IB101(pMEB669) aerobic

B.


50
40
30
20
10
0
Average Activity
0
8
16
24
32
40
48
Time (hours)
IB101(pMEB618) control
IB101(pMEB618) aerobic

As in the IB101(pMEB669) deletional mutant, strain IB101(pMEB618) showed almost no transciptional activity of the *osu* promoter, despite exposure to environemtnal stress for 48 hours (Figure 3.17B). These data indicate that the site of a regulatory sequence within the promoter is likely located between the IB101(pMEB715) and IB101(pMEB669) promoter fragments.

To test the possibility of an OsuR role in oxygen induction, the deletion mutants were evaluated in the *osuR* mutant, IB393. First, the effects of environmental stress on xylosidase activity of IB393 containing the full length promoter was measured. As expected, the results of this study showed that *osuA* transciptional activity was maintained in this strain, just as in strain IB101(pMEB856) (Figure 3.18). In subsequent studies, the remaining deletions were tested in IB393. For both strains IB393(pMEB715) and IB393(pMEB669), we observed little to no significant transciptional activity, compared to the control (Figure 3.19A and B). In response to oxygen exposure, *osuA* activity was not detected in either strain as was originially anticipated.

In order to compare this oxygen induction data for all deletion mutants, a summary figure was created that represented transcriptional activity of each deletion and condition measured at 12 hours post-oxygen exposure (Figure 3.20). Of particular note in this figure are the high levels of activity observed in the aerobically-grown strains IB101(pMEB856) and IB101(pMEB715), as well as a decrease in activity in strain IB393(pMEB856). It is evident from this summary figure that although activity in the aerobically-grown strain IB393(pMEB856) was significantly higher than the anaerobic control, this activity was decreased compared the wildtype. Furthermore, activity was lost in strain IB101(pMEB669), as well as the remaining IB393 deletion mutants (Figure 3.20).

Figure 3.18. Activity of IB393(pMEB856) glucose grown cells following exposure to air. Two separate overnight cultures of *B. fragilis* strains were prepared in 5 ml TYG with appropriate antibiotics, and were grown anaerobically at 37°C. Strains then were subcultured in 100 ml TYG (no resazurin, no antibiotics) to an OD of approximately 0.3 (A_{550}). One flask of culture was added to aerobic shaker, while a control culture was grown anaerobically. Growth was continually measured in both samples at the following timepoints: 10 min, 20 min, 40 min, 60 min, 1 h (T_1), 2 h, 4 h, 12 h, 24 h, and 48 h. Data are represented as the mean of three independent experiments.

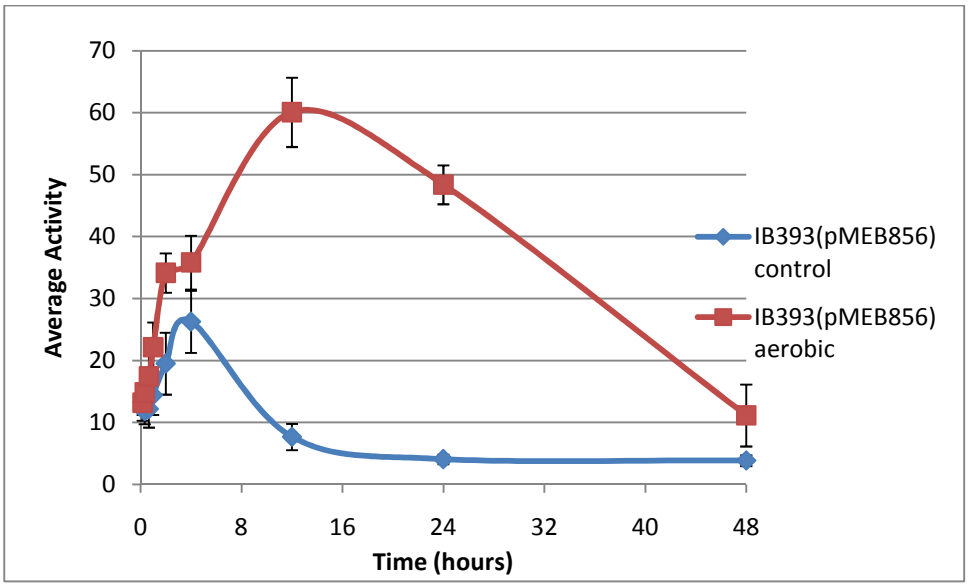
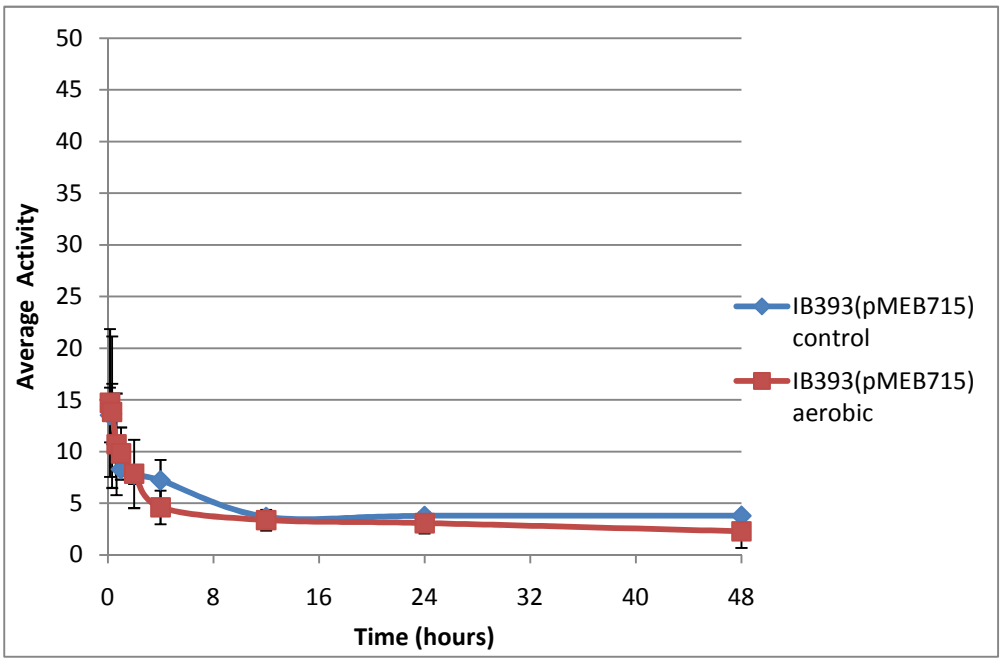

70
60
50
40
30
20
10
0
Average Activity
0
8
16
24
32
40
48
Time (hours)
IB393(pMEB856) control
IB393(pMEB856) aerobic

Figure 3.19. Activity of IB393(pMEB715) (A) and IB393(pMEB669) (B) glucose grown cells following exposure to air. Two separate overnight cultures of *B. fragilis* strains were prepared in 5 ml TYG with appropriate antibiotics, and were grown anaerobically at 37°C. Strains then were subcultured in 100 ml TYG (no resazurin, no antibiotics) to an OD of approximately 0.3 (A_{550}). One flask of culture was added to aerobic shaker, while a control culture was grown anaerobically. Growth was continually measured in both samples at the following timepoints: 10 min, 20 min, 40 min, 60 min, 1 h (T_1), 2 h, 4 h, 12 h, 24 h, and 48 h. Data are represented as the mean of three independent experiments.

A.


50
45
40
35
30
25
20
15
10
5
0
Average Activity
0
8
16
24
32
40
48
Time (hours)
IB393(pMEB715) control
IB393(pMEB715) aerobic

B.

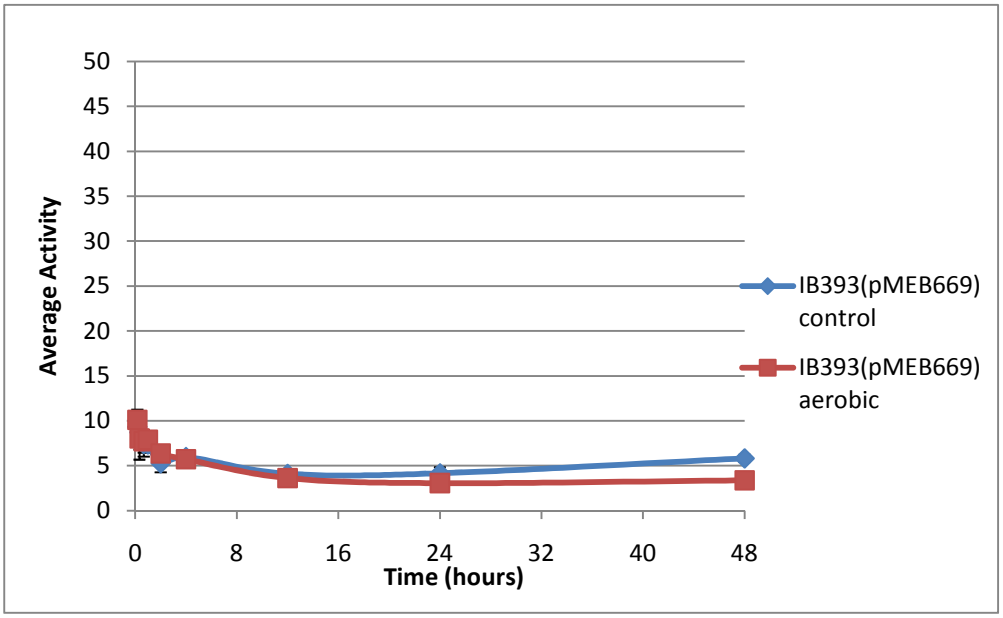
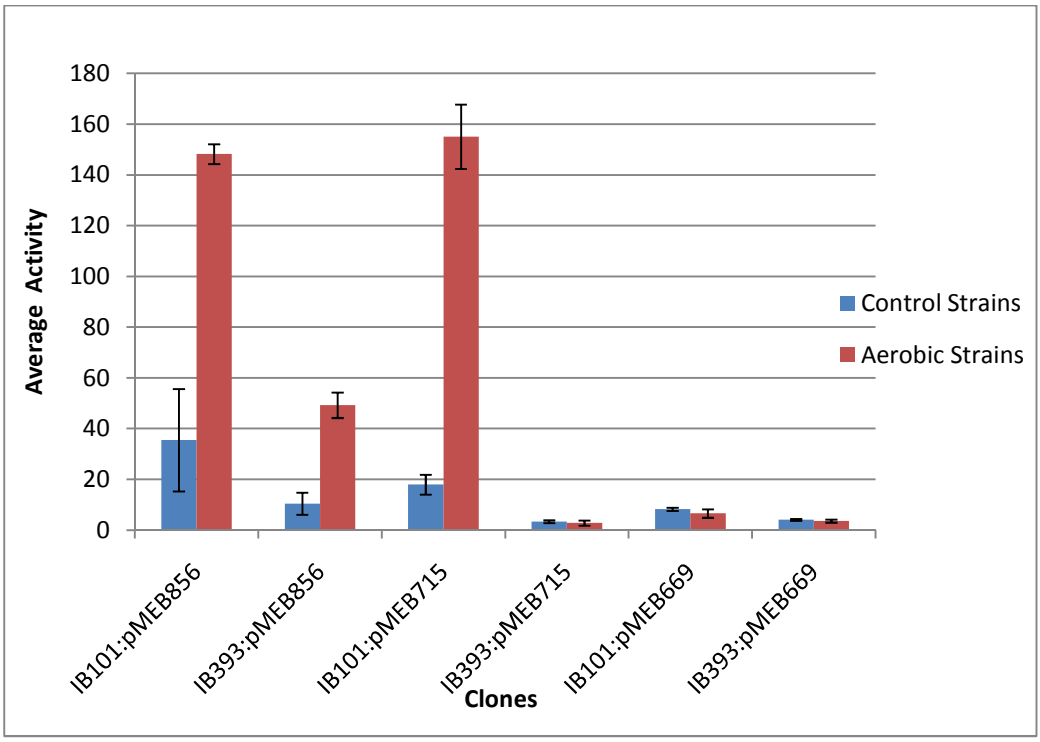

50
45
40
35
30
25
20
15
10
5
0
Average Activity
0
8
16
24
32
40
48
Time (hours)
IB393(pMEB669) control
IB393(pMEB669) aerobic

Figure 3.20. Activity of all strains at 12 hrs of growth following exposure of mid-log phase glucose grown cells to oxygen. Summary data was compiled after oxygen induction experiments were performed, as described previously. Data are represented as the mean of three independent experiments.


180
160
140
120
100
80
60
40
20
0
Average Activity
Control Strains
Aerobic Strains
IB101:pMEB856
IB393:pMEB856
IB101:pMEB715
IB393:pMEB715
IB101:pMEB669
IB393:pMEB669
Clones

CHAPTER FOUR

DISCUSSION

This study has characterized the primary starch utilization operon in the obligate anaerobe *B. fragilis*, and identified putative regulatory sequences associated with the organism's oxidative stress response and starch metabolism. Despite being obligate anaerobes, *Bacteroides* species represent some of the most aerotolerant anaerobes known, and they have evolved a complex oxidative stress response. Previous studies found expression of the *osu* operon to be important for survival during oxygen exposure; *osu* expression was shown to be oxygen responsive, and expression of *osuA* increased almost 20-fold when exposed to air (39). The same study also identified a gene, *osuR*, which encodes a transciptional activator of this operon, both of which are responsive to maltose and other maltooligosaccharides. Based on these findings, the current study was undertaken in order to elucidate regulatory regions within the *osuA* promoter.

Building on the hypothesis that these regulatory sites would exist as independent cis-acting regions of the promoter, mutational studies were performed to identify regions that respond to environmental stress and maltose. The results of this study demonstrate that *osu* may not be regulated independently at two different sites. In fact, it was found that the regulatory regions for both anaerobic maltose utilization, as well as persistence during oxidative stress, are likely found within the same approximately 50 bp region of the *osuA* promoter. Furthermore, a previous study by Spence *et al*. suggests the possibility of an additional regulator (39), an idea which will be examined below. While our current study supports previously published works that indicate the role of *osu* in the OSR of *B. fragilis*, new evidence from this study also

illustrates some dicrepancies with previous data, and suggests a new model of transciption during times of oxygen exposure and anaerobic polysaccharide utilization during growth.

4.1 Xylosidase/Arabinosidase Assay Optimization for *osu*

In order to better establish a standardized, reproducible method for measuring activity of the *osu* promoter, a whole cell colorimetric assay, utilizing the *Bacteroides* xylosidase gene, was optimized for use in this study. Both length of assay and volume of cells in the assay were examined. While the original protocol called for a 5 ml sample of cell culture, and a one hour incubation length, we found this system grossly overestimated the volume and length of time necessary to achieve meaningful reproducible results. In section 3.1 above, the optimization procedure was described for this assay. In studies that examined the cell volume removed from culture, we found that 5 ml samples produced aborbance values (A_{405}) that were beyond the linear range of the assay so that aerobic and anaerobic cultures were vitually indistinguishable. The 5 ml sample assays showed absorbances 3-4 fold higher than 2.5 ml samples for anaerobically grown cultures, and nearly twice as high for the aerobic culture (Figure 3.1). Based on these findings, the assay volume was reduced to 2.5 ml for all assays performed in this study.

In the next experiment, assay length was examined and it was found that the aerobic wildtype sample displayed an almost 50% higher absorbance value in the 30 minute assay, but lower average units of activity (Figure 3.2A, B). Both the anaerobic cultures (grown in TYG and TYM media) showed similar trends in absorbance and activity. Thus, the 15 minute xylosidase

assay was determined to be the more reproducible parameter for measuring xylosidase activity in the *B. fragilis* strains and deletional mutants used throughout this project.

4.2 The *osuA* Promoter and Starch Utilization

The *osu* operon, previously identified as a series of genes encoding a putative starch utilization system which was upregulated by aerobic exposure (37), has been shown to be required for starch and glycogen utilization during anaerobic growth (39). Starch is a complex plant-derived polysaccharide consisting of branched chains of glucose molecules joined by α-(1,4) glycosidic bonds. These carbohydrates, provided by the host's diet, serve as an important source of carbon and energy for the bacterial microflora of the human intestinal tract, including the *Bacteroides* species (10). Glycogen is a compound that serves as a secondary long-term energy storage molecule in animal cells. In studies to elucidate the potential regulatory sequence of the promoter associated with the *osu* operon, maltose, the subunit of starch or glycogen, was used to induce transciption activity, as this disaccharide represented a sufficient inducer of starch utilization.

The experiments described in Chapter Three demonstrated that maltose was indeed a strong inducer of *osu* in the wildtype strain (Figure 3.5), as well as strains which contained the full length promoter including IB101(pMEB856) and the 141 bp deletion mutant IB101(pMEB715) (Figures 3.9 and 3.10, respectively). The consistent level of transcriptional activity maintained between the strains IB101(pMEB856) and IB101(pMEB715) deletion mutants indicates that there was no loss of regulatory promoter function and thus, the regulatory sequence was not contained here. Further examination of the deletion mutants

IB101(pMEB669) and IB101(pMEB618) yielded data that supports the possibility of a regulatiory site in the approximately 50 bp region between the 715 and 669 deletion fragments. Maltose induction studies of strain IB101(pMEB669) showed almost no transciptional activity during the 4 hour experiment (Figure 3.11A). Similar results were found when studying strain IB101(pMEB618) (Figure 3.11B). Loss of activity in these clones indicate the likelihood that a site of transciptional regulation in response to maltose/starch utilization lies within the 715 deletion fragment, between the start of the 715F primer site and the 669F primer site (Figure 4.1).

Results for the maltose induction experiments using deletion mutants in strain IB393 show a different pattern of transcription. The strain IB393(pMEB856) maintained a low level of *osu* transciptional activity consistent with the wildtype containing the full length promoter region (Figure 3.12), with both strains displaying a growth-phase dependent activity over time. At its highest level, the activity measured in this mutant was more than 50% lower than that observed in the wildtype strain. Furthermore, subsequent investigations of the remaining deletion mutants in the *osuR* mutant strain show a complete loss of transciptional activity for the *osu* promoter even in the presence of maltose (Figures 3.13, 3.14). The difference between this *osuR* mutant data and the wildtype could be explained by the presence of an additional weak promoter in the region downstream from the 856F primer start site but upstream from 715 (Figure 4.1).

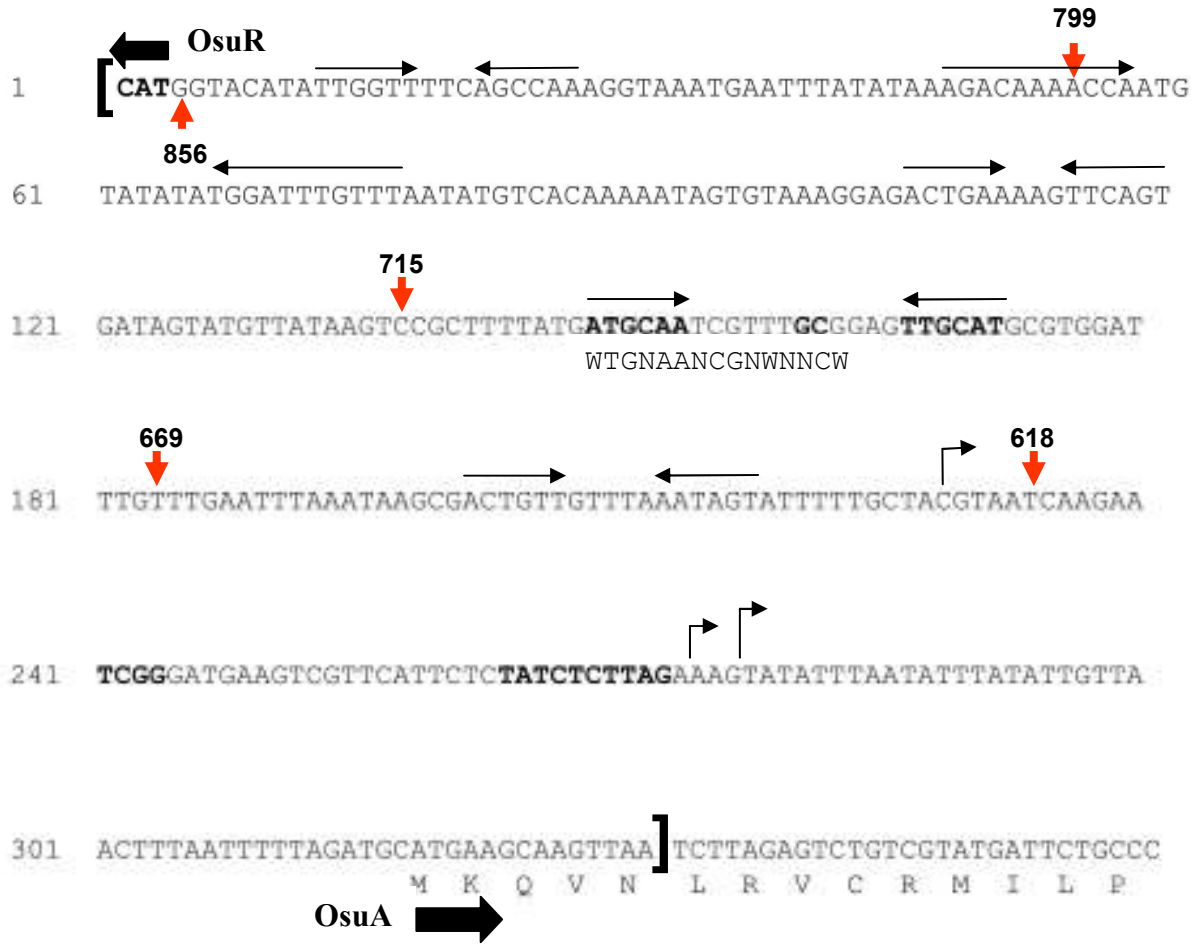
In addition, in samples supplemented with glucose, *osu* activity was decreased by approximately 50% over the time course (Figures 3.9, 3.10), when compared to induction by maltose in the wildtype IB101(pFD1078) strain (Figures 3.5). The fact that glucose does not repress activity below the xylose grown cells suggests that there is no catabolite repression mediated by glucose. That activity in glucose-grown culture is greater than that observed in

xylose-grown cultures in the wildtype, but not in the *osuR* mutant, seems to support this assertion. However, the difference in strain IB393 is small and may not be significant.

4.3 The *osuA* Promoter and Oxidative Stress

Although the primary function of the *osu* operon is in starch binding and utilization in *B. fragilis*, it also plays a role in the oxidative stress response (OSR) of this organism. Many of the proteins that have been identified in the OSR of *B. fragilis* are involved in protection from oxygen radicals through detoxification processes, and so further study will be necessary to better understand the specific role of *osu* in this system. One idea revolves around the observation that many genes involved in carbohydrate utilization and energy generation were induced by oxygen (41). It is possible that cells are attempting to maximize chances to gain energy in order to maintain their oxidative stress protective mechanisms. Oxidative stress was used in our research to attempt to elucidate the sepcific regulatory regions of the *osuA* promoter that control transcription of this operon in response to environmental stressors.

Figure 4.1 Sequence analysis of *osuA* promoter region showing deletions used in this study. Bold arrows show *osuR* and *osuA* gene start sites, while thin arrows with 90° angles show transcriptional start sites within the *osu* promoter sequence. Other thin arrows show regions of dyad symmetry.


OsuR
799
1
CATGGTACATATTGGTTTTCAGCCAAAGGTAAATGAATTTATATAAAGACAAAACCAATG
856
61
TATATATGGATTTGTTTAATATGTCACAAAAATAGTGTAAAGGAGACTGAAAAGTTCAGT
715
121
GATAGTATGTTATAAGTCCGCTTTTATGATGCAATCGTTTGCGGAGTTGCATGCGTGGAT
WTGNAANCGNWNNCW
669
618
181
TTGTTTGAATTTAAATAAGCGACTGTTGTTTAAATAGTATTTTTGCTACGTAATCAAGAA
241
TCGGGATGAAGTCGTTCATTCTCTATCTCTTAGAAAGTATATTTAATATTTATATTGTTA
301
ACTTTAATTTTTAGATGCATGAAGCAAGTTAATCTTAGAGTCTGTCGTATGATTCTGCCC
M K Q V N L R V C R M I L P
OsuA

In the current study, cultures of deletion mutants were exposed to oxygen for up to 48 hours. Initial trials with the plasmid pMEB856 in IB101 revealed activity similar to the wildtype strain, IB101(pFD1078), as expected (Figure 3.15). Examination of the deletion mutant, IB101(pMEB715), also showed oxygen induced activity levels consistent with the wildtype, throughout the 48 hour experiment (Figure 3.16). Analysis of the remaining deletion mutants however, showed a loss of this high transcriptional activity. Strains IB101(pMEB669) and IB101(pMEB618) displayed low background activity for the 48 hour experimental duration (Figures 3.17A and B). Despite our initial hypothesis that oxygen and maltose regulatory regions would exist as distinct units of the promoter, these findings suggest that both sites are likely found within the approximately 50 bp segment of DNA downstream of the 715 site.

To determine whether this trend held true for the *osuR* mutant, IB393, oxidative stress studies were performed on the same deletion mutants in this strain. Oxygen exposure of strain IB393(pMEB856) showed an interesting pattern of activity, with the aerobic sample displaying the highest activity values during the first 24 h (Figure 3.18). However, even the highest activity for the aerobic samples of this strain were less than 50% of maximal activity achieved by the wildtype strain. Both strains IB393(pMEB715) and IB393(pMEB669) showed very little transcriptional activity during these experiments, a result that was different than that found in the wildtype deletion mutant strains (Figure 3.20). Taken with the previous maltose induction studies, these data point to the important role of *osuR* in activation of transciption of the *osu* promoter and subsequent four-gene operon under all conditions teseted.

4.4 Regulation of *osu*

The OSR employed by *B. fragilis* encompasses a wide array of genes upregulated in response to stress involved with persistence in oxygen. Many genes that have been identified using micrarray data have been shown to be under the control of the peroxide responsive global regulator, OxyR (30). While OxyR displays high sensitivity to even low concentrations of oxygen, and it induces its regulon rapidly in response to oxidative stress (31), the *osu* operon has been found to be induced by starch and oxygen independent of OxyR control regulation (39). Furthermore, the results of this study have demonstrated that the full length *osu* promoter is indeed activated by exposure to oxygen and maltose, and is likely tightly associated with the transciptional regulator, *osuR*.

As an activator of *osu* transcription, the OsuR protein has been shown to have significant homology to a family of transciptional regulators known as LacI (42). LacI-type regulators characteristically act as repressors such that, in the absence of a particular metabolite, they are able to bind to a specific sequence of the gene/operon promoter, and prevent transciption of that gene by RNA polymerase. When a particular metabolite is present, these regulators undergo a conformational change that reduces the affinity of the repressor for the specific binding site, thereby removing the repression and allowing transcription to begin (42, 16, 20). Although the family of LacI regulators typically act as repressors, OsuR acts as a transcriptional activator of the *osu* operon. There is a precedent, however, for other members of this LacI-type family of regulators to activate rather than repress. In *Lactococcus lactis*, a Gram-positive organism used widely in the industrial production of fermented dairy products, the maltose operon regulator, MalR, belongs to the LacI-type regulator family and has been shown to activate transcription of maltose utilization genes (1). Additionally, *Bacillus subtilis* contains a catabolite control protein,

CcpA, which mediates glucose control and the expression of carbon utilization genes. However, this LacI-GalR family member activates the transcription of these genes by binding a DNA operator sequence known as the catabolite response element (CRE), causing relief from the carbon catabolite repression (CCR) of certain systems (25, 47).

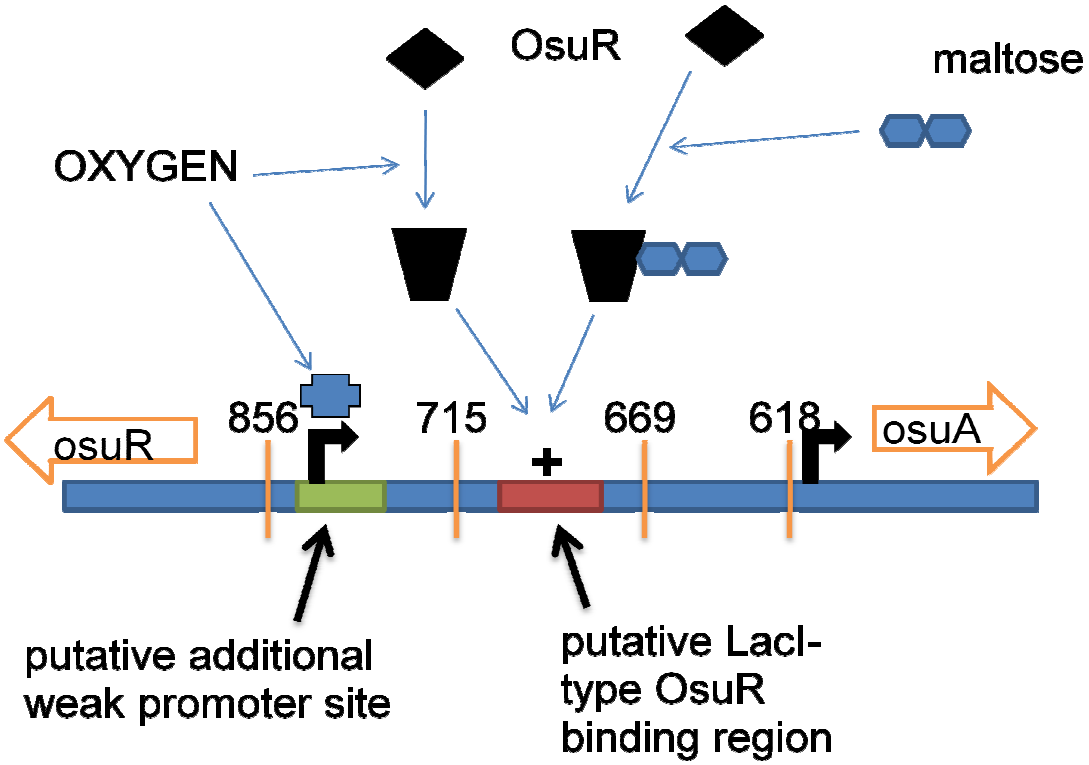
Analysis of the *osu* promoter sequence in this study revealed a LacI-type binding sequence approximately 10 bp downstream of the start site for the 715 fragment (Figure 4.1). Consistent with this finding is the data reported in the Results section of this work that outline the loss of transcriptional activity despite the presence of maltose or oxygen, in strains that no longer contain this region, namely IB101(pMEB669) and IB101(pMEB618). It is possible that OsuR is required for binding to this region, in order to activate the promoter and thus facilitate transcription of the subsequent *osuA* gene. Taken as a whole, our data suggest a model of *osu* transcription in the presence of oxygen and maltose (Figure 4.2). During anaerobic growth in media supplemented with maltose, the disaccharide enters the cell and binds to OsuR. The formation of this maltose-OsuR complex causes a conformational change in the protein and OsuR is then able to bind with the putative LacI-type region of the *osu* promoter located between the 715 and 669 sites. This binding event activates expression of the *osu* operon, as well as the maltose utilization operon and possibly the expression of other enzymes involved in starch utilization and degradation within the *B. fragilis* genome.

In the presence of oxygen however, data presented in this thesis suggest a variation on this model of *osu* transciption (Figure 4.2). As oxygen molecules are passed into the cell, their presence causes some other metabolite or transcription factor to bind to an additional weak promoter region between the 856 and 715 sites within the *osu* promoter. As a result, transcription is initiated for the *osu* operon. Further studies will be needed to map the region of

the promoter between the 856 and 715 primer sites, in order to confirm or rule out the possibility of such an additional weak promoter in this region. Furthermore, the fact that 669 deletion mutants were inactive for all conditions studied suggests that, in the absence of the low activity promoter, binding of OsuR, or some other unidentified factor, to the putative LacI-type OsuR binding site within the promoter region is critical for any activity, whether maltose or oxygen induced. In addition, the fact that deletions examined in strain IB393 showed less than 50% of the maximal activity achieved in wildtype deletions seems to be consistent with this theory. While the exact role of OsuR in the regulation and control of the *osu* operon is still unclear, it seems evident that it is an important player in the expression of this operon, as well as the survival and potential pathogenicity of *B. fragilis* and the organism's OSR.

In conclusion, the model that emerged after characterizing the promoter region of this oxidative starch utilization operon was found to be a more complex picture of *osu* control and regulation than was originially anticipated. After mapping the promoter and identifying any additional regions of regulation, future studies of the *osu* starch utilization operon could include identification of the aerobic transcription factor responsible for regulation and gene expression. Utilizing DNA Affinity Capture techniques, it would be possible to peform "pull down" assays to identify which transciption factors bind the smallest region of the *osuA* promoter that responds to aerobic induction. Finally, studies to examine the critical role OsuR seems to play in maltose and oxygen induction would provide further insight into the role of the *osu* operon and its regulator in the oxidative stress response of *B. fragilis*.

Figure 4.2. Model depicting the putative regulation and control of the *osu* promoter region during exposure to oxygen or maltose in *B. fragilis*. Expression of the *osu* operon in media supplemented with maltose may be controlled by the LacI-type, OsuR regulator binding to a motif between 715 and 669. Expression of the *osu* operon during exposure to oxygen may be controlled by OsuR, as well as an additional weak promoter upstream between 856 and 715. OsuR, or some other unidentified transcription factor, is likely critical for activation by binding to the putative OsuR binding site, as loss of this site results in a complete loss of activity, whether starch or oxygen induced.


OsuR
maltose
OXYGEN
856
715
669
618
osuR
osuA
putative additional weak promoter site
putative LacI-type OsuR binding region

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