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Characterization of the Function and Interaction of Proteins Involved in Exopolysaccharide Synthesis in *Streptococcus thermophilus*, *Streptococcus iniae*, and *Lactococcus lactis* subsp. *cremoris*

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CHARACTERIZATION OF THE FUNCTION AND INTERACTION OF PROTEINS INVOLVED IN EXOPOLYSACCHARIDE SYNTHESIS IN *STREPTOCOCCUS THERMOPHILUS*, *STREPTOCOCCUS INIAE*, AND *LACTOCOCCUS LACTIS* SUBSP. *CREMORIS*

by

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ABSTRACT

Characterization of the Function and Interaction of Proteins Involved in Exopolysaccharide Synthesis in *Streptococcus thermophilus*, *Streptococcus iniae*, and *Lactococcus lactis* subsp. *cremoris*

by

Angela D. Cefalo, Doctor of Philosophy

Utah State University, 2012

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Amino acid residues that are important for metal binding and catalysis in Gram-positive phosphotyrosine phosphatases were identified in *Streptococcus thermophilus* Wzh/EpsB proteins. The Wzh protein from *S. thermophilus* MR-1C was purified after heterologous expression and tested for phosphatase activity against synthetic phosphotyrosine and phosphoserine/threonine peptides. The purified Wzh protein was able to remove phosphate from both phosphotyrosine peptides tested and the phosphatase activity of Wzh was dramatically reduced by the presence of the phosphotyrosine phosphatase inhibitor sodium vanadate at concentrations of 1, 5, and 10 mM. Purified Wzh had no activity against the synthetic phosphoserine/threonine peptide. These results established that Wzh functions as a phosphotyrosine

phosphatase. By using the yeast two-hybrid system, strong intraspecific protein interactions were detected in *S. thermophilus* MR-1C, *Streptococcus iniae* 9066, and *Lactococcus lactis* subsp. *cremoris* JRF1 between the putative transmembrane activation protein (Wzd, CpsC, and EpsA, respectively) and the putative protein tyrosine kinase (Wze, CpsD, and EpsB, respectively). Weaker protein interactions take place forming a dimer between two identical protein tyrosine kinases and between the protein tyrosine kinase and phosphotyrosine phosphatase (Wzh, CpsB, and EpsC, respectively) in these species. Protein-protein interactions involving a *S. thermophilus* MR-1C Wzd/Wze fusion protein and Wzd and Wze indicated that these proteins may form multi-protein complexes. All combinations of the *S. thermophilus* Wzh, Wzd, Wze, Wzg (regulation), CpsE (glycosyl-1-phosphate transferase), CpsS (polymerization), CpsL (unknown), CpsW (regulation), and CpsU (membrane translocation) proteins were analyzed for protein-protein interactions but no additional interactions were discovered. For each of the intraspecific interactions detected, interspecific interactions were also detected when one protein was from *S. iniae* and the other was from *S. thermophilus*. Interactions were also observed between two protein tyrosine kinases when one protein was from either of the *Streptococcus* species and the other from *L. lactis* subsp. *cremoris*. These results and sequence comparisons performed in this study support the conclusion that interactions among the components of the tyrosine kinase/phosphatase regulatory system are conserved in the family Streptococcaceae. Interspecific protein-protein interactions

suggest that functional regulatory complexes can be formed in naturally occurring and genetically engineered recombinant strains.

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PUBLIC ABSTRACT

Characterization of the Function and Interaction of Proteins Involved in Exopolysaccharide Synthesis in *Streptococcus thermophilus*, *Streptococcus iniae*, and *Lactococcus lactis* subsp. *cremoris*

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Many microorganisms produce capsules of repeating sugar units that surround the cell called exopolysaccharides. The synthesis of these capsules is a complex process that involves numerous protein components. A better understanding of the way these proteins function and interact with one another will benefit many industrial processes by aiding in the construction of bacterial strains with enhanced properties and could also lead to new treatment strategies against microbial pathogens in which capsule production is important in their ability to cause disease. In this study, the function of one of the proteins involved in the regulation of capsule synthesis in the dairy starter culture *Streptococcus thermophilus* MR-1C is clearly established. The research in this study also provides insight into the protein-protein interactions involved in capsule production and their conservation among Gram-positive bacteria in the family Streptococcaceae using the

dairy starter cultures *S. thermophilus* MR-1C and *Lactococcus lactis* subsp. *cremoris* JRF1, and a commensal strain of the fish pathogen *Streptococcus iniae* 9066. Experimental data obtained on the ability of the proteins involved in capsule synthesis to interact with counterparts from a different species suggest that the transfer of genes between the streptococci species and to some extent between streptococci and lactococci could form functional regulatory complexes. This would be a necessary requirement for efficient capsule production in starter strains that have been genetically modified to improve their functional characteristics for industrial uses or in naturally occurring recombinant strains.

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Angela D. Cefalo

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LIST OF DEFINITIONS

AA	amino acid
AD	activation domain
ASA	acetyl-salicyclic acid
BD	binding domain
β-Gal	β-galactosidase
CA	colanic acid
CAMP	cationic antimicrobial peptide
CPS	capsular polysaccharides
EM	electron microscope
EPS	exopolysaccharides
IL	interleukin
INF	interferon
kDa	kilodalton
LAB	lactic acid bacteria
LB	Luria-Bertani
LMW-PTP	low-molecular-weight acid phosphotryosine protein phosphatase
LPS	lipopolysaccharide
M	metal ion
NT	nucleotide
OM	outer membrane

PTK	protein tyrosine kinase
PTP	phosphotyrosine phosphatase
PVC	polyvinyl chloride
RS	restriction site
TNF	tumor necrosis factor
Ugd	UDP-glucose dehydrogenase
W	water molecule
X-gal	bromo-chloro-indolyl-galactopyranoside
YPD	yeast peptone dextrose

CHAPTER 1

INTRODUCTION

Many microorganisms produce capsules of repeating sugar units that surround the cell. These capsules are collectively termed exopolysaccharides (EPS) and can be tightly associated with the cell wall as capsular polysaccharides (CPS) or liberated into the medium as an unattached loose slime (ropy EPS). EPS is produced by both Gram-negative and Gram-positive bacteria and EPS producers can be found in many different niches. The size and composition of EPS polymers are very diverse and are often strain dependent. Due to the unique properties of these polymers, their possible uses in industry, the roles they play in the disease processes of pathogens, and their ability to increase survival of bacteria in adverse environmental conditions, much research has been done in this area. However, many aspects about the complex processes involved in EPS production and export remain unknown. The functions of some proteins involved in EPS production are unclear and little is known about how these proteins interact with one another to produce EPS. A better understanding of the process involved in EPS production could benefit industry by allowing the genetic manipulation of EPS producing cultures to improve their functionality and could benefit medicine by identifying new targets for the design of antimicrobial drugs. The primary research goals of this dissertation are as follows:

1. Establish that the *Streptococcus thermophilus* Wzh protein functions as a phosphotyrosine phosphatase.
2. Investigate protein-protein interactions involved in EPS biosynthesis in *S. thermophilus* MR-1C.

3. Determine if the same protein-protein interactions take place during EPS production in *Streptococcus iniae* 9066, and *Lactococcus lactis* subsp. *cremoris* JRF1.
4. Investigate interspecific protein-protein interactions to determine if functional regulatory complexes could be formed in naturally occurring and genetically engineered recombinant strains.

To achieve the first goal tyrosine phosphatase and serine/threonine phosphatase kits from Promega (Madison, Wisconsin, USA) were used. These kits provided a convenient non-radioactive method to study phosphatase activity and have been used to successfully characterize similar bacterial phosphatases. The yeast two-hybrid system is a well established method used to study protein-protein interactions and was used to accomplish the other three research goals.

The *S. thermophilus* Wzh protein has been thought to function as a phosphotryosine phosphatase (PTP) due to its protein sequence similarity with known PTPs but this activity has never been demonstrated. This is the first study to analyze the direct interaction of some of the proteins involved in EPS biosynthesis. This is the first study to investigate whether these proteins require their cognately encoded counterparts in order to form protein-protein interactions or whether they can form interspecific protein-protein interactions.

The literature review will detail what is presently known about bacterial exopolysaccharide capsules. The first section will briefly discuss the complex and diverse structure of EPS. The many diverse functions of EPS, such as in protection from

adverse environmental conditions, the survival of pathogens, biofilm formation, protection from bacteriophage infection, symbiotic relationships with plants, or energy storage, will be reviewed.

The next section of the literature review will detail how EPS clusters are named and organized. To provide an example of this organization in Gram-negative systems the *Escherichia coli* K-12 *wca* operon is presented. The *cps* operons of streptococci and the *cps* and *eps* operons found in lactic acid bacteria (LAB) will provide examples for Gram-positive systems. These examples will detail important information on the organization of the EPS genes found in *S. thermophilus*, *S. iniae*, and *L. lactis* subsp. *cremoris* that are used in the experiments contained in this dissertation. The chimeric structure of *eps/cps* gene clusters due to horizontal gene transfer events will also be discussed in this section.

The literature review will also discuss the steps needed for EPS biosynthesis. EPS can be synthesized either extracellularly by transglycosylases or intracellularly by glycosyltransferases. After intracellular assembly, translocation and polymerization of the repeat sugar units can take place through either a Wzy-dependent or a Wzy-independent pathway. Finally, what is known about the enzymes and mechanism involved in the attachment of capsules to the cell surface is discussed.

Next, the literature review will present what is currently known about the protein tyrosine kinase (PTK)/PTP regulatory system that controls exopolysaccharide production. Protein organization of this regulatory system is different in Gram-negative and Gram-positive bacteria. Previous research has suggested that the effects of tyrosine phosphorylation of the PTK on EPS production varies from system to system; either

promoting high molecular mass polysaccharide synthesis or reducing it. The current viewpoint is that cycling between phosphorylated and non-phosphorylated forms of the PTK is essential for efficient high molecular weight polysaccharide synthesis.

The functional properties of EPS are influenced by both its chemical composition and its molecular mass. Natural or genetically modified polysaccharides that can be used in food, medical applications, or other industries are popular research topics. The last section of the literature review will present uses for EPS in bioremediation, as cryoprotectants, and as food additives. It will also discuss the use of EPS in the prevention of biofilm formation and the benefits of EPS on human health.

The third chapter of this dissertation presents research that clearly establishes *S. thermophilus* Wzh functions as a PTP that could remove phosphate from phosphotyrosine residues in two test peptides and therefore is likely to have this same function with the PTK Wze and other proteins important in EPS biosynthesis. Protein sequence alignments of *S. thermophilus* Wzh/EpsB (different naming schematics) proteins with known Gram-positive PTPs identified that Wzh contained conserved amino acid residues that are important in metal binding and catalysis. The *wzh* gene from *S. thermophilus* MR-1C was cloned into *Escherichia coli* BL21 (DE3) cells to allow the production of a His-tagged fusion protein. The Wzh fusion protein was purified and tested for PTP activity against synthetic phosphotyrosine peptides alone and in combination with the PTP inhibitor sodium vanadate. The purified Wzh protein was functional in releasing phosphate from both of the synthetic phosphotyrosine peptides tested. The activity of Wzh was dramatically decreased in the presence of sodium vanadate. The purified Wzh

was also tested for activity against a synthetic phosphoserine/threonine peptide but no phosphatase activity was detected.

The fourth chapter of this dissertation identifies interactions that take place among the protein components involved in EPS biosynthesis in *S. thermophilus* MR-1C using the yeast two-hybrid system. These protein-protein interactions are important to the efficient synthesis of high molecular weight EPS. This research demonstrated that there is a strong interaction between the PTK Wze and the transmembrane activation protein Wzd, a weaker interaction between two identical PTK Wze proteins, and a weaker interaction between the PTK Wze and the PTP Wzh. A Wzd/Wze fusion protein was created and protein-protein interactions of this fusion protein with Wzd and Wze may indicate that these proteins form multi-protein complexes. All other combinations of Wzh, Wzd, Wze, Wzg (regulation), CpsE (glycosyl-1-phosphate transferase), CpsS (polymerization), CpsL (unknown), CpsW (regulation), and CpsU (membrane translocation) failed to identify any other protein-protein interactions.

The fifth chapter of this dissertation uses the yeast two-hybrid system to identify the intraspecific and interspecific protein interactions in *S. thermophilus*, *S. iniae* and *L. lactis* subsp. *cremoris* that would indicate that the protein-protein interactions of the PTK/PTP regulatory system are conserved in family Streptococcaceae and that intraspecific gene exchanges have the potential to form functional recombinants. As was found in *S. thermophilus*, a strong protein-protein interaction was detected between the PTK and the transmembrane protein, between two identical PTK proteins, and between the PTK and the PTP in *S. iniae* and *L. lactis* subsp. *cremoris*. Interspecific interactions

were detected for each of the intraspecific interactions when when one protein was from *S. iniae* and the other was from *S. thermophilus*. Interspecific interactions were also observed between two PTKs when one protein was from either of the *Streptococcus* species and the other from *L. lactis* subsp. *cremoris*.

The last chapter of this dissertation will address limitations of this research and suggest a direction for future research on the biosynthesis of EPS. Taken together the results of the experiments contained in this dissertation have increased the understanding of the protein functions and interactions important in the biosynthesis of EPS production. These results also suggest that functional regulatory complexes could be created in genetically engineered recombinants aiding in the construction of strains with enhanced properties.

CHAPTER 2

LITERATURE REVIEW

BACTERIAL EXOPOLYSACCHARIDE CAPSULES: FUNCTIONS, SYNTHESIS, REGULATION, AND APPLICATIONS

1. Structure of exopolysaccharides

Exopolysaccharides (EPS) are highly hydrated and complex structures that consist of repeating units of monosaccharides joined by glycosidic linkages. EPS come in a wide variety of compositions and configurations due to the diversity of the sugar components and the variety of linkages between the sugars (Roberts 1996). The molecular mass, structure, and yield of EPS vary greatly even between bacterial strains of the same species (De Vuyst et al. 2001). *Streptococcus pneumoniae* strains produce over 90 distinct capsular types that vary in sugar composition and complexity of the glycosidic linkages (Yother 2011). More than 80 distinct extracellular polysaccharides have been found in different strains of *Escherichia coli* (Whitfield and Roberts 1999). EPS can be classified as either homopolysaccharides or heteropolysaccharides. The homopolysaccharides contain repeating units of only one type of monosaccharide and include cellulose, dextrans, mutan, alternan, pullulan, levan and curdlan (Monsan et al. 2001). The heteropolysaccharides contain repeated subunits of different monosaccharides, derivatives of monosaccharides, or substituted monosaccharides (De Vuyst et al. 2001). A common feature among the capsules of *S. pneumoniae* and many other species is that the capsules are mostly negatively charged due to the presence of

acidic sugars, phosphate, or pyruvate. The remainder of the capsules are usually neutral in their charge (Kamerling 2000).

2. Functions of EPS

2.1. EPS protects bacteria in adverse environmental conditions

EPS protects bacteria from adverse conditions in the surrounding environment. Bacteria that produce EPS have the advantage of binding water in low moisture environments and protecting themselves from desiccation (Grilli-Caiola et al. 1993, 1996; Ophir and Gutnick 1994; Potts 1999; Obadia et al. 2007). Mucoid strains of many pathogens, such as *E. coli* and *Pantoea stewartii*, are more resistant to drying than isogenic non-mucoid strains. In fact, it has been found that exposure to desiccation conditions actually increases the expression of genes that encode the biosynthetic enzymes needed for the production of the EPS colanic acid (CA) in *E. coli* (Ophir and Gutnick 1994). Obadia et al. (2007) found that cells of *E. coli* K-12 with increased expression of the *wzb* gene encoding a phosphotyrosine phosphatase produced more CA than wild type cells and were more resistant to desiccation. A strain that was deficient in the expression of the protein tyrosine kinase Wzc_{ca} and unable to produce CA was more susceptible to desiccation than wild type cells. This resistance was influenced by the size of the EPS polymer produced. Wild type cells that produced CA with a wide distribution of sizes were more resistant to desiccation than strains expressing the non-phosphorylated form of Wzc_{ca}, which produced a polymer mix with a narrow size distribution. It was determined that a polymer mix ranging from 600 kDa to 2,000 kDa conferred the best resistance against desiccation. The specific mechanism involved in this resistance is not

well understood, but several possibilities have been investigated. The hydrophilic nature of EPS allows a layer of water to be retained around the cell protecting the integrity of macromolecules and preventing damage to the cell wall due to the cellular shrinking normally associated with desiccation (Grilli-Caiola et al. 1993, 1996). External protective compounds that aid in the survival of desiccated cells such as water stress proteins can be retained by EPS (Potts 1999). In conditions where water is limited, the production of damaging reactive oxygen species can be increased. The EPS from *Burkholderia cenocepacia* and *Pseudomonas aeruginosa* can scavenge and neutralize these reactive oxygen species preventing cellular damage (Pasquier et al. 1997; Bylund et al. 2006).

EPS can protect many different bacterial cells from heat, acid, and osmotic stress. For example, *E. coli* O157:H7 cells that were unable to produce CA, a type of EPS, due to insertional mutagenesis of the *wca* (Fig. 2.1) operon were less tolerant to acid and heat stress than the isogenic wild-type cells. The growth of wild-type cells was not markedly affected at pH 4.5, but the growth of the mutant cells was completely inhibited at the same pH (Mao et al. 2001). The strong negative charge of CA may neutralize protons and act as a buffer at the cell surface to prevent the accumulation of positively charged chemical groups on the cell envelope or the penetration of ions into the cells. Without the protective CA layer, it is believed that the penetration of protons and change in intracellular pH impaired cell metabolism and caused cell death. D-values are defined as the time at a specific temperature that is required to inactivate one log of the bacterial

population. The CA deficient mutants had significantly lower D-values than the wild type cells at 55 and 60 ^{o}C (Mao et al. 2001).

The influence of several heat shock proteins on the regulation of CA induction suggests a role for CA in the heat shock response. For example, it has been found that overexpression of the DjlA co-chaperone of *E. coli* triggers the synthesis of CA. The interaction of DjlA with the molecular chaperone DnaK is necessary for the activation of the *wca* operon. The induction of *wca* (Fig. 2.1) may occur by modulation of the activity of the RcsB/RcsC phosphotransferase signaling pathway by the interaction of DjlA and DnaK (Kelly and Georgopoulos 1997; Genevaux et al. 2001). The CA deficient mutants were also more susceptible to osmotic and oxidative stresses (Chen et al. 2004).

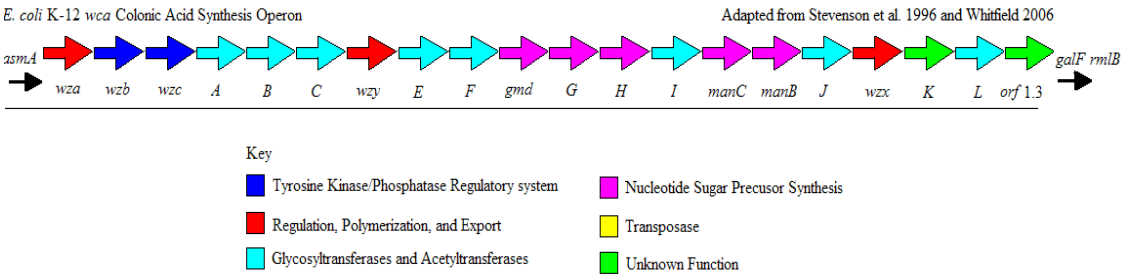

Fig. 2.1. Organization of the *wca* operon in *Escherichia coli* K-12. The ORFs and direction of transcription are indicated by the arrows. Proposed function of the genes is indicated by the color of the arrow.

EPS protects microorganisms in cold environments by forming and maintaining microhabitats around the cells (Decho 1990). The research of Kim and Yim (2007) demonstrated the cryoprotective abilities of EPS produced by the Antarctic bacterium *Pseudoalteromonas arctica*. When the EPS from this organism was used as a cryoprotectant for *E. coli* cells, it was found that the survival ratio increased to more than

90% as compared to a survival ratio of around 43% for cells prepared with 20% (v/v) glycerol.

Capsular and ropy EPS has been shown to sequester cations, including Fe^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+}, thereby concentrating helpful metal ions in the microenvironment around the cells. The negatively charged groups in EPS can bind cations and protect the bacterial cell against toxic metals (Ordax et al. 2010). Proteinaceous particles have been shown to adhere to EPS causing them to be concentrated around the cell. This would allow efficient proteolytic digestion of the particles and facilitate the absorption of the resulting amino acids into the cells (Qin et al. 2007). Bacterial EPS have been shown to be powerful emulsifiers that improve the solubility of substrates in the environment making them more bioavailable (Martínez-Checa et al. 2002, 2007; Iyer et al. 2006; Ta-Chen et al. 2008). An interesting new function in enzyme stabilization for EPS was investigated by Qin et al. (2007). They determined that the EPS from the deep-sea organism *Pseudoalteromonas* sp. SM9913 stabilized the protease produced by this bacterium and effectively prevented it from auto-digesting. This would enhance digestion of proteinaceous particles.

2.2. EPS are important for the survival of pathogens

Mutations that negatively affect capsule synthesis in pathogenic organisms usually result in a decreased ability to cause invasive infections and attenuation of pathogenicity (Watson and Musher 1990; Morona et al. 2004, 2006; Locke et al. 2007; Nelson et al. 2007; Brunner et al. 2010). The EPS capsules of many human and plant pathogens are important for the initial attachment of the bacterial cells to host tissue.

Bacteria can regulate capsule biosynthesis to allow for varying degrees of capsule production under different environmental conditions or stages of growth. For example, in *S. pneumoniae* cells that produce a smaller amount of capsule the adhesive molecules are more exposed. This promotes colonization by strengthening the contact with epithelial cells and allowing uptake of the pathogen. However, a larger capsule would be beneficial in survival and evasion of the host immune system allowing systematic dissemination of the pathogen (Hammerschmidt et al. 2005).

The mechanisms by which production of EPS helps pathogenic bacteria evade the immune system are not completely understood, but several possibilities have been investigated. The negative surface charge of the capsule may be important. A study done by Nelson et al. (2007) indicates that the capsule of *S. pneumoniae* is essential for escaping the lumenal mucus and permitting the bacteria access to the host cellular receptors on the epithelial surface of the nasal mucosa, thereby allowing colonization. As the bacteria escape the lumenal mucus, they evade clearance by mucocilliary flow. The mechanism behind this may involve electrostatic repulsion between the highly negatively charged muco-polysaccharides and the negatively charged EPS of the bacterial capsule (Nelson et al. 2007). Miller and Neely (2005) and Locke et al. (2007) have shown that capsule production in *Streptococcus iniae* prevents phagocytosis by macrophages. Mutants deficient in capsule production were bound and internalized much more efficiently by macrophages than capsular wild type cells. The wild type cells had a 76% survival rate from phagocytosis, but the capsule deficient mutants had only a 13-29% survival rate. Interestingly, cells with mutations causing overproduction of the capsule

were even more resistant to phagocytosis than their wild type counterparts. It has been determined that monocytes adhere significantly less to cells with negative surface charges. The greater the negative charge of the EPS, the greater the resistance to the phagocytic cells of the immune system (Swiatlo et al. 2002). In a similar manner, EPS may protect soil bacteria from ingestion by amoebas and flagellated soil protozoa (Moxon and Kroll 1990). Research in plants, indicates that polyanionic EPS may suppress response to pathogens by binding available calcium ions that function as a second messenger in pathogen perception. Chelation by the EPS would stop the calcium influx from the apoplast to the cytosol that is necessary to the hypersensitive response. It is unknown whether the chelation of calcium ions by polyanionic EPS would suppress vertebrate innate immunity (Grant et al. 2000; Lecourieux et al. 2006; Aslam et al. 2008).

EPS can function in evasion of the immune system by the molecular mimicry of host cells. The sialic acid containing capsular polysaccharide (CPS) of *Neisseria meningitidis* can protect the bacteria from non-specific host defense mechanisms in the absence of a specific antibody. The sialic acid capsule mimics the sialic acid found decorating host cells. This mimicry allows the capsule to interfere with complement-mediated killing by preventing the activation of complement via the alternative pathway and inhibiting opsonophagocytosis (Jarvis and Vedros 1987; Jarvis 1994; Vogel et al. 1997; Kahler et al. 1998). *Klebsiella pneumoniae* uses molecular mimicry to evade macrophages by incorporating the immunologically inert sugar fucose in CPS (Wu et al. 2007). The structural similarities between capsule and host tissue polysaccharides can

cause poor antibody responses to such capsules by the immune system (Kahler et al. 1998; Wu et al. 2007).

EPS may help cells elude the immune system by acting as a physical barrier or by hiding opsonins bound to the bacterial cell wall, preventing their recognition by phagocytic cells (Musher 1992). The ability of EPS producing cells to circumvent phagocytosis by macrophages could be explained by the masking of host cell binding sites (Locke et al. 2007). In *Streptococcus pyogenes*, the hyaluronic capsule blocks the binding of specific antibodies to the GRAB protein on the surface of bacterial cells, possibly through steric or electrostatic hindrance (Kahler et al. 1998; Dinkla et al. 2007). Anti-lipoteichoic acid antibodies present in normal human serum cannot recognize encapsulated *Enterococcus faecalis* cells but will readily bind to non-encapsulated strains (Hufnagel et al. 2005; Thurlow et al. 2009). Melin et al. (2009) found that certain capsular serotypes of *S. pneumoniae* were able to inhibit complement associated cell lysis and opsonophagocytosis by decreasing the deposition of human complement C3. Capsules can protect bacteria from becoming caught in neutrophil extracellular traps where they would be killed by antimicrobial peptides (Wartha et al. 2007). The capsules of many pathogens are necessary for intracellular survival in human phagocytic and non-phagocytic cells. The mechanism behind intracellular survival involves increased resistance to cationic antimicrobial peptides (CAMPs) present in the phagocytic cells. The capsule may interfere with the interaction of CAMPs with the surface of the bacterial cells (Campos et al. 2004; Spinosa et al. 2007). Recently, it was demonstrated that non-capsular mutants of *Porphyromonas gingivalis* increased the production of interleukin

(IL)-1b, IL-6, and IL-8 in human gingival fibroblasts compared to wild type capsular cells. The induction of these proinflammatory cytokines could explain why non-capsular cells are contained in localized abscesses by the immune system whereas wild type cells evade this defense mechanism and cause spreading phlegmonous infections that lead to destructive periodontal diseases. The CPS of *P. gingivalis* may prevent the recognition of immune inducing agents on the surface of the bacterial cell by Toll-like receptors on the fibroblasts or it may lower the expression of inflammatory cytokines by actively modulating the immune responses of the fibroblasts (Brunner et al. 2010). Calcium ions are essential for the differentiation and proper functioning of the cellular components of the human immune system. The chelation of calcium ions by polyanionic EPS could suppress immune system responses allowing for better survival of pathogens (Grant et al. 2000; Lecourieux et al. 2006; Aslam et al. 2008; Oh-hora and Rao 2008).

2.3. EPS are important in biofilm formation

The EPS capsules of many human and plant pathogens are important for the initial attachment of the bacterial cells to host tissue and in the formation of biofilms. Boddicker et al. (2006) found that biofilm formation was significantly reduced, flat, and unstructured in a strain of *K. pneumoniae* with a mutation in the capsule synthesis locus. Biofilms are in part created through lectin-ligand interactions that involve cell-surface polysaccharide molecules (Jenkinson 1994; Mora et al. 2008). This ability to form biofilms allows bacteria to colonize many different ecological niches while protecting them from harmful substances or organisms. Biofilms can form on various surfaces including silicon, latex, polycarbonate, stainless steel, glass, polyvinyl chloride (PVC),

and plant materials (Beuchat 2002; Lehner et al. 2005). Biofilm formation is an important step in the formation of dental carries by allowing adherence of *Streptococcus mutans* to the surface of teeth (Cerning 1990; Whitfield and Keenleyside 1995). Bacteria in biofilms are resistant to many antimicrobial compounds including antibiotics. *Pseudomonas* species can form an alginate rich biofilm in the lungs of cystic fibrosis patients that provides a physical barrier to antibiotics (Worlitzsch et al. 2002). Hill et al. (2005) tested clinical isolates of *P. aeruginosa* from the lungs of cystic fibrosis patients for the antibiotic susceptibility of cells grown in a biofilm verses cells grown in a planktonic state. All 10 antibiotics tested were less effective against the cells grown in a biofilm than on cells grown in an aerobic planktonic state. Hill et al. (2005) also showed that cells growing in a biofilm were less susceptible to two or more antibiotics used in combination than cells grown in aerobic planktonic cultures. Biofilms can cause nosocomial infections by colonization of indwelling medical devices such as catheters and endotracheal tubing (Boddicker et al. 2006). Fouling of industrial pipes and equipment by biofilms causes economic losses and can result in the spread of food borne illnesses (Rättö et al. 2006; Skandamis et al. 2009).

2.4. EPS and bacteriophage infection

EPS may protect some bacteria from lytic phage infection. Moineau et al. (1996) found that 27 distinct lactococcal phages were unable to propagate on six different EPS producing strains of *Lactococcus lactis*. EPS tightly associated with the cell surface may mask phage receptor sites on the surface of the bacterial cell. EPS production in *Sinorhizobium meliloti* has been shown to inhibit phage absorption (Defives et al. 1996).

However, Broadbent et al. (2003) and Rodríguez et al. (2008) found no relationship between CPS and phage attack in *Streptococcus thermophilus*. In fact, CPS may be directly involved in phage-host interactions in some cases. Specific phages have been isolated that only infect strains of *E. coli* and *Vibro cholerae* that posses an EPS capsule (Albert et al. 1996; Scholl et al. 2001).

2.5. EPS are important in establishing symbiotic relationships with plants

EPS are important in the interaction of bacteria with plants. The research conducted by Jones et al. (2008) demonstrated the importance of the EPS succinoglycan in the establishment of the symbiotic relationship of *S. meliloti* with plants. Mutants that lack the ability to produce succinoglycan cannot form mature root nodules. An increase in expression of ribosomal components, translational factors, and protein degradation machinery was seen in *Medicago truncatula* (alfalfa) roots inoculated with succinoglycan producing wild type cells of *S. meliloti*. This increase was not seen when the roots where inoculated with a succinoglycan deficient mutant. However, the roots inoculated with this mutant did express a large number of plant defense genes that would most likely cause a termination of the colonization of the plant by *S. meliloti*. These results suggest that plants make profound metabolic adjustments to prepare their roots for invasion by *S. meliloti* when succinoglycan is sensed early on in the infection process. Acidic polysaccharides have been shown to be important for a number of other rhizobia to establish symbiosis (Djordjevic et al. 1987; Laus et al. 2005).

2.6. EPS in energy storage

Most bacteria are unable to catabolize the sugar polymers in EPS, and therefore it cannot function in energy storage (Cerning 1990; De Vuyst and Degeest 1999). However, *S. mutans* is not only able to breakdown dextran, a class of exopolysaccharides produced by lactic acid bacteria (LAB); it can then use the oligosaccharides that are produced (Colby and Russel 1997).

3. EPS gene clusters

3.1. Nomenclature

At the present time, sequence comparisons and functional studies of *eps* genes and gene clusters can be difficult due to the confusion incurred by the lack of a unified system for genetic nomenclature in EPS producing bacteria. For example, the terms CPS and EPS are often used interchangeably to designate a capsular EPS and the genes for EPS synthesis have been designated *eps*, *cps, cap, exo, gum,* or named after the species. In the bacterial polysaccharide gene nomenclature system proposed by Reeves et al. (1996), homologous genes are assigned the same symbol without regard to species or strain differences. In this system, most genes are given names in the form of *w**** with the genes in a cluster having the first three characters in common. If the function of a gene is the same as that of a gene located in another cluster, then the same name is used for both even if it means that genes with different three letter designations will exist in one cluster. This system proposes that all genes of any block defined by the same first two letters are of the same general type. Genes for homologous proteins involved in saccharide processing are given names in the form of *wz** and genes involved in the

synthesis of saccharide precursors have names related to the pathway (Reeves et al. 1996). For example, in this approach the *S. thermophilus* regulatory genes *epsA-D*, which are present in all *S. thermophilus eps* gene clusters, are designated as *wzg*, *wzh*, *wzd*, and *wze*, respectively. The homologues to the *S. thermophilus* NCFB 2393 *cpsE* and *cpsF* genes that encode glycosyltransferases would be designated as *wchA* and *wchF*, respectively. The *S. thermophilus* Sfi6 *epsM*, MR-1C *cpsU*, and Sfi39 *epsJ* genes show homology to the conserved flippase gene, *wzx*. The *S. thermophilus* Sfi6 *epsJ*, MR-1C *cpsS*, and Sfi39 *epsF* genes have significant homology to the conserved EPS polymerase gene *wzy* (Broadbent et al. 2003). The advantages of this system are that each distinctive gene will have a unique name, but that name will identify it as a bacterial surface polysaccharide gene, genes with the same function will have the same name, all genes in a pathway will have names that relate to the pathway, and families of saccharide processing genes will have distinctive names. The use of this system should diminish the confusion created by the use of the same name for genes of different functions and giving genes of the same function different names. This would make it easier for researchers to identify homologous genes in different species or strains and would facilitate analysis of those genes. One of the disadvantages of this method is that not all genes in a cluster will have the same three-letter symbol making identification of genes that belong in the same cluster somewhat more difficult. Difficulties may occur as the old names are replaced by new names (Reeves et al. 1996). This dissertation uses names associated with the protein and nucleotide files deposited in GenBank and associated with the literature to avoid confusion by the renaming of established proteins or genes.

3.2. General organization

In both Gram-positive and Gram-negative bacteria, the enzymes needed for the synthesis and transportation of EPS are encoded by genes clustered in large operons (Whitfield and Roberts 1999). However, more genes are required for EPS synthesis than are generally found in EPS gene clusters. For example, some of the nucleotide sugar precursors are produced by enzymes encoded by genes that have functions other than in EPS synthesis. Mutation of these genes located outside the EPS gene clusters may still result in EPS-deficient phenotypes (Whitfield and Keenleyside 1995).

In general, the beginning of each EPS gene cluster encodes proteins involved in membrane translocation and regulation (Stevenson et al. 1996; Stingele et al. 1996; De Vuyst and Degeest 1999; Iannelli et al. 1999; De Vuyst et al. 2001; Jolly and Stingele 2001). Many bacteria have a tyrosine kinase/phosphatase regulatory system located in the first portion of the EPS gene cluster. This system also functions in the determination of polymer chain length (Vincent et al. 1999; Morona et al. 2000a; Bender and Yother 2001; Wugeditsch et al. 2001; Bender et al. 2003; Ferreira et al. 2007). This region is followed by a central portion containing genes for glycosyltransferases. The genes needed for sugar nucleotide precursor synthesis can be located in this central region or at the end of the cluster. Downstream of the central region are genes that encode proteins responsible for polymerization and export of the repeat unit (Stevenson et al. 1996; Stingele et al. 1996; De Vuyst and Degeest 1999; Iannelli et al. 1999; De Vuyst et al. 2001; Jolly and Stingele 2001).

3.3. The wca operon of E. coli K-12

One of the most studied operons of this type in Gram-negative cells is associated with the production of CA in *E. coli* K-12. The genes on this operon all have the same transcriptional orientation and appear to be part of a single large transcriptional unit. The *wca* operon consists of 20 different genes, which either have known functions or predicted functions based on homology studies (Fig. 2.2) (Stevenson et al. 1996; Whitfield 2006). The *wza* gene encodes an outer membrane (OM) lipoprotein that is essential for the formation of high molecular weight CPS on the surface of the cells (Drummelsmith and Whitfield 2000; Nesper et al. 2003; Beis et al. 2004a, 2004b). The *wzb* gene encodes an acid phosphatase and the *wzc* a protein tyrosine kinase (PTK) (Vincent et al. 1999, 2000; Wugeditsch et al. 2001; Doublet et al. 2002; Grangeasse et al. 2002; Paiment et al. 2002; Obadia et al. 2007). The *manB*, *manC*, *gmd, fcl,* and *gmm* genes encode enzymes needed for nucleotide precursor synthesis. The first sugar added to the CA polymer is glucose and the initiating glucose-1-phosphate transferase is encoded by *wcaJ*. The remaining glycosyltransferases are encoded by the *wcaA, wcaC, wcaE, wcaL,* and *wcaI* genes. The *wcaB* and *wcaF* genes encode acetyltransferases that are responsible for the addition of acetyl groups to the final polymer. It appears that the *wzy* gene encodes a polymerase that functions in the polymerization of the repeat units. The *wzx* gene product is thought to function in the export of the polymer outside the cell. The functions of the *wcaK* and *wcaM* gene products are not known (Stevenson et al. 1996; Whitfield 2006).

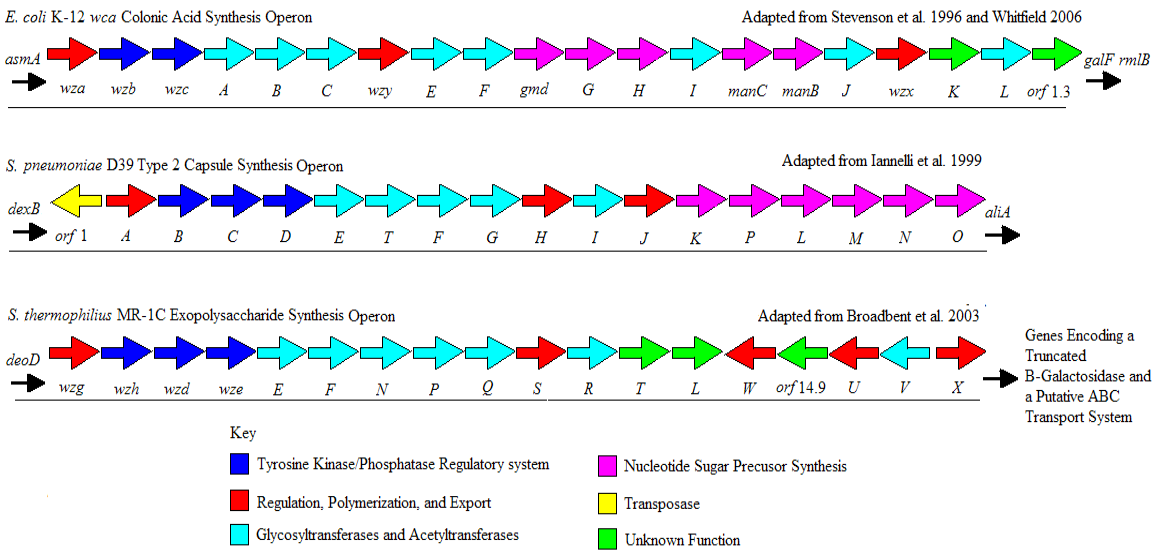

Fig. 2.2. Organization of the EPS gene clusters in *E. coli* K-12, *Streptococcus pneumoniae* D39, and *Streptococcus thermophilus* MR-1C. The ORFs and direction of transcription are indicated by the arrows. Proposed function of the genes is indicated by the color of the arrow.

3.4. The cps operon of the streptococci

One of the best-studied EPS operons in Gram-positive bacteria is involved in the production of the type 2 capsule in *S. pneumoniae* D39. This operon consists of 18 open reading frames, 17 of which have the same direction of transcription. These 17 genes are thought to be transcribed as a single unit due to the location of the upstream promoter and the location of a transcriptional terminator downstream of the last gene. The transcriptional orientation of *orf1* is opposite that of the rest of the genes in the cluster (Fig. 2.2). The *orf1* gene product shows homology to several transposases and is probably part of an insertion sequence (Iannelli et al. 1999). The *cpsA, cpsB, cpsC, cpsD,* and *cpsE* genes are highly conserved among different species of streptococci and insight into their functions can be gained by analyzing the mutational studies performed in

Group B streptococci. In general, CpsA proteins in these systems are thought to be important in regulating EPS synthesis. The *cpsIaA* gene product of *S. agalactiae* functions in the regulation of capsule production as an activator of gene transcription. The inactivation of *cpsIaA* resulted in reduced amounts of polysaccharide production and reduced expression of the type Ia gene cluster (Cieslewicz et al. 2001). The deletion of *cps2A* in *S. pneumoniae* did not observably alter the level of Cps2D, but a decrease in Cps2D phosphorylation and a reduction in encapsulation occurred. This suggests that Cps2A could have functions other than or in addition to transcriptional control (Bender et al. 2003). The *cps2B* gene encodes a novel manganese-dependent phosphatase (Bender and Yother 2001). When the *cpsIaB* of *S. agalactiae* was inactivated, only very small amounts of cell-associated capsule were produced (Cieslewicz et al. 2001). The gene products of *cps2C* and *cps2D* are part of a tyrosine kinase system that functions in determination of polymer chain length and regulation (Bender and Yother 2001; Morona et al. 2000a, 2002; Bender et al. 2003). Mutations in the *S. agalactiae cpsIaC* and *cpsIaD* genes resulted in fewer polysaccharides on the cell surface and a reduction in polysaccharide chain length (Cieslewicz et al. 2001). The glucose-1-phosphate transferase is encoded by *cps2E* (Iannelli et al. 1999). Disruption of the *cpsIaE* gene of *S. agalactiae* resulted in a non-capsular phenotype (Cieslewicz et al. 2001). The remaining glycosyltransferases are encoded by *cps2T, cps2F, cps2G*, and *cps2I*. The *cps2H* gene appears to encode a polysaccharide polymerase and the *cps2J* gene a repeat unit transporter. The last six genes of the cluster, *cps2K*, *cps2P*, *cps2L*, *cps2M*, *cps2N*, and *cps2O,* function in nucleotide sugar precursor biosynthesis (Iannelli et al. 1999).

3.5. Genetics of EPS production in lactic acid bacteria

A better understanding of capsule production by LAB could be greatly beneficial to the dairy industry. Unlike those found in *E. coli* and *S. pneumoniae*, the EPS gene clusters of the LAB *S. thermophilus* and *Lactococcus lactis* have no orf's with homology to genes required for the synthesis of nucleotide sugar precursors. All the enzymes needed for precursor synthesis are located elsewhere in the genome (Stingele et al. 1996; van Kranenburg et al. 1997; Broadbent et al. 2001, 2003). For example, capsule production in *S. thermophilus* MR-1C may involve at least 19 different genes distributed over a 44 kb region of the chromosome (Broadbent et al. 2001). Not all of the *S. thermophilus* genes are in the same transcriptional orientation. The direction of *cpsW*, *orf 14.9*, *cpsU* (*wzx*), and *cpsV* are in the opposite orientation of the other open reading frames (Fig. 2.2). Functions for the *S. thermophilus* MR-1C genes are based on homology to those of known functions in other organisms (Broadbent et al. 2001, 2003). The *wzg, wzh, wzd,* and *wze* genes are highly conserved among *S. thermophilus* strains and are homologous to *S. pneumoniae cps2A, cps2B, cps2C* and *cps2D* respectively. The Wzg and CpsW proteins of MR-1C are predicted to function in the regulation of capsule biosynthesis. The *wzd* and *wze* genes likely encode a tyrosine kinase system that functions in regulation and the determination of polymer chain length. The *wzh* gene may encode the cognate tyrosine phosphatase. The *cpsE, cpsF, cpsN, cpsP, cpsQ, cpsR, cpsT,* and *cpsV* genes are predicted to encode glycosyltransferases that function in assembly of the repeating sugar units (Stingele et al. 1996; Bourgoin et al. 1999; Broadbent et al. 2001, 2003). The *cpsE* gene encodes a galactosyltransferase that

catalyzes the first step in the synthesis of the repeat unit by transferring galactose-1-phosphate to the carrier lipid (Stingele et al. 1996; Almiron-Roig et al. 2000). Inactivation of *cpsE* in MR-1C resulted in a non-capsular phenotype, similar to what was observed in *S. agalactiae* (Low et al. 1998; Cieslewicz et al. 2001). The *cpsU* (*wzx*) and *cpsX* genes encode proteins that are predicted to function in the transport of the repeating units across the cell membrane. CpsS (Wzy) is predicted to function in polymerization of the repeating sugar units to form the final EPS polymer. The genes *cpsL* and *orf14.9* encode proteins with unknown functions (Stingele et al. 1996; Bourgoin et al. 1999; Broadbent et al. 2001, 2003). Structurally similar *eps* gene clusters are found in other EPS producing *S. thermophilus* strains but the glycosyltransferase genes typically differ. These vary in the number of genes and their arrangement within the gene cluster (Broadbent et al. 2003). Homologues of some of the *eps* genes are present in EPS$^-$ strains of *S. thermophilus* such as TA061 (Welker and Broadbent 2002a, 2002b, 2002c; Welker, Broadbent, and Cefalo unpublished results).

In many mesophilic LAB, such as *L. lactis*, the *eps* gene cluster is plasmid-encoded, which may explain the high rate of conversion to an EPS$^-$ phenotype seen in this species. This conversion in starter strains is problematic for the dairy industry (van Kranenburg et al. 1997, 1999). The *eps* gene clusters in thermophilic LAB, such as S. *thermophilus*, are all chromosomally encoded and located adjacent to the *deoB* and *deoD* genes in the genome (Stingele et al. 1996; Almiron-Roig et al. 2000; Broadbent et al. 2001; Germond et al. 2001). The fact that the *eps* gene cluster is chromosomally encoded in *S. thermophilus* makes it more stable than the *eps* gene cluster in *L. lactis* (Broadbent

et al. 2001). Phenotypic instability of the *S. thermophilus eps* gene cluster has been associated with insertion elements that are located within or adjacent to the genes responsible for EPS production (Bourgoin et al. 1999). In *S. thermophilus* Sfi39, a spontaneous, non-ropy mutant was produced by IS*905* transposition into the *epsF* gene of this strain (Germond et al. 2001). The loss of EPS producing ability of *S. thermophilus* has also been attributed to general genomic instability (Bourgoin et al. 1999). Recent results obtained from comparative genetic analysis of *S. thermophilus* CNRZ368 and MR-1C indicate that point mutations rather than integration of IS elements appear to be responsible for inactivation of the *eps* genes in CNRZ368 (Bourgoin et al. 1999; Broadbent et al. 2001; Welker and Broadbent 2002c; Welker, Broadbent, and Cefalo unpublished results).

3.6. Horizontal gene transfer

The chimeric structure found in many of the *eps/cps* gene clusters of pathogenic as well as non-pathogenic bacteria has likely resulted from horizontal gene transfer events (Bourgoin et al. 1996, 1999; Coffey et al. 1998; Pluvinet et al. 2004; Hols et al. 2005; Tyvaert et al. 2006; Delorme et al. 2007; Rasmussen et al. 2008; Liu et al. 2009; Eng et al. 2011). Different studies have found evidence suggesting lateral gene transfer of gene sized or sub-gene regions in the *eps* gene clusters of *S. thermophilus*. This evidence supports the belief that *S. thermophilus eps* gene clusters evolved partly through DNA exchanges with other species of *Streptococcus* as well as with LAB, such as *L. lactis*, during co-culture in milk (Guédon et al. 1995; Bourgoin et al. 1996, 1999; Pluvinet et al. 2004; Hols et al. 2005; Tyvaert et al. 2006; Rasmussen et al. 2008; Liu et al. 2009;

Eng et al. 2011). In *S. thermophilus* all 3 gene transfer mechanisms (i.e. conjugation, transduction, and natural competence) have been shown to be active resulting in gene content that is 20% variable with 8% likely to be derived from recent horizontal gene transfers (Burrus et al. 2002; Bolotin et al. 2004; Hols et al. 2005; Blomqvist et al. 2006; Ammann et al. 2008; Rasmussen et al. 2008; Fontaine et al. 2010; Eng et al. 2011). A prominent feature of the *Streptococcus eps*/*cps* gene clusters, including those of *S. thermophilus* and *S. iniae*, are insertion sequence elements (IS*981*, IS*1191*, and IS*S1*) which could facilitate genetic exchange of genes or partial genes between species (Guédon et al. 1995; Bourgoin et al. 1996, 1999; Pluvinet et al. 2004; Hols et al. 2005; Tyvaert et al. 2006; Lowe et al. 2007; Rasmussen et al. 2008; Eng et al. 2011). The insertion sequence elements that are found in both the genomes of *S. thermophilus* and *L. lactis* share a high degree of nucleotide sequence identity (at least 98%) suggesting that horizontal transfer has recently occurred between these LAB during co-culture in milk during yogurt or cheese manufacture. Research has identified horizontal gene transfers of *eps/cps* genes between *Lactococcus* and *Streptococcus* involving genes or sub-gene regions for glycosyltransferases whose function may not rely on protein-protein interactions rather than of the *wzh*, *wzd* or *wze* genes (Guédon et al. 1995; Bourgoin et al. 1996, 1999; Broadbent et al. 2003; Pluvinet et al. 2004; Tyvaert et al. 2006; Rasmussen et al. 2008; Liu et al. 2009; Eng et al. 2011).

4. Biosynthesis of EPS

4.1. Extracellular synthesis of EPS

Only a few polysaccharides such as dextran, levan, and alternan are synthesized extracellularly by transglycosylases. For example, the homopolysaccharide dextran produced by *Leuconostoc mesenteroides* is made by dextransucrase (a type of glucansucrase). This enzyme splits sucrose by cleaving the glycosidic bond, liberating fructose and glucose. Dextransucrase then transfers the glucose residue to the reducing end of a dextran chain. The energy needed to form the new glycosidic bonds in the dextran chain is derived from the enzymatic splitting of sucrose. Glucansucrases are generally located extracellularly, but they can also be detected in a cell-associated form. The expression of some glucansucrases requires induction by sucrose, while others are expressed constitutively (Cerning 1990; Janeček et al. 2000; Monsan et al. 2001; van Hijum et al. 2006; Kumar et al. 2007).

4.2. Intracellular synthesis of repeating sugar units

Heteropolysaccharides are made intracellularly and then exported. In the model for intracellular synthesis of EPS, the repeating sugar units are assembled by sugar specific glycosyltransferases (Cerning 1990, 1995; Stingele et al. 1996, 1999; De Vuyst and Degeest 1999; Jolly and Stingele 2001; Kumar et al. 2007; Guo et al. 2008). The assembly of the repeating unit takes place on the inner face of the cytoplasmic membrane from intracellular precursors by sequential transfer of sugar nucleotide diphospho-precursors to a carrier lipid, which in many cases is undecaprenyl phosphate. The first step in the assembly of the repeating sugar unit is catalyzed by a glycosyl-1-phosphate

transferase that transfers a sugar-1-phosphate to the lipid carrier. The subsequent glycosyltransferases catalyze the addition of sugar residues by glycosidic linkage formation (Fig. 2.3) (Johnson and Wilson 1977; Cerning 1990, 1995; Whitfield and Valvano 1993; Stingele et al. 1996, 1999; De Vuyst and Degeest, 1999; Whitfield and Roberts 1999; Jolly and Stingele 2001; Cartee et al. 2005; Kumar et al. 2007).

In the Wzy-dependent pathway for translocation and polymerization of EPS, the repeating sugar units are translocated across the membrane and polymerized on the outside of the cell to produce high molecular weight polysaccharides (Cerning 1990, 1995; Stingele et al. 1996, 1999; De Vuyst and Degeest 1999; Jolly and Stingele 2001;

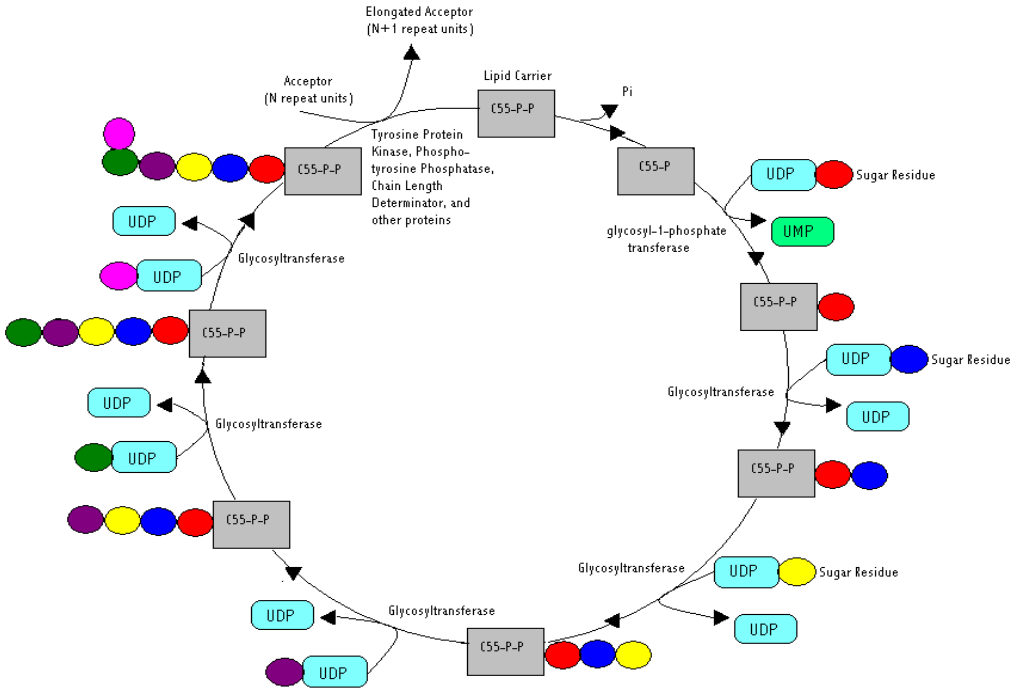

Fig. 2.3. Model for the assembly of the EPS basic repeat unit. UDP stands for uridine diphosphate and UMP stands for uridine monophosphate. C55 represents the carrier lipid undecaprenyl phosphate. Adapted from Stingele et al. (1996, 1999), De Vuyst and Degeest (1999), and Broadbent et al. (2003).

Guo et al. 2008; Yother 2011). In the Wzy-dependent pathway used in the assembly of *E. coli* CA, group 1, and group 4 capsules; it is thought that the putative flippase (Wzx) flips the lipid-linked repeat units to the periplasmic face of the inner membrane. Then the putative polymerase (Wzy) generates a long-chain polymer by successive transfer of the growing chain linked to the carrier lipid to the reducing end of a single repeat unit in the periplasm (Liu et al. 1996; Drummelsmith and Whitfield 1999; Feldman et al. 1999; Valvano 2003; Whitfield 2006). The terminal stages of capsule assembly involve the OM lipoprotein Wza. Wza is a multimeric complex composed of eight subunits arranged in a tetramer of dimers that forms a ring-like structure in the membrane. This structure is thought to form a pore through which the CPS may cross from the periplasm through the OM (Drummelsmith and Whitfield 2000; Nesper et al. 2003; Beis et al. 2004a, 2004b; Collins et al. 2007). The periplasmic region of Wza interacts with the periplasmic domain of the autophosphorylating PTK Wzc (Figure 2.4). This interaction causes significant conformation changes to both proteins at the junction (Collins et al. 2006, 2007). In both the electron microscope (EM) and x-ray structure of the Wza octamer alone, the central cavity is open to the extracellular environment, but closed to the periplasm (Beis et al. 2004b; Dong et al. 2006). The EM structure of the Wza octamer when interacting with the Wzc tetramer shows the Wza channel in its open configuration forming a pore that leads into the large central cavity from the periplasm. Therefore, the interaction of Wzc oligomers and Wza is critical to the export regulation of CPS (Collins et al. 2007). In mutants that lack a *wza* gene, no detectable capsule is produced; but there is also no intracellular polymer production. This evidence supports a type of feedback

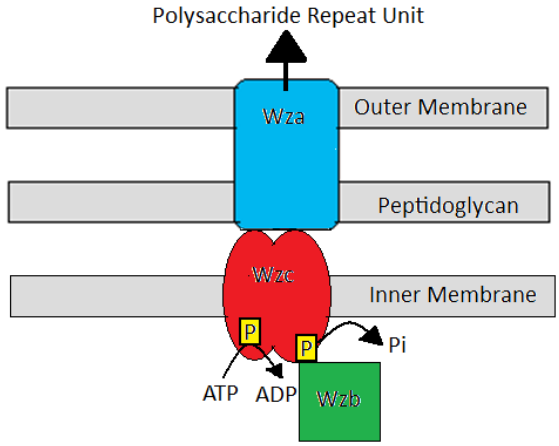

Fig. 2.4. The arrangement of the outer membrane channel Wza, the protein tyrosine kinase Wzc, and the phosphotyrosine phosphatase Wzb in *Escherichia coli*. Wzc first undergoes autophosphorylation and then the phosphorylated Wzc causes transphosphorylation of another Wzc protein in the oligomer. Wzc is then dephosphorylated by Wzb. The cycling between phosphorylation and dephosphorylation forms of Wzc is essential for high-level polymerization. The polymer is exported through the outer membrane by the multimeric Wza complex in its open form. Modified from Nesper et al. (2003) and Dong et al. (2006).

mechanism that couples polymer synthesis and export (Drummelsmith and Whitfield 2000; Nesper et al. 2003). Although Wza is essential for the formation of high molecular weight CPS on the surface of *E. coli* cells, evidence suggests that this OM channel does not recognize the specific type of EPS polymer being transported and that it is highly conserved between strains (Reid and Whitfield 2005). For example, an essentially identical Wza protein is employed by *E. coli* and *K. pneumoniae* even though the EPS that they produce is of a different chemical composition and structure (Rahn et al. 1999). It is thought that water acts to ensure that the polar protein side chains and sugar hydroxyl groups make hydrogen bonds in Wza's large polar cavity, therefore negating the need for specific recognition between the Wza protein and the carbohydrates in EPS (Dong et al.

2006). Most of the heteropolysaccahride capsules of the streptococci, including *S. thermophilus*, are thought to be produced in a manner similar to *E. coli* group 1 and 4 capsules. These Gram-positive bacteria would of course lack the machinery needed to export CPS though an OM (Whitfield and Roberts 1999; Broadbent et al. 2003; Yother 2011).

The biosynthesis of group 2 and 3 capsules in *E. coli* occurs by a different mechanism that is independent of Wzx and Wzy. For these capsules, initiation occurs on an unknown endogenous acceptor with extension occurring by the action of progressive glycosyltransferases. The polymer is then exported by an ABC transporter (Whitfield 2006). In the production of the *S. pneumoniae* type 3 simple disaccharide capsule, a single enzyme is responsible for forming all the glycosidic linkages and for polymerization. This enzyme alternates adding the different sugars to the non-reducing end of a polysaccharide chain. Exactly how termination and size determination is achieved is not known (Dillard et al. 1995; Arrecubieta et al. 1996; Cartee et al. 2000; Whitfield 2006; Yother 2011).

4.3. Attachment of the capsule to the cell surface

Little is known about the enzymes and mechanisms involved in the attachment of capsules to bacterial surfaces. In Gram-negative cells, CPS is believed to be anchored to the cellular surface by a covalent attachment to phospholipid or lipid-A molecules (Kuo et al. 1985; Whitfield and Valvano 1993). However, Fresno et al. (2006) found that the association of the *K. pneumoniae* capsule to the cell surface is through ionic interactions with the lipopolysaccharide (LPS). They suggest a possible mechanism where divalent

ions such as Mg^{2+} or Ca^{2+} could play a role in the ionic interaction between glucuronic acid in the CPS and the negatively charged carboxyl groups of the galacturonic acid residues in the oligosaccharide core. In support of this, the presence of galacturonic acid in the core oligosaccharide was needed to attach the capsule to the cell surface. The OM protein Wzi of *E. coli* K30 is thought to be involved at some level in linking group 1 CPS to the cell surface, because in the absence of this protein CPS is still made and exported but much of it is secreted into the surrounding medium (Rahn et al. 2003). The capsules of the Gram-positive staphylococci and streptococci are usually covalently linked to the cell wall peptidoglycan but the enzymes involved in this process are unknown (Yeung and Mattingly 1983; Fournier et al. 1984; Sorensen et al. 1990; Deng et al. 2000). Ropy EPS is released onto the cell surface and then into the medium because of the lack of attachment to the cellular surface (Roberts 1996).

5. Regulation of EPS synthesis by tyrosine phosphorylation

5.1. Tyrosine phosphorylation in Gram-negative systems

Although once considered rare, tyrosine phosphorylation is recognized today as a key regulatory device in prokaryotic systems that functions in virulence, stress response, and DNA metabolism (Chow et al. 1994; Jagtap and Ray 1999; Petranovic et al. 2007). Bacterial protein tyrosine kinases (PTKs) have been found to be involved in EPS synthesis where they regulate both the length and the amount of polymer produced. Advances in genome sequencing have allowed the identification of this type of protein-modifying enzyme in a wide variety of Gram-negative bacteria including *E. coli*, *K. pneumoniae*, *Acinetobacter johnsonii*, and *Burkholderia cepacia* (Duclos et al. 1996;

Grangeasse et al. 1998; Vincent et al. 1999, 2000; Wugeditsch et al. 2001; Preneta et al. 2002; Nakar and Gutnick 2003; Ferreira et al. 2007; Obadia et al. 2007). One of the best-studied regulatory systems of this type is the Wzc PTK and the Wzb phosphotyrosine phosphatase (PTP) of *E. coli* K-12. Wzc and Wzb function in the transport of the extracellular polysaccharide CA out of the cell and into the medium (Vincent et al. 1999, 2000; Wugeditsch et al. 2001; Doublet et al. 2002; Grangeasse et al. 2002; Paiment et al. 2002; Obadia et al. 2007). The typical Gram-negative PTK is expressed as a single protein and consists of two main domains. These domains are organized in the cell as a periplasmic N-terminal domain flanked on both sides by a transmembrane α-helix, and a C-terminal cytoplasmic domain. The C-terminal cytoplasmic domain harbors the ATP-binding sites in the form of Walker A (GXXGXGK[T/S], where X is any amino acid) and Walker B (hhhhD, where h is a hydrophobic residue) motifs, and the tyrosine phosphorylation sites (Walker et al. 1982; Doublet et al. 2002; Obadia et al. 2007). The N-terminal domain of Wzc is similar in topology to Wzz, a protein that influences LPS O antigen chain length (Morona et al. 2000b). Wzc_{ca} contains six different sites for phosphorylation; five of these are located in a tyrosine cluster, between residues 708 and 715, at the very end of the C-terminal portion of the molecule. The last phosphorylation site is located upstream at Y569 (Vincent et al. 2000; Doublet et al. 2002; Grangeasse et al. 2002). A model for tyrosine phosphorylation has been proposed that consists of a two-step cooperative mechanism in which Wzc_{ca} is phosphorylated by both intramolecular and intermolecular events. In the first step, an unknown effector molecule triggers the phosphorylation of Y569. Phosphorylation of Y569 causes a significant

increase in the protein kinase activity and promotes the intermolecular phosphorylation of the five tyrosine residues at the C-terminal end of another Wzc_{ca} molecule (Grangeasse et al. 2002). It has been suggested that this could be due to a rotational change in the tyrosine side chain upon becoming cis-phosphorylated that would make the catalytic site accessible to the tyrosine cluster or other substrates for transphosphorylation (Lee et al. 2008). The tyrosine residues are phosphorylated at the expense of ATP molecules that bind to the Walker A and B motifs at the C-terminal domain of Wzc_{ca} (Grangeasse et al. 2002). The C-terminal tyrosine cluster is necessary for CA synthesis. Obadia et al. (2007) found that little or no CA was produced if the tyrosine cluster was deleted or if the residues were changed to glutamic acid to mimic phosphorylation.

Both the phosphorylated and dephosphorylated forms of Wzc_{ca} are essential for the production of CA. The dephosphorylated form of Wzc_{ca} promotes the synthesis of CA. When Wzc_{ca} becomes phosphorylated, the production of CA is blocked and the transportation of the polymer across the membrane is promoted (Vincent et al. 2000; Collins et al. 2006; Obadia et al. 2007). Upon dephosphorylation of Wzc_{ca} by the PTP Wzb, the production of CA is restored (Vincent et al. 1999, 2000). Obadia et al. (2007) found that strains modified to overproduce Wzb synthesized a larger amount of the CA polymer that had the same size distribution as the wild type cells. Wzb is a small cytoplasmic protein of the low-molecular-weight acid phosphotyrosine protein phosphatase (LMW-PTP) family (Vincent et al. 1999; Obadia et al. 2007). The phosphate-binding loop of PTPs contain the conserved active motif, $C(X)_5RS$. Located further downstream is an essential aspartate residue that acts as a general acid (Kennelly

and Potts 1999). Hagelueken et al. (2009) produced crystal structures of Wzb that indicate the active site is marked by a reactive cysteine residue (Cys13). The importance of Cys13 is demonstrated by the fact that if this residue is mutated to serine Wzb phosphatase activity is eliminated (Wugeditsch et al. 2001). In the proposed mechanism for Wzb, the phosphate is positioned such that the phosphorus oxygen bond is in line with the S atom of Cys13 in the active site. This is the correct orientation for bimolecular nucleophilic substitution, which is the mechanism Wzb uses to remove phosphate from phosphotyrosine containing substrates. The binding of phosphate to the active site leads to an ordering not only of the residues that are directly bound to the phosphate but also of the nucleophilic Cys13 and Tyr117, which are adjacent to phosphate ligands and believed to be important for substrate binding (Hagelueken et al. 2009).

Wzb can very rapidly dephosphorylate the tyrosine residues at the C-terminus of Wzc_{ca}, but it may lack the ability to dephosphorylate Y569. If Wzb does remove the phosphate from Y569, it does so at a much slower rate. It is therefore possible, that another PTP could be involved in removing phosphate from Y569 (Grangeasse et al. 2003). The PTP from *K. pneumoniae* is able to dephosphorylate Wzc from *E. coli*, suggesting that the action of the phosphatase is not specific to its proximally encoded kinase (Preneta et al. 2002). In fact, several different Wzb proteins have been shown to dephosphorylate substrates other than Wzc. It is conceivable that the Wzb protein is involved in the dephosphorylation of enzymes involved in nucleotide sugar precursor synthesis or glycosyltransferases (Bugert and Geider 1997; Grangeasse et al. 1998; Vincent et al. 1999; Nakar and Gutnick 2003).

This mechanism implies that Wzc_{ca} subunits interact to undergo intermolecular phosphorylation. To support this hypothesis it has been demonstrated that *E. coli* Wzc has the ability to form oligomers. In *E. coli* K30, Wzc forms tetramers in the inner membrane of the cell. In a structure that has been described to look like an extracted molar tooth, four periplasmic domains of different Wzc molecules interact to form a "crown" with the cytoplasmic tyrosine autokinase domains forming the "roots". No interaction between the cytoplasmic tyrosine autokinase domains was observed, but such an interaction is predicted from the protein's transphosphorylation ability (Doublet et al. 2002; Collins et al. 2006). Mutant proteins of Wzc_{ca} that cannot undergo phosphorylation still have the ability to form oligomers similar to the wild-type protein. This implies that the phosphorylation state of Wzc_{ca} is not essential for oligomerization. The periplasmic domain of Wzc is involved in a protein-protein interaction with the OM lipoprotein Wza forming a complex that spans the periplasmic space. The interaction of Wzc_{ca} and Wza_{ca} is needed for CA synthesis, therefore Wzc_{ca} could have functions interacting with the biosynthetic machinery and coupled to the export pathway (Nesper et al. 2003; Reid and Whitfield 2005; Collins et al. 2007). The phosphorylated form of Wzc_{ca} influences the size of the CA polymer. Obadia et al. (2007) identified a proline rich region in the periplasmic domain of Wzc_{ca} that is crucial for determining the extent of polymerization and the amount of polymer produced. Although a direct interaction between Wzc and Wzy has not been reported to date, it is possible that the PTK could affect the way the Wzy polymerase functions; and thereby influence the amount and size of the produced polymer (Whitfield and Larue 2008).

There is some evidence that phosphorylation of Wzc_{ca} Y569 stimulates the phosphorylation of UDP-glucose dehydrogenase (Ugd). The tyrosine phosphorylation of Ugd would then lead to elevated synthesis of UDP-glucuronic acid, one of the building blocks of CA synthesis. In support of this, when the Y71 in Ugd is changed to phenylalanine Ugd can no longer be phosphorylated by Wzc_{ca} and there is a reduction in the synthesis of CA (Grangrasse et al. 2003; Lacour et al. 2008). Wzc may also be involved in the phosphorylation of the glycosyltransferase, WcaJ. A tyrosine residue that undergoes phosphorylation and seems to be important for proper protein function has been identified in WcaJ from *K. pneumoniae*. *K. pneumoniae* cells that contain a gene mutation that results in the replacement of Y5 with phenylalanine in the WcaJ protein produce about 50% less CPS than wild type cells. The loss of Y5 in WcaJ increased the LD_{50} by 200 fold in a mouse peritonitis model compared with wild type cells. It is conceivable that other glycosyltransferases are phosphorylated by Wzc_{ca} during CA synthesis (Obadia et al. 2007; Lin et al. 2009).

Finally, it has been demonstrated that Wzc_{ca} has ATPase activity and that the ATPase activity is enhanced by the intramolecular phosphorylation of Wzc_{ca}. It is possible that the energy produced from the hydrolysis of ATP by Wzc_{ca} could be used for CA synthesis and that both the kinase and ATPase activity of this protein are necessary to control the biosynthesis and/or export of polysaccharides (Obadia et al. 2007; Soulat et al. 2007). Recently, phosphorylation of *K. pneumoniae* Wzc was also observed on a serine residue located in the Walker A motif and on a serine and a threonine residue located in the periplasmic loop. The role of phosphorylation at these residues is not clear

but it may affect kinase activity, ATP binding, K antigen chain length, or signal transduction (Lin et al. 2009).

5.2. Tyrosine phosphorylation in Gram-positive systems

The best-classified system of tyrosine phosphorylation in Gram-positive bacteria involves the CpsB, CpsC, and CpsD proteins that function in CPS production in *S. pneumoniae* (Morona et al. 2000a, 2002; Bender and Yother 2001; Bender et al. 2003; Yother 2011). It has been postulated that the CpsB-CpsC-CpsD-ATP complex may affect capsular synthesis by indirect or direct enhancement of the activity of the polymerase, allowing efficient synthesis of the polysaccharide repeat units (Bender and Yother 2001; Morona et al. 2003, 2004).

CpsB is a novel manganese-dependent PTP belonging to the polymerase histidinol phosphatase family that regulates capsule production by removal of the phosphates from the PTK CpsD and by inhibiting the phosphorylation of tyrosine residues (Morona et al. 2000a, 2002; Bender and Yother 2001). High-resolution crystal structures of the phosphate-complexed and the ligand free but metal bound form of Cps4B from *S. pneumoniae* TIGR4 indicate that this PTP has three metal ions (M1, M2, and M3) bound to the active site that are coordinated by conserved amino acids and water molecules (W1, W2, and W3) (Hagelueken et al. 2009; Kim et al. 2011). Contradictory results have been presented on which metal ion occupies each of the three sites. Hagelueken et al. (2009) modeled the three metal sites as manganese although the weaker electron density peak of M3 suggested that it might be a different ion from M1 and M2. However, Kim et al. (2011) found that their samples of Cps4B contained mostly Fe and

Mg ions and they assigned M1 and M2 as Fe ions and M3 as a Mg ion. As these ions were not intentionally added to the buffer solutions for purification and crystallization it is thought that they are picked up by the recombinant enzymes in the expression medium. A unique chemical mechanism for the metal-dependent PTPs from Gram-positive bacteria has been proposed which employs a metal-bound water molecule or hydroxyl ion as a nucleophile (Wilcox 1996; Hagelueken et al. 2009; Kim et al. 2011). In this mechanism, the incoming phosphotyrosine binds to the active site of Cps4B via arginine residues, M2, and M3. A strong ionic interaction of the phosphate group with arginine in the active site helps align the phosphorous atom directly over the nucleophile and displaces the negative charge on the phosphorous atom allowing for the subsequent nucleophilic attack. W1 serves as a ligand for both M1 and M2 and most likely represents a shared hydroxyl ion that is deprotonated and acts as the nucleophile. The positively charged arginine residues and metal ions surrounding the active site stabilize the developing negative charges during the S_N2 displacement. The positive potential around the active site would also stabilize the tyrosinate that results from cleavage of the phosphodiester bond (Gerrantana et al. 2001; Hagelueken et al. 2009; Kim et al. 2011). When the phosphate ion binds to the active site it replaces all three metal-bound water molecules. The chemistry of this proposed mechanism is similar to that of the phosphotriesterases (Benning et al. 2001; Elias et al. 2008; Hagelueken et al. 2009; Kim et al. 2011).

In typical Gram-positive EPS systems, the transmembrane activation domain (CpsC) and the nucleotide-binding domain (CpsD) are separate proteins, in contrast to

being contained in one protein as in *E. coli* (Morona et al. 2000a; Doublet et al. 2002; Yother 2011). The nucleotide binding domain contains the Walker A and B motifs and the phosphorylation sites but the autophosphorylation of CpsD needs the presence of CpsC (Morona et al. 2000a, 2003; Bender and Yother 2001; Doublet et al. 2002). A model has been proposed in which the phosphorylation of tyrosine on CpsD acts to negatively regulate CPS production. It is thought that the dephosphorylated form of CpsD interacts with CpsC and ATP, possibly facilitating a conformational change in CpsC that results in the promotion of CPS synthesis. In this state, interactions between CpsC and other proteins may be promoted, allowing polymerization and biosynthesis to proceed at the maximal level. When CpsD autophosphorylates tyrosine residues at the expense of the bound ATP, it causes a dissociation from CpsC that decreases the amount of CPS production to a minimal level and promotes the transfer of the CPS polymer to an undefined cell wall-CPS ligase (Morona et al. 2000a, 2003, 2004). The phosphorylation event may take place through a cooperative mechanism involving the transphosphorylation between the tyrosine residues in the $(YGX)_4$ repeat domain of CpsD (Morona et al. 2003).

It was thought that mutations that changed the tyrosine residues of the $(YGX)_4$ repeat domain to phenylalanine residues would result in an increase in capsule production. The introduced mutations actually resulted in a decrease in cell wall associated capsule production, but an altered more mucoid phenotype in which the polysaccharides had a lower molecular weight. Morona et al. (2003) proposes that these mutations increase the dephosphorylated form of CpsD and could affect its role in

polymerization thereby resulting in the lower molecular weight polysaccharides. It is also possible the mutations to the $(YGX)_4$ repeat domain altered the structural conformation or the protein-protein interactions of CpsD. For CpsD to function there is a minimum requirement of two of these YGX domains. These results imply that switching between phosphorylated and dephosphorylated forms of CpsD is required in this system for efficient, high molecular weight polysaccharide synthesis. The dephosphorylated form of CpsD promotes polysaccharide polymerization and the phosphorylated form promotes transfer of the polymer to the cell wall ligase (Morona et al. 2000a, 2003, 2004). Cieslewicz et al. (2001) proved the importance of the autophosphorylating PTK CpsIaD on capsule function in group B streptococci. Cells containing a *cpsIaD* deletion mutation had a 91% reduction in the amount of cell associated polysaccharide as measured by immunoassay. The average chain length of the polysaccharide produced by the allelic replacement of *cpsIaD* was about one-half of that produced by the wild-type cells. The deletion of the *cpsD* gene in *S. iniae* by allelic exchange mutagenesis abolished capsule production, increased the length of coccus chains, and caused an over 100-fold attenuation in pathogenicity as compared to that of the wild type parental strain (Locke et al. 2007). *S. pneumoniae* cells with a mutation that caused the inactivation of *cpsD* were able to adhere more strongly to cells in the nasopharynx, possibly due to the increased exposure of important pneumococcal surface structures such as adhesins. However, these mutants had decreased ability to persist in the nasopharynx and were more susceptible to early clearance by the immune system. They also appeared to be deficient in the ability to migrate from the lungs to the blood stream and to cause

systemic disease (Morona et al. 2004). Cells with mutations in the *cpsC* gene of *S. pneumoniae* D39 attached only about half as much CPS to the cell wall as the wild type cells. This suggests a role for CpsC in the cell wall attachment of CPS, possibly through interactions with other proteins involved in CPS biosynthesis such as the cell wall CPS ligase. The *cpsC* mutants were unable to enter the bloodstream, indicating the importance of CPS attachment to the cell wall in the ability of this organism to cause invasive disease (Morona et al. 2006).

In *S. pneumoniae* D39, the loss of Cps2B resulted in increased tyrosine phosphorylation of Cps2D and an increase in capsule production. Although the display and function of the capsule in these mutants was apparently normal, the activity of Cps2B was critical for the survival of D39 during colonization and systemic infections. The regulation of the capsule or other factors controlled though CpsB activity might be altered in these mutants in the animal environment. It is possible that other phosphorylated proteins may be subject to Cps2B control (Bender et al. 2003). However, Bender et al. (2003) only measured the cell wall associated CPS produced by their mutants. A clearer view of this mutation is suggested by Morona et al. (2006). They found that in the absence of CpsB there was a decrease in CPS biosynthesis but an increase in the in the proportion of total CPS produced that is attached to the cell wall compared to the wild type cells. The loss of CpsB increased the amount of phosphorylated CpsD, causing a reduction in the biosynthesis of CPS and an increase in the attachment of the polymer to the cell wall. Mutation of CpsB negatively affected the

ability of *S. pneumoniae* to cause invasive disease (Bender et al. 2003; Morona et al. 2004, 2006).

There is evidence for the ability of Gram-positive PTKs to induce phosphorylation of other proteins. For example, the *Bacillus subtilis* PTK YwqD is able to phosphorylate Ugd, but only in the presence of the transmembrane protein YwqC. This suggests that interaction between the proteins containing the nucleotide binding domain and the transmembrane domain may influence the phosphorylation of other important proteins involved in EPS production in Gram-positive bacteria (Mijakovic et al. 2003). Olivares-Illana et al. (2008) created a chimeric protein that united the cytoplasmic domain of the transmembrane activator protein (CapA) with the PTK (CapB) from *S. aureus*. They proposed a novel mechanism for the regulation of polysaccharide synthesis and export by PTKs. Their results suggest that the kinase domains associate into an octameric ring when unphosphorylated, with each tyrosine tail interacting with the active site of the neighboring subunit. This octameric ring structure would extend to the transmembrane domains and constrain their action. Upon phosphorylation the kinase domains dissociate and the constraint on the transmembrane domains is released causing a conformational change. The kinase domains are then free to phosphorylate additional endogenous substrates. This switch in conformation, affects the interactions of the transmembrane domain with other proteins such as the polysaccharide unit polymerase, the flippase, or the lipid sugar transferase. It is possible that the affinity of the machinery to the polysaccharide is altered. In this way, the cycling between phosphorylated and non-phosphorylated forms regulates the switch between polymerization and export of

polysaccharides. This could be a general model for the regulation of polysaccharide synthesis due to the high conservation among polysaccharide co-polymerases (Olivares-Illana et al. 2008).

Minic et al. (2007) produced cells with mutations in the *epsB*, *epsC*, *epsD*, or *epsE* genes in *Streptococcus thermophilus* CNRZ1066. They observed that the cells that had mutations in *epsC*, *epsD,* or *epsE* produced no detectable capsule. As was seen in the *S. pneumoniae* system, the EpsC protein of *S. thermophilus* was needed for EpsD phosphorylation to occur. Experimental results from these mutants suggest that the glycosyltransferase activity of EpsE requires EpsC and EpsD, but is negatively affected by EpsB. The possibility of protein interactions between EpsC, EpsD, and EpsE was evident. It is possible that EpsE is regulated by the phosphorylation of one of its tyrosine residues by the PTK EpsD in conjunction with EpsC. In support of this, Y200 has been shown to be necessary for the glycosyltransferase activity of EpsE. EpsE could then be subject to dephosphorylation by the PTP EpsB (Morona et al. 2000a; Minic et al. 2007).

5.3. PTK activity varies from system to system

The biological function and molecular mechanism of PTK activity appear to vary from strain to strain. PTK autophosphorylation has been found to either reduce or promote high molecular mass polysaccharide production. It is the dephosphorylated form of Wzc_{ca} that promotes CA synthesis in *E. coli* K-12 (Vincent et al. 2000). It has been shown that the phosphorylation of Wzc_{cps} is essential for the assembly of group I CPS in *E. coli* K30. However, in this system the phosphorylated form of Wzc appears to promote the synthesis of the group I CPS (Wugeditsch et al. 2001). Divergent results on

the effect of the autophosphorylation state of *S. pneumoniae* CpsD have been observed. In strain RX1, the dephosphorylated form of CpsD seems to promote CPS synthesis, whereas the opposite situation is found in strain D39 (Bender et al. 2003; Morona et al. 2003). The current view is that the autophosphorylation of bacterial PTKs does not function in an on/off switch type mechanism, but that the cycling between phosphorylated and unphosphorylated forms is necessary for polysaccharide synthesis (Bechet et al. 2009). The two-step mechanism for Wzc_{ca} phosphorylation in *E. coli* K12 involves the phosphorylation of Y569 (Grangeasse et al. 2002). A similar reaction is not present in Gram-positive bacteria or in some Gram-negative bacteria. For example, in *E. coli* K30 the phosphorylation of Wzc_{cps} on Y569 is not necessary for the production of group 1 capsules (Paiment et al. 2002). The Wzc_{ca} of *E. coli* K-12 has been shown to be structurally independent due to its PTK ability (Doublet et al. 2002). However, the Wzc_{cps} of *E. coli* K-30 is unable to autophosphorylate at the C-terminal end and requires the presence of the N-terminal domain to be phosphorylated (Wugeditsch et al. 2001). In some Gram-positive bacteria, such as *S. pneumoniae*, interaction with the membrane peptide CpsC is needed for the PTK CpsD to become phosphorylated, but in *B. subtilis* the PTK YwqD alone is able to autophosphorylate (Morona et al. 2000a; Mijakovic et al. 2003).

6. Industrial uses of EPS

6.1. EPS and bioremediation

EPS can be used in bioremediation. The EPS of cyanobacteria have been shown to bind several toxic metals and can be used to remove these contaminates from the

environment (De Philippis et al. 2007; Sharma et al. 2008). One of the problems encountered in the bioremediation of hydrocarbons is the bioavailability of these poorly soluble compounds. EPS can act as an emulsifier to solubilize hydrocarbons allowing them to be more readily degraded. For example, the sulfate and uronic acid containing EPS from *Halomonas eurihalina* has the ability to emulsify a variety of hydrocarbons including *n*-tetradecane, *n*-hexadecane, *n*-octane, xylene, light and heavy mineral oils, petrol, and crude oil. In fact, the EPS from this organism was more efficient at emulsification of crude oil than the chemical surfactants Tween 20, Tween 80, and Triton X-100 (Martínez-Checa et al. 2002, 2007). A marine isolate of *Enterobacter cloacae* that produces EPS containing high amounts of uronic acid, fucose, and sulfate can emulsify a variety of hydrocarbons, vegetable oils, and mineral oils (Iyer et al. 2006). The EPS of *Gordonia alkanivorans* was able to enhance the cell floating of four non-floating strains used in diesel bioremediation. The floating cell behavior enhances the cell contact with the substrate resulting in the more efficient utilization of diesel. Addition of the EPS from this organism enhanced the biodegradation of diesel by 40-45% and has an emulsification index of over 63%. This EPS could be used as a powerful biostimulant to help improve the degradation efficiency of the existing oil-utilizing bacteria in polluted environments (Ta-Chen et al. 2008).

6.2. EPS as a cryoprotectant

The EPS of the Antarctic bacterium *P. arctica* could have industrial uses as a powerful cryoprotectant (Kim and Yim 2007). The ability of a capsule to protect bacteria from the damage incurred by freezing has been demonstrated in frozen dairy desserts.

Hong and Marshall et al. (2001) demonstrated that encapsulated *S. thermophilus* survived longer in reduced fat ice cream stored at -29 ºC than their non-encapsulated mutants. The enhanced survival of lactose hydrolyzing *S. thermophilus* in the ice cream could be beneficial to individuals who are lactose intolerant. *S. thermophilus* cultures produce β-galactosidase, which hydrolyzes lactose to the absorbable monosaccharides glucose and galactose. If this enzymatic activity is retained in the intestinal tract it could provide lactose deficient individuals with sufficient enzyme to assist in digestion of lactose.

6.3. Uses of EPS as food additives

The use of EPS producing starter stains such as *Weissella cibaria* and *Lactobacillus plantarum* can improve the rheological properties of sourdough bread. When compared to bread made by EPS^- strains the glucans produced by these organisms increased dough viscosity, increased bread volume, and reduced the firmness of the final product. The production of EPS by LAB used in the production of sourdough could be used to replace the current additives used for improving the textural qualities and the shelf life of baked goods (Cagno et al. 2006; Katina et al. 2009; Galle et al., 2010).

EPS producing cultures have a wide variety of uses in the dairy industry. The water binding properties of EPS help control syneresis and improve the stabilizing and viscosifying properties of fermented dairy products (Cerning 1990, 1995; De Vuyst and Degeest 1999). The use of EPS producing cultures could reduce the need for the addition of other stabilizers such as gelatin, modified starch, or carrageenan. EPS producing cultures have been used in yogurt, sour cream, Mozzarella cheese, soft cheese, and whipped toppings to improve rheological properties (Perry et al. 1998; Hassan et al.

1996, 2004; Broadbent et al. 2003). Low-fat cheese and yogurt produced with EPS^+ starter cultures have a better taste, texture, and mouth-feel than those manufactured with starter strains that lack the ability to produce EPS (Broadbent et al. 2001; Duboc and Mollet 2001). Even though EPS have no taste of their own, they increase the time the milk product spends in the mouth, causing an enhanced perception of taste (Duboc and Mollet 2001). Milk fat plays an important role in moisture retention of cheese by entrapping and binding water in serum channels (McMahon et al. 1993; Oberg et al. 1993; McMahon and Oberg 1998). Low-fat cheeses have fewer fat globules to break up the protein matrix, resulting in less space for water retention. This causes low-fat cheese to have a rubbery-like texture, poor melt properties, and to become less pliable upon cooking (McMahon et al. 1993; Low et al. 1998; McMahon and Oberg 1998; Broadbent et al. 2001). Polysaccharides can perform a similar function as fat molecules by breaking up the protein matrix forming a serum cavity (Perry et al. 1997). The formation of these cavities allows the entrapment of water molecules and their distribution throughout the curd matrix resulting in a softer body (Perry et al. 1997). Significantly higher moisture content is seen in low-fat cheese that has been manufactured using an EPS^+ *S. thermophilus* starter culture than in low-fat cheese manufactured using an EPS^- commercial strain (Perry et al. 1997, 1998; Low et al. 1998; Petersen et al. 2000). The results obtained by Low et al. (1998) established that the increase in moisture retention observed in low-fat cheeses in their experiments was due exclusively to *S. thermophilus* MR-1C EPS production. Curd yield was significantly higher in cheese made with the EPS producing strains MR-1C or MTC360 when compared to cheese manufactured using

the EPS$^-$ strains TA061 or DM10 (Petersen et al. 2000). Low-fat cheese manufactured using *S. thermophilus* strains such as MR-1C that produce a capsular type of EPS had similar melting and shredding properties as that manufactured with an EPS$^-$ strain. A major concern in the cheese industry is that the incorporation of EPS producing starter cultures will increase the whey viscosity and have detrimental effects on the recovery of whey products. The viscosity of whey manufactured with the ropy MTC360 strain was significantly higher and required longer ultrafiltration times than cheese manufactured with EPS$^-$ starter strains. The ultrafiltration times and whey viscosity of cheese manufactured with the MR-1C strain were comparable to that of cheese manufactured with EPS$^-$ strains. The ropy EPS$^+$ strains of *S. thermophilus*, such as MTC360, increased the moisture content of the cheese by nearly 7% when compared to cheese made with the EPS$^-$ strain TAO61. An increase in moisture content of this magnitude caused the cheese to be sticky, to shred poorly, and to expel serum during the melt test (Peterson et al. 2000). Incorporation of the capsular EPS$^+$ starter culture MR-1C increased the moisture content by 1.5%, which significantly increased the cheese yield without adversely affecting the melting or shredding properties or affecting whey viscosity (Perry et al. 1997, 1998; Low et al. 1998; Peterson et al. 2000; Broadbent et al. 2001). These results suggest that the use of capsular but not ropy EPS$^+$ *S. thermophilus* starter cultures, like MR-1C, could improve the quality of low-fat cheese. However, a fast acid producing strain is needed for Mozzarella cheese production and *S. thermophilus* MR-1C lacks this ability (Broadbent et al. 2001). Zisu and Shah (2004) used a capsular *S. thermophilus* culture to make low-fat Mozzarella cheese. Although the increase in moisture in this

cheese was only 1.25%, it was sufficient to reduce hardness, chewiness, and springiness and to improve the meltability of the low-fat cheese. Recently, the ropy strain *L. lactis* subsp. *cremoris* JFR1 was used to produce low-fat Cheddar cheese that had similar texture, melting characteristics, and viscoelastic properties to full fat Cheddar cheese (Awad et al. 2005; Hassan et al. 2005). The changes in porosity of these cheeses were less pronounced than those of other low-fat cheeses that underwent major changes in the size and distribution of pores during ripening. In full fat cheese, the large pores contained fat globules and in the JFR1 reduced fat cheese they contained either fat or EPS (Hassan and Awad 2005). The EPS molecules form filaments that can interact with milk proteins and aid in the formation of protein aggregates altering the texture and viscosity of fermented dairy products (Ayala-Hernandez et al. 2008). The reduced fat cheese made with an EPS^+ culture contained more continuous protein filaments than the cheese without EPS. In full fat cheese, the protein filaments provide resistance during heating, causing a more uniform melting. The layered structure of the protein filaments in the low-fat cheese made without EPS is probably responsible for the rubbery texture typical of most low-fat cheeses (Hassan and Awad 2005). Ropy EPS improves the rennet coagulation properties, reduces the cheese making time, and increases moisture, thereby increasing the efficiency of low fat cheddar cheese making (Rynne et al. 2007).

6.4. Use of EPS to prevent biofilm formation

The treatment of abiotic surfaces with EPS significantly inhibits mature biofilm development by a broad range of bacteria and has a long lasting effect (Valle et al. 2006). For example, the EPS produced by *Lactobacillus acidophilus* A4 has the ability to

prevent biofilm formation by pathogenic *E. coli* O157:H7. Polystyrene wells and PVC surfaces that contained 1mg/ml of released EPS from *L. acidophilus* had an 87% reduction in biofilm formation by *E. coli* O157:H7. Under similar conditions, biofilm formation was decreased by 94% on PVC surfaces. The biofilm formation of several other Gram-negative and Gram-positive pathogens, such as *Salmonella enteritidis*, *Salmonella typhimurium*, *Yersina enterocolitica*, *P. aeruginosa*, *Listeria monocytogenes*, and *Bacillus cereus,* was significantly decreased on both polysterene and PVC treated with EPS from *Lb. acidophilus*. The EPS from this organism would be a food-grade material, so it may have applications for biofilm prevention in food processing plants (Kim et al. 2009).

6.5. EPS and human health

The EPS from food grade LAB may help prevent chronic gastritis. The administration of the non-steroidal anti-inflammatory drug acetyl-salicyclic acid (ASA) can have negative effects on the gastrointestinal system including dyspepsia, abdominal pain, and the formation of gastric ulcers. Researchers found that if mice were preventively treated with fermented milk containing the EPS producing *S. thermophilus* strain CRL 1190 they did not develop chronic gastritis when administered ASA. The treated mice had reduced levels of cells that secrete the proinflammatory cytokine interferon (INF)-γ and increased numbers of cells that secrete the regulatory cytokine IL-10. IL-10 is known to inhibit the production of INF-γ. The same effect was seen if purified EPS suspended in autoclaved milk was administered, but not if the EPS was suspended in water or if the milk was administered alone. This suggests that EPS-milk

protein interactions may be responsible for the gastroprotective effect (Rodríguez et al. 2009). EPS significantly attenuated experimental colitis in rats, which could make it a promising therapeutic for the treatment of inflammatory bowel disease (Şengül et al. 2005). EPS has been shown to have cholesterol-lowering activities (Nakajima et al. 1992). The EPS from certain bifidobacteria, lactobacilli, and streptococci have been reported to be beneficial in preventing ulcers. Bacterial cell wall polysaccharides had a similar effect especially if the rhamnose concentration was greater than 60%, suggesting that rhamnose may be involved in the anti-ulcer effect (Nagaoka et al. 1994).

EPS have been shown to have anticarcinogenic properties. Mutagenic heterocyclic amines are found in foods such as cooked beef and fried hamburger. Up to 89% reduction of the mutagenicity of the heterocyclic amine 3-amino-1,4-dimethyl-5*H*-pyrido-[4,3-*b*]indole was observed in the presence of an EPS from *Bifidobacterium longum*. The antimutagenicity of the EPS was dose dependent and may relate to the ability of this EPS to bind the mutagens. The acidic moieties in many EPS may interact with the high alkalinity of most mutagens, forming stable complexes that prevent the conversion of promutagen to ultimate mutagen in the intestinal tract (Sreekumar and Hosono 1998). Kitazawa et al. (1991) found that the growth of Sarcoma-180 tumors could be inhibited by the intraperitoneal injection of lyophilized *L. lactis* subsp. *cremoris* KVS 20 cells in mice. This same strain was not effective against the Sarcoma-180 tumor in vitro suggesting that the ability of this strain to prevent tumor proliferation was mediated through immune activity. Kitazawa et al. (1991) postulated that the slime material produced by this strain may be the principal component in the antitumoral effect.

A later study showed that the EPS from *L. lactis* subsp. *cremoris* KVS 20 induced B-cell dependent mitogenic activity (Kitazawa et al. 1992). An EPS producing strain of *Lb. delbrueckii* subsp. *bulgaricus* used in yogurt manufacture has been shown to induce host-mediated antitumor activity. After fractionation of the EPS into neutral and acidic components, it was determined that the acidic fraction was a phosphopolysaccharide that had mitogenic activity for murine B-lymphocytes. It was determined that the phosphate groups act as a trigger for the mitogenic induction by EPS (Kitazawa et al. 1998). Most of the studies on antitumor activity of EPS have been done with intraperitoneal injection, but Zubillaga et al. (2001) was able to demonstrate that the water-soluble EPS from kefir grains had antitumoral properties when administered orally. It is thought that the immune enhancement by the orally administered EPS involves the participation of T-cells and not B-cells.

EPS have been shown to enhance proliferation of T-lymphocytes, stimulate macrophage activation, and to induce production of the cytokines (Forsén et al. 1987; Kitazawa et al. 1998). The EPS from a strain of *Bacillus licheniformis* and *Geobacillus thermodenitrificans* seem to improve immune surveillance of peripheral blood mononuclear cells toward Herpes simplex virus-2. The EPS from these organisms causes a proinflammatory response that is believed to hinder the replication of the virus in human peripheral blood mononuclear cells by stimulating the production cytokines, such as INF- γ, INF-α, tumor necrosis factor (TNF)-α, IL-12, and IL-18 by T-helper type 1 cells (Arena et al. 2006, 2009). The EPS of *L. lactis* subsp. *cremoris* SBT 0495 was able

to enhance the production of specific antibodies in mice when administered intraperitoneally, suggesting that it may act as an adjuvant (Nakajima et al. 1995).

Sulphated polysaccharides can interfere with the absorption and penetration of enveloped viruses such as herpes simplex virus, human cytomegalovirus, and human immunodeficiency virus into host cells as well as inhibit various retroviral reverse transcriptases (Baba et al. 1988; Hayashi et al. 1996; Matsuda et al. 1999; Zhu et al. 2004).

Low molecular weight dextrans from *L. mesenteroides* are widely used in the pharmaceutical industry as blood plasma extenders and blood flow improvers (Cerning 1990). The deep-sea hydrothermal vent organism *Altermonas infernus* produces an EPS with anticoagulant properties (Colliec et al. 2001).

7. Conclusions

EPS help bacteria to survive in unique ecological niches and to evade host immune response. The production of EPS is a complex process that is not fully understood and likely involves many protein interactions and regulatory mechanisms. Tyrosine phosphorylation, a mechanism that at one time was unheard of in bacterial systems, plays a prominent role in the production and export of EPS. The phosphorylation of PTKs and their dephosphorylation by PTPs is important for efficient high molecular weight EPS synthesis and for regulating the polymer length and quantity of EPS produced. Due to roles in pathogenesis, possible health benefits, and industrial applications of EPS, it is expected that copious amounts of new research in this area will be done in the future and many new EPS gene clusters will be characterized. The

research contained in this dissertation adds new insight into the complex process of EPS biosynthesis by establishing that the *S. thermophilus* Wzh protein functions as a phosphotyrosine phosphatase, investigating protein-protein interactions in *S. thermophilus* MR-1C, determining if the same protein-protein interactions take in *Streptococcus iniae* 9066, and *Lactococcus lactis* subsp. *cremoris* JRF1, and investigating interspecific protein-protein interactions to determine if functional regulatory complexes can be formed in naturally occurring and genetically engineered recombinant strains.

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CHAPTER 3

STREPTOCOCCUS THERMOPHILUS WZH FUNCTIONS AS A PHOSPHOTYROSINE PHOSPHATASE

Abstract

Amino acid residues that are important for metal binding and catalysis in Gram-positive phosphotyrosine phosphatases were identified in the *Streptococcus thermophilus* MR-1C Wzh protein using sequence comparisons. His-tagged fusion Wzh proteins were purified from *Escherichia coli* cultures and tested for phosphatase activity against synthetic phosphotyrosine and phosphoserine/threonine peptides. Purified Wzh released 2316.5 ± 138.7 pmol PO_4 min^{-1} μg^{-1} from phosphotyrosine peptide-1 and 2345.7 ± 135.2 pmol PO_4 min^{-1} μg^{-1} from phosphotyrosine peptide-2. The presence of the phosphotyrosine phosphatase inhibitor sodium vanadate decreased purified Wzh activity by 45-50% at 1 mM, 74-84% at 5 mM, and by at least 88% at 10 mM. Purified Wzh had no detectable activity against the phosphoserine/threonine peptide. These results clearly establish that *S. thermophilus* MR-1C Wzh functions as a phosphotyrosine phosphatase that could function to remove phosphate groups from proteins involved in exopolysaccharide biosynthesis including the protein tyrosine kinase Wze and priming glycosyltransferase.

1. Introduction

Although once considered rare, tyrosine phosphorylation is recognized today as a key regulatory device in prokaryotic organisms that functions in exopolysaccharide production, virulence, stress response, and DNA metabolism (Chow et al. 1994; Jagtap and Ray 1999; Petranovic et al. 2007). The phosphorylation and dephosphorylation of bacterial protein tyrosine kinases (PTKs) is necessary for exopolysaccharide biosynthesis and assembly in a wide variety of Gram-positive and Gram-negative bacteria including *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Acinetobacter johnsonii*, *Burkholderia cepacia*, and *Staphylococcus aureus* (Duclos et al. 1996; Grangeasse et al. 1998; Vincent et al. 1999, 2000; Morona et al. 2000; Bender and Yother 2001; Wugeditsch et al. 2001; Preneta et al. 2002; Soulat et al. 2007; Ferreira et al. 2007). The dephosphorylation of PTKs is accomplished by phosphotyrosine phosphatases (PTPs), many of which are well-characterized (Grangeasse et al. 1998; Vincent et al. 1999; Bender and Yother 2001; Morona et al. 2002; Preneta et al. 2002; Ferreira et al. 2007; LaPointe et al. 2008; Hagelueken et al. 2009; Kim et al. 2011). The best-characterized Gram-positive PTP is the CpsB protein of *S. pneumoniae*. CpsB is a novel metal-dependent PTP that belongs to the polymerase histidinol phosphatase family and regulates capsule production by the removal of the phosphates from the transmembrane/periplasmic kinase complex CpsCD and by inhibiting phosphorylation of tyrosine residues (Morona et al. 2002; Bender and Yother 2001; Hagelueken et al. 2009; Kim et al. 2011). Bender et al. (2003) created a deletion mutation in Cps2B of *S. pneumoniae* D39 that resulted in the increased phosphorylation of the tyrosine residues

on the PTK Cps2D and increased capsule production. However, Bender et al. (2003) only measured the cell wall associated CPS produced by their mutants. Morona et al. (2006) found that in the absence of CpsB there was a decrease in CPS biosynthesis but an increase in the proportion of total CPS produced that is attached to the cell wall compared to the wild type cells. The loss of CpsB increased the amount of phosphorylated CpsD, causing a reduction in the biosynthesis of CPS and an increase in the attachment of the polymer to the cell wall. In both the above cases, the mutation of CpsB negatively affected the ability of *S. pneumoniae* to colonize and cause invasive disease in mice. These results suggest that switching between phosphorylated and non-phosphorylated forms of the PTK is needed for efficient, high molecular weight polysaccharide synthesis (Bender et al. 2003; Morona et al. 2003, 2004, 2006).

It is possible that other phosphorylated proteins will be subject to Cps2B control. Experimental research done on *Streptococcus thermophilus* CNRZ1066 by Minic et al. (2007) demonstrated that the hypothetical PTP EpsB negatively affects the glycosyltransferase activity of EpsE. Their results indicate that EpsE may be regulated by the phosphorylation of one of its tyrosine residues by the PTK EpsD in conjunction with EpsC. In support of this, they identified a tyrosine residue (Y200) that is necessary for the glycosyltransferase activity of EpsE and could perhaps be subject to phosphorylation by EpsD and subsequently to dephosphorylation by EpsB. It is feasible that other glycosyltransferases or proteins involved in EPS biosynthesis may be regulated by the phosphorylation and dephosphorylation of tyrosine (Mijakovic et al. 2003; Minic et al. 2007).

Recently, high-resolution crystal structures of both the phosphate-complexed and the ligand free but metal bound form of Cps4B from *S. pneumoniae* TIGR4 have been determined. The data from structural studies and site-directed mutagenesis have provided insights into the active site and possible conformational changes of Cps4B. Cps4B has three metal ions (M1, M2, and M3) bound to the active site that are coordinated by conserved amino acids and water molecules (W1, W2, and W3) (Hagelueken et al. 2009; Kim et al. 2011). It is unknown, which metal ions occupy the M1, M2, and M3 sites in Cps4B as contradictory results have been presented. Hagelueken et al. (2009) found that the addition of Cu^{2+}, Co^{2+} and Mn^{2+} ions to reactions containing Cps4B increased the activity by more than 10-fold whereas Fe^{2+}, Ni^{2+} and Zn^{2+} lead to a much lower activation of the enzyme. The three metal ions were modeled as manganese although the weaker electron density peak of M3 suggested that it might be a different ion from M1 and M2. However, Kim et al. (2011) found that their samples of Cps4B contained mostly Fe and Mg ions, but not other metal ions such as Ni^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Co^{2+}. They assigned M1 and M2 as Fe ions and M3 as a Mg ion. As these ions were not intentionally added to the buffer solutions for purification and crystallization it is thought that they are picked up by the recombinant enzymes in the expression medium. This may have a profound effect on metal loading in purified enzymes.

A unique chemical mechanism for the metal-dependent PTPs from Gram-positive bacteria has been proposed which employs a metal-bound water molecule or hydroxyl ion as a nucleophile (Wilcox 1996; Hagelueken et al. 2009; Kim et al. 2011). In this mechanism, the incoming phosphotyrosine binds to the active site of Cps4B via arginine

residues, M2, and M3. A strong ionic interaction of the phosphate group with arginine in the active site helps align the phosphorous atom directly over the nucleophile and displaces the negative charge on the phosphorous atom allowing for the subsequent nucleophilic attack. W1 serves as a ligand for both M1 and M2 and most likely represents a shared hydroxyl ion that is deprotonated and acts as the nucleophile. The positively charged arginine residues and metal ions surrounding the active site stabilize the developing negative charges during the S_N2 displacement. The positive potential around the active site would also stabilize the tyrosinate that results from cleavage of the phosphodiester bond (Gerrantana et al. 2001; Hagelueken et al. 2009; Kim et al. 2011). When the phosphate ion binds to the active site it replaces all three metal-bound water molecules. The chemistry of this proposed mechanism is similar to that of the phosphotriesterases (Benning et al. 2001; Elias et al. 2008; Hagelueken et al. 2009; Kim et al. 2011).

The *S. thermophilus* Wzh/EpsB protein is homologous to known PTPs and believed to function in removing the phosphate groups from the PTK Wze/EpsD as well as from the priming glycosyltransferase and possibly other proteins (Broadbent et al. 2003; Minic et al. 2007). However, the PTP activity of the Wzh/EpsB protein in *S. thermophilus* has never been directly demonstrated or verified. In this study, several *S. thermophilus* Wzh/EpsB protein sequences were analyzed to verify that they contain the conserved amino acids identified by Hagelueken et al. (2009) and Kim et al. (2011) that are thought to be necessary to the function of the Gram-positive metal-dependent PTPs. The Wzh protein from *S. thermophilus* MR-1C was purified after heterologous

expression in *E. coli* and analyzed for tyrosine phosphatase activity and for serine/ threonine phosphatase activity using synthetic phosphopeptides. The activity of Wzh was tested in the presence of the PTP inhibitor sodium vanadate.

2. Materials and methods

2.1. Computational analysis of protein sequences

Protein sequences were obtained for the PTPs of several Gram-positive bacteria including several strains of *S. thermophilus* (Table 3.1). Alignments were performed using the program ClustalW2 (Larkin et al. 2007) that is located at the website http://www.ebi.ac.uk/Tools/clustalw2/index.html. *S. pneumoniae* TIGR4 Cps4B used by Hagelueken et al. (2009) and Kim et al. (2011) was included in the alignment and the amino acids and motifs that were identified as being important for metal binding and catalysis in this species were labeled. The conservation of these important amino acids and motifs in other Gram-positive species was analyzed.

2.2. Bacterial strains, media, and growth conditions

S. thermophilus MR-1C was cultured in M17 broth containing 0.5% lactose and incubated at 37 ^{o}C without aeration (Terzaghi and Sandine 1975). *Escherichia coli* DH5α (Invitrogen, Carlsbad, California, USA) and BL21(DE3) (Novagen, Madison, Wisconsin, USA) were grown at 37 ^{o}C in Luria-Bertani (LB) broth with shaking. LB agar plates containing ampicillin at a concentration of 50 μg/ml was used for the selection of *E. coli* transformants. Bacterial strains were stored at -80 °C in 20% glycerol.

Table 3.1. GenBank accession numbers of the organisms used for computational analysis of protein sequences.

Organism	Phosphotyrosine Phosphatase	GenBank Accession Number
Streptococcus pneumoniae TIGR4	Cps4B	NC_003028.3
Streptococcus pneumoniae D39	Cps2B	NC_008533.1
Streptococcus pneumoniae CDC1873-00	CpsB	NZ_ABFS01000001.1
Streptococcus thermophilus MR-1C	Wzh	AF448249.1
Streptococcus thermophilus MR-2C	Wzh	AY061649.1
Streptococcus thermophilus MTC360	Wzh	AF434993.1
Streptococcus thermophilus MTC330	EpsB	AF430847.1
Streptococcus thermophilus CNRZ1066	EpsB	NC_006449.1
Streptococcus oralis SK610	CpsB	AJKQ01000025.1
Streptococcus sanguinis ATCC 49296	CpsB	NZ_AEPO01000010.1
Streptococcus macedonicus ACA-DC 198	CpsB	NC_016749.1
Streptococcus iniae 9066	CpsB	HQ698911.1
Streptococcus bovis ATCC 700338	CpsB	NZ_AEEL01000013.1
Streptococcus salivarius CCHSS3	CpsB	NC_015760.1
Streptococcus infantis SK1302	CpsB	NZ_AEDY01000066.1
Streptococcus agalactiae A909	CpsB	NC_007432.1
Streptococcus gordonii str. *Challis* substr. CH1	Wzh	CP000725.1
Staphylococcus aureus 5C	Cap5C	U81973.1
Lactococcus lactis subsp. *cremoris* JRF1	EpsC	HQ665557
Lactobacillus rhamnosus ATCC 9595	Wzb	AY659976
Bacillus subtilis 168	YwqE	NP_391505

2.3. Plasmid construction

The *wzh* gene of *S. thermophilus* MR-1C (accession number AF448249.1) was amplified using PCR and sequence specific primers that contained restriction sites for cloning. The forward primer had the sequence 5' TCTAGAAAAAGAAGGAGGAAAA TAAGTGATTGACGTTCAC 3' and the reverse primer had the sequence 5' GTCGACT AAATATTGATTTTCTAGTAATG 3'. The PCR product containing the *wzh* gene was ligated into pGEM5Z (Promega, Madison, Wisconsin, USA) using a pGEM®-T Vector System according to the manufacturer's instructions (Promega, Madison, Wisconsin, USA) after which the ligation mixture was electroporated (2500 V, 200 Ω, 25 μF) into *E. coli* DH5α cells using a Gene Pulser® II Electroporation System (Bio Rad, Hercules, California, USA), transformants carrying the *wzh* gene identified, and the cloned gene in selected transformants sequenced to identify exact matches to the *wzh* gene. The gene

inserts were removed from the pGEM5Z vector and ligated into the pET-22b vector (Novagen, Madison, Wisconsin, USA) using the *Xba*I site created by the forward primer and the *Sal*I site created by the reverse primer to allow the production of His-tagged fusion proteins. The pET-22b plasmid containing the *wzh* insert (pET-22b/*wzh*) was first built in DH5α cells and then recovered. The construct was then electroporated into *E. coli* BL21(DE3) cells to allow high level induced protein expression. The pET-22b/*wzh* plasmid was recovered from BL21(DE3) cells and sequenced to confirm the accuracy of the *wzh* expression construct.

2.4. DNA sequencing

All DNA sequencing reactions were done at the Utah State University Center for Integrated BioSystems using an ABI prism 3730 DNA analyzer and Taq FS terminator chemistry. To sequence inserts in pGEM5Z the universal T7 and Sp6 primers as well as the *wzh* specific primers listed above were used. To sequence inserts in pET-22b the universal T7 promoter and T7 terminator primers were used.

2.5. Wzh fusion protein production, purification, and quantification

For protein induction, 300 ml of LB broth containing ampicillin at a concentration of 50 μg/ml was inoculated with *E. coli* BL21(DE3) cells containing the pET-22b/*wzh* plasmid to an OD_{600} 0.1 from an overnight culture. The culture was grown at 37 ^{o}C with shaking until the OD_{600} reached 0.5–1.0 and then protein production was induced by adding IPTG to a final concentration of 1mM for 3 hours (pET System Manuel, Novagen,

Madison, Wisconsin, USA). Aliquots of the uninduced and induced cells were taken for analysis of protein induction by SDS-PAGE electrophoresis.

The Wzh protein was purified using the PrepEase® histidine-tagged protein purification mini kit-high specificity (USB, Santa Clara, California, USA). The instructions for purification of His-tagged proteins from *E. coli* under native conditions provided with the kit were modified to allow for the recovery of *S. thermophilus* Wzh in a phosphate free buffer. Bacterial cells were harvested by centrifugation at 6,000 x g for 15 min at 4 °C and the pellet was resuspended in 3 ml equilibration buffer (10 mM Tris pH 8.0 and 300 mM NaCl). Lysozyme was added to a final concentration of 1 mg/ml and the solution was left on ice for 30 min. The suspension was then sonicated using a cell disruptor (model W-220 F; Heat Systems-Ultrasonics, Inc., Farmingdale, New York, USA) on ice using setting 2 for 5 x 10 second bursts with a 15 second cooling period between each burst. DNase I was added to the lysate at a concentration of 5 µg/ml and it was left on ice for 15 minutes. To remove cellular debris, the lysate was centrifuged at 10,000 x g for 30 minutes at 4 °C and then the supernatant was transferred to a clean tube. A PrepEase® Ni-TED column was equilibrated by adding 320 µl of equilibration buffer. The clarified lysate was then added to the column and allowed to drain by gravity flow. The column was washed three times with 320 µl of equilibration buffer and then the protein was eluted by adding 600 µl of elution buffer (10 mM Tris pH 8.0, 300 mM NaCl, 20% glycerol, and 250 mM imidazole) 100 µl at a time and collecting the fractions. The protein content in eluted fractions was quantified by using a NanoDrop™ 8000 system (Thermo Scientific, Wilmington, Delaware, USA). The fraction containing the

greatest concentration of His-tagged *S. thermophilus* Wzh protein was analyzed using SDS-PAGE electrophoresis and then stored at -80 °C.

2.6. Assay of the phosphatase activity of S. thermophilus MR-1C Wzh

The activity of the purified Wzh protein from *S. thermophilus* MR-1C was quantified using the Promega (Madison, Wisconsin, USA) tyrosine phosphatase and serine/threonine phosphatase assay systems according to the manufacturer's instructions. Both systems measure the release of phosphate from phosphorylated peptide substrates.

The tyrosine phosphatase assays were performed at 37 ^{o}C for 10 minutes in the presence of synthetic phosphotyrosine peptides (Phosphotyrosine peptide-1: END(pY)INASL or Phosphotyrosine peptide-2: DADE(pY)LIPQQG. Sodium vanadate solution was added to the reactions in concentrations of 1, 5, and 10 mM to test the activity of Wzh in the presence of a PTP inhibitor (LaPointe et al. 2008). A 0.1 M activated sodium vanadate solution was prepared by adding 0.64 g of sodium orthovanadate to 35 ml of ultrapure water while stirring until completely dissolved. The pH of the solution was adjusted to 10 with 1 N HCl or 1 N NaOH and then boiled until it became colorless and remained colorless upon cooling. The phosphate released from 0.1 μg of purified Wzh from the synthetic phosphotyrosine peptides was calculated from absorbance measurements (A_{600}) of three separate trials taken after adding the molybdate dye additive that complexes with free phosphate to form a green color. The absorbance measurements where then compared to a calibration curve determined with known concentrations of free phosphate (Fig. 3.1). Using this information, the activity of Wzh

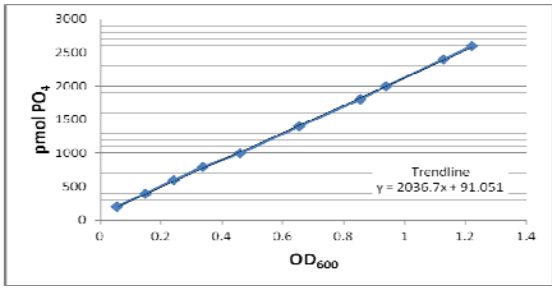

Fig. 3.1. Phosphate calibration curve. A phosphate standard (1 mM) was diluted so that samples contained 200, 400, 600, 800, 1000, 1400, 1800, 2000, 2400, and 2600 pmol of PO_4. The molybdate dye additive was added and the OD600 was taken. This curve was used to determine the pmol of PO_4 released from synthetic phosphopeptides by the PTP Wzh from *Streptococcus thermophilus*.

alone and in the presence of activated sodium vanadate was expressed in released pmol PO_4 min^{-1} μg^{-1}.

The serine/threonine phosphatase assays were performed at 37 ^{o}C for 10-30 minutes in the presence of the synthetic phosphopeptide RRA(pT)VA. Purified Wzh was added to the reactions in concentrations ranging from 0.1-2 µg and activity was determined as described for the tyrosine phosphatase assays.

The sodium vanadate solution (at 1, 5, and 10 mM) and elution buffer did show any background absorbance above that of the dye additive itself. Reactions that contained all components including the synthetic phosphopeptides but lacking Wzh and reactions that contained all the components including Wzh but lacking synthetic phosphopeptides were set up as negative controls. Reactions that contained all components plus phosphotyrosine peptide-1 without Wzh had a background OD_{600} of 0.34, reactions that contained all components plus phosphotyrosine peptide -2 without Wzh had a background OD_{600} of 0.14, and reactions that contained all components plus the phosphoserine/threonine peptide without Wzh had a background OD_{600} of 0.18.

These values where subtracted from the reactions containing these peptides and Wzh to correct for background absorbance. The reaction that contained all reaction components plus Wzh without either of the phosphotyrosine peptides did not have background absorbance above that of the dye additive itself. Reactions that contained the *S. thermophilus* MR-1C Wze (PTK) that was cloned and purified by the same method as Wzh were also tested. Purified Wze was added to reactions with all synthetic phosphopeptides at a concentration of 1 μg to confirm that any activity seen was not due to any contaminating *E. coli* proteins from the purification procedure.

3. Results

3.1. Computational analysis of protein sequences

Recent research has identified conserved amino acid residues that are necessary for the proper function of Gram-positive PTPs (Shi 2004; LaPointe et al. 2008; Hagelueken et al. 2009; Kim et al. 2011). To confirm if these conserved amino acids are present in the Wzh proteins of *S. thermophilus*, the predicted Wzh/EpsB proteins from strains MR-1C, MR-2C, MTC330, MTC360, and CNRZ1066 were aligned with several PTPs from other species of streptococci including the well-studied *S. pneumoniae*. Also included in the alignment are other Gram-positive PTP's, including those from *Staphylococcus aureus* 5C, *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus rhamnosus* ATCC 9595, and *Bacillus subtilis* 168, to illustrate any amino acids that may be conserved not just among the streptococci but among all Gram-positive PTP's (Fig. 3.2).

Four motifs that contain several conserved amino acids including histidine, aspartate, and glutamate residues that are important in binding divalent cations and in

coordination of the catalytic site have been identified in PTPs (Aravind and Koonin 1998; Shi 2004; LaPointe et al. 2008; Hagelueken et al. 2009; Kim et al. 2011). These motifs are labeled with the conserved amino acid residues indicated in bold type (Fig. 3.2). High-resolution crystal structures of *S. pneumoniae* TIGR4 Cps4B have more clearly identified amino acid residues that are responsible for coordination of metal and phosphate ions, therefore making them essential to the catalytic mechanism of Gram-positive PTPs (Hagelueken et al. 2009; Kim et al. 2011). M1 in Cps4B is coordinated by two axial ligands (Glu80 and Asp199) and three equatorial ligands (His5, His7, and W1) (Hagelueken et al. 2009). Glu80, Asp199, His5, and His7 are highly conserved in all compared PTPs, including *S. thermophilus*, and are shadowed in light grey (Fig. 3.2). M2 is coordinated by the amino acids Glu80, Glu108, and His136 and by W1 and W2. Glu80 and W1 serve as bidentate ligands for M1 and M2 (Hagelueken et al. 2009). The amino acid residues Glu80, Glu108, and His136 that are needed to coordinate M2 are conserved in all the compared PTPs and are shadowed in light grey (Fig. 3.2). M3 is coordinated by His42, Asp14, His201, and W3 (Hagelueken et al. 2009). The amino acid residues that coordinate M3 are conserved in all the compared PTPs and are shadowed in light grey (Fig. 3.2). The phosphate ion is bound to the active site of Cps4B by interactions involving the metal ions as it replaces all three metal-bound solvent molecules as well as with the amino acid residues Arg139 and Arg206 (Hagelueken et al. 2009). Asp139 and Arg206 are conserved in all of the compared PTP's and are shadowed in light grey (Fig. 3.2).

Kim et al. (2011) identified two active site loops in both Cps4B from *S. pneumoniae* TIGR4 and YwqE from *B. subtilis* that are important in the catalytic mechanism of PTPs from Gram-positive organisms. The first loop encompasses Arg44-Pro51 of Cps4B (Asn44-Glu51of YwqE) and contains the sequence motif [(G/P)x(Y/F) with x being any amino acid]. The sequence in this loop, RKGMFETP, is highly conserved for all of the PTPs from streptococci species and is labeled and shadowed in dark grey. The PTPs from *Staph. aureus* 5C, *L. lactis* subsp. *cremoris* JRF1, *Lb. rhamnosus* ATCC 9595, and *B. subtilis* subsp. *subtilis* str. 168 vary in the sequence in this loop but still contain the motif [(G/P)x(Y/F)] (Fig. 3.2). Loop II extends from Leu168-Arg176 in CpsB (Ala168-Lys173 of YwqE) and contains the sequence motif [(G/P)x1-2FGx0-1(K/R)] (Kim et al. 2011). The sequence in this loop is more variable between compared PTPs than that of loop I and is labeled and shadowed in dark grey. The sequence motif [(G/P)x1-2FGx0-1(K/R)] is not conserved in all of the compared PTPs, although it is present in all of the *S. thermophilus* proteins (Fig. 3.2).

3.2. Purification and determination of the phosphatase activity of S. thermophilus MR-1C Wzh

IPTG-induced *E. coli* BL21 (DE3) cultures containing the pET-22b/*wzh* plasmid overproduced a 28 kDa protein (Fig. 3.3) corresponding to the predicted size of the *S. thermophilus* MR-1C Wzh protein (Broadbent et al. 2003). The purified Wzh protein was eluted in the highest concentration in fraction 2 and formed a single band of the same molecular mass as that in the IPTG-induced culture (Fig. 3.3).

The purified *S. thermophilus* Wzh protein released phosphate equally from both synthetic phosphotyrosine peptides with 2316.5 ± 138.7 pmol PO_4 min^{-1} μg^{-1} being

Fig. 3.2. Alignment of the PTPs from various Gram-positive bacteria. Included in this alignment are the PTPs from *Streptococcus pneumoniae* TIGR4 (Cps4B, SP TIGR4), *Streptococcus pneumoniae* D39 (Cps2B, SP D39), *Streptococcus pneumoniae* CDC1873-00 (CpsB, SP CDC1873-00), *Streptococcus thermophilus* MR-1C (Wzh, ST MR-1C), *Streptococcus thermophilus* MR-2C (Wzh, ST MR-2C), *Streptococcus thermophilus* MTC360 (Wzh, ST MTC360), *Streptococcus thermophilus* MTC330 (EpsB, MTC330), *Streptococcus thermophilus* CNRZ1066 (EpsB, ST CNRZ1066), *Streptococcus oralis* SK610 (CpsB, SO SK610), *Streptococcus sanguinis* ATCC 49296 (CpsB, SSan ATCC49296), *Streptococcus macedonicus* ACA-DC 198 (CpsB, SM ACA-DC198), *Streptococcus iniae* 9066 (CpsB, SIni 9066), *Streptococcus bovis* ATCC 700338 (CpsB, SBATCC 700338), *Streptococcus salivarius* CCHSS3 (CpsB, SSal CCHSS3), *Streptococcus infantis* SK1302 (CpsB, SInf SK1302), *Streptococcus agalactiae* A909 (CpsB, SA A909), *Streptococcus gordonii* str. *Challis* substr. CH1 (Wzh, SG CH1), *Staphylococcus aureus* 5C (Cap5C, SAur 5C), *Lactococcus lactis* subsp. *cremoris* JRF1 (EpsC, LL JRF1), *Lactobacillus rhamnosus* ATCC 9595 (Wzb, LR ATCC9595), and *Bacillus subtilis* 168 (YwqE, BS 168). The four PTP motifs are labeled with the conserved histidine, aspartate, and glutamate residues indicated in bold type. The conserved amino acids Glu80, Asp199, His5, His7, Glu108, His136, His42, Asp14, His201, Asp139, and Asp206 involved in binding metal and phosphate ions as identified by Hagelueken et al. (2009) are shadowed in light grey. The conserved loops identified by Kim et al. (2001) are shadowed in dark grey. Loop I contains the conserved motif [(G/P)x(Y/F)] and loop II has the conserved motif [(G/P)x1-2FGx0-1(K/R)]. Amino acids that are identical in all sequences of the alignment are indicted by an asterisk, conserved substitutions are indicated by two dots, and semi-conserved substitutions are indicated by one dot.

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                    Motif I                  Motif II     Loop I
SP TIGR4            MIDIHSHIVFDVDDGPKSREESKALLAESYRQGVRTIVSTSHRRKGMFETPEEKIAENFL 60
SP D39              MIDVHSHIVFDVDDGPKSREESKALLAESYRQGVRTIVSTSHRRKGMFETPEEKIAENFL 60
SP CDC1873-00       MIDIHSHIVFDVDDGPKSREESKALLAEAYRQGVRTIVSTSHRRKGMFETPEEKIAENFL 60
ST MR1C             MIDVHSHIVFDVDDGPKTLEESLDLIGESYAQGVRKIVSTSHRRKGMFETPEDKIFANFS 60
ST MR2C             MIDVHSHIVFDVDDGPETLEESLDLIGESYAQGVRKIVSTSHRRKGMFETPEDKIFANFK 60
ST MTC360           MIDVHSHIVFDVDDGPETLEESLDLIGESYAQGVRKIVSTSHRRKGMFETPEDKIFANFK 60
ST MTC330           MIDVHSHIVFDVDDGPETLEESLDLIGESYAQGVRKIVSTSHRRKGMFETPEDKIFANFK 60
ST CNRZ1066         MIDVHSHIVFDVDDGPETLEESLDLIGESYAQGVRKIVSTSHRRKGMFETPEDKIFANFK 60
SO SK610            MIDIHSHIVFDVDDGPKSIEESKKLLREAYSQGVRTIVSTSHRRKGMFETPEEKIATNFL 60
SSan ATCC49296      MIDIHSHIVFDVDDGPKSIEESKKLLREAYSQGVRTIVSTSHRRKGMFETPEEKIATNFL 60
SM ACA-DC198        MIDIHSHIVFDVDDGPTTIEESLALVGESYRQGVRTIVSTSHRRKGMFETPEDKIFANFS 60
SIni 9066           MIDIHSHIVFDVDDGPLTIDDSLALIGESYRQGVRTIVSTSHRRKGMFETPEDDIYNKFL 60
SB ATCC700338       MIDIHSHIVFDVDDGPKTIEESLDLIGESYRQGVRTIVSTSHRRKGMFETPEDKIFANFS 60
SSal CCHSS3         MIDVHSHIVFDVDDGPKTLEESLDLIGESFAQGVRTIVSTSHRRKGMFETPEDKIFANFK 60
SInf SK1302         MIDIHSHIVFDVDDGPKDKAESIALLEEAYAQGVRTIVSTSHRRKGMFETPEEDIARNFK 60
SA A909             MIDIHSHIVFDVDDGPKTLEESLSLIEESYRQGVRIIVSTSHRRKGMFETPEDIIFKNFS 60
SG CH1              MIDIHSHIVFDVDDGPKTIEDSRALLEESYRQGVRTIISTSHRRKGMFETPEAKIEENFK 60
SAur 5C             MIDIHNHILPNIDDGPTNETEMMDLLKQATTQGVTEIIVTSHHLHPRYTTPIEKVKSCLN 60
LL JRF1             MIDIHCHILPGIDDGAKTSGDTLTMLKSAIDEGITTITATPHHN-PQFNNESPLILKKVK 59
LR ATCC9595         MIDVHCHMLPGIDDGSKDLTTSLELAQAAVADGITHALMTPHHMNGRYTNHATDVIRMTD 60
BS 168              MIDIHCHILPAMDDGAGDSADSIEMARAAVRQGIRTIIATPHHNNGVYKNEPAAVREAAD 60
                    ***:* *::  :***.        :   :  :*:     *.*:    : .    :
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SP TIGR4          QVREIAKEVASDLVIAYGAEIYYTPDVLDKLEKKRIPTLN-DSRYALIEFSMNTPYRDIH 119
SP D39            QVREIAKEVADDLVIAYGAEIYYTPDVLDKLGKKRIPTLN-DSRYALIEFSMNTPYRDIH 119
SP CDC1873-00     QVREIAKEVASDLVIAYGAEIYYTPDVLDKLGKKRIPTLN-DSRYALIEFSMNTPYRDIH 119
ST MR1C           KVKAEAEALYPDLTIYYGGELYYTSDIVEKLEKNLIPRMH-NTQFALIEFSARTSWKEIH 119
ST MR2C           KVKAEAEALYPDLTIYYGGELYYTSDIVEKLEKNLIPRMH-NTQFALIEFSARTSWKEIH 119
ST MTC360         KVKAEAEALYPDLTIYYGGELYYTSDIVEKLEKNLIPRMH-NTQFALIEFSARTSWKEIH 119
ST MTC330         KVKAEAEALYPDLTIYYGGELYYTSDIVEKLEKNLIPRMH-NTQFALIEFSARTSWKEIH 119
ST CNRZ1066       KVKAEAEALYPDLTIYYGGELYYTSDIVEKLEKNLIPRMH-NTQFALIEFSARTSWKEIH 119
SO SK610          KVREMAKEVADDLIIAYGAEIYYTPDVVEKLEKKLIPTLN-DSRYALIEFSMNTPYRDMH 119
SSan ATCC49296    KVREMAKEVANDLIIAYGAEIYYTPDVVEKLEKKLIPTIN-DSRYALIEFSMNTPYRDIH 119
SM ACA-DC198      QVKEAAEAKYEGLEILYGGELYYSSDILERLEQRQVPRMN-DTRFALIEFSMTTPWKEIH 119
SIni 9066         HLKREAEKEYEGLTILYGGELYYTEDILDKLAQNLIPRMN-DTRFALIEFSMNTPWRDIH 119
SB ATCC700338     QVKEAAEAKYEGLEILYGGELYYSSDILEKLERGQVPKMN-NTRFALIEFSMTTSWKDIH 119
SSal CCHSS3       KVKEEAEALYPDLTIFYGGELYYTSDIVEKLEKNLIPRMH-NTQFALIEFSARTSWKEIH 119
SInf SK1302       VVKELAKEIAPDFTILYGAEIYYSSDALNKLENQQIPQLN-GTRYALIEFSMNTPYREIH 119
SA A909           IVKHEAEKRFEHLQILYGGELYYTSDMLEKLKLKQIPTLN-NTKFALIEFSMQTSWKDIH 119
SG CH1            QVQELAKEIADDLTILYGAEIYYTSDILDKLEQEKIPRLA-GSQYALIEFSMITPYKEIH 119
SAur 5C           HIESLEEVQALNLKFYYGQEIRITDQILNDIDR-KVINGINDSRYLLIEFPSNEVPHYTD 119
LL JRF1           EVQNIIDEHQLPIEVLPGQEVRIYGDLLKEFSEGKLLKAAGTSSYILIEFPSNHVPAYAK 119
LR ATCC9595       EFQDELDRRNIPLTVFPCQEVRINGQLLEAIDHNDILTCDVSGHYVLIEFPSDDVPLYTQ 120
BS 168            QLNKRLIKEDIPLHVLPGQEIRIYGEVEQDLAKRQLLSLN-DTKYILIEFPFDHVPRYAE 119
                   ..        : .   *:   : . :   :       : ****.

                     ______Motif III_______                    Loop II
SP TIGR4          SALSKILMLGITPVIAHIERYDALENNEKRVRELIDMGCYTQVNSSHVLKPKLFGERYKF 179
SP D39            SALSKILMSGITPVIAHIERYDALGNNEKRVRELIDMGCYTQVNSSHVLKPKLFGERYKF 179
SP CDC1873-00     SALNKILMLGITPVIAHIERYDALENNEKRVRELIDMGCYTQVNSSHVLKPRLFGERYKF 179
ST MR1C           SGLSNVLRAGVTPIVAHIERYDALEENADRVREIINMGCYTQVNSSHVLKPKLFGDKDKV 179
ST MR2C           SGLSNVLRAGVTPIVAHIERYDALEENADRVREIINMGCYTQVNSSHVLKPKLFGDKDKV 179
ST MTC360         SGLSNVLRAGVTPIVAHIERYDALEENADRVREIINMGCYTQVNSSHVLKPKLFGDKDKV 179
ST MTC330         SGLSNVLRAGVTPIVAHIERYDALEENADRVREIINMGCYTQVNSSHVLKPKLFGDKDKV 179
ST CNRZ1066       SGLSNVLRAGVTPIVAHIERYDALEENADRVREIINMGCYTQVNSSHVLKPKLFGDKDKV 179
SO SK610          KGLSNILMLGITPVIAHIERYDALENNEKRVRELIDMGCYTQVNSSHVLKPKLFGETYKF 179
SSan ATCC49296    KGLSNILMLGITPVIAHIERYDALENNEKRVRELIDMGCYTQVNSSHVLKPKLFGETYKF 179
SM ACA-DC198      TALSNVIMLGITPVVAHIERYNALEFNEERVKELINMGGYTQINSSHVLKPKLFGDKYHQ 179
SIni 9066         TALSQVIMLGITPIIAHIERYDALALNAKRVQELINMGCYTQVNSSHVLKAKLFGDSLKV 179
SB ATCC700338     TALSNVIMLGITPVVAHIERYNVLEFNEERVKELINMGCYTQINSSHVLKPKLFGDKYRQ 179
SSal CCHSS3       TGLSNVLRAGVTPIVAHIERYDALEENADRVREIINMGCYTQVNSSHVLKPKLFGDKDKV 179
SInf SK1302       NALTNVLMLGITPVIAHIERYDALENNEKRVRDLINMGCYTQINSSSILKPKLFGDTYKF 179
SA A909           TALSNVLMLGITPVVAHIERYNALENQKERVKEIINMGCYTQINSSHILKQKLFNDKHKR 179
SG CH1            TALSNVLRLGVTPVVAHIERYHCLENDEKKVRDLINMGCYTQINSSSVLKPKLFGDTYKF 179
SAur 5C           QLFFELQSKGFVPIIAHPERNKAISQNLDILYDLINKGALSQVTTA-----SLAGISGKK 174
LL JRF1           ELFYNIKLEGLQPILVHPERNSGIIENPDILFDFIEQGVLSQITAS-----SVTGHFGKK 174
LR ATCC9595       NMLFEVMQRGMIPVIVHPERNTRLMKHPGLLYQMVERGAFAQVTAS-----SYVGTFGKK 175
BS 168            QLFYDLQLKGYIPVIAHPERNREIRENPSLLYHLVEKGAASQITSG-----SLAGIFGKQ 174
                    : .:   *  *::.* **   :  .   : .::: *  :*:.:.       .   :

                        _____Motif IV_________
SP TIGR4          MKKRAQYFLEQDLVHVIASDMHNLDGRPPHMAEAYDLVTQKYGEAKAQELFIDNPRKIVM 239
SP D39            MKKRAQYFLEQDLVHVIASDMHNLDGRPPHMAEAYDLVTQKYGEAKAQELFIDNPRKIVM 239
SP CDC1873-00     MKKRAQYFLEQDLVYVIASDMHNLDGRPPHMAEAYDLVTQKYGEAKAQELFIDNPRKIVM 239
ST MR1C           RKKRVRYFLEKNLVHMVASDMHNLGPRPPFMKDAYEIVKKNYGPKRAKNLFIENPKTLLE 239
ST MR2C           RKKRVRFFLEKNLVHMVASDMHNLGPRPPFMKDAYEIVKKNYGSKRAKNLFIENPKTLLE 239
ST MTC360         RKKRVRFFLEKNLVHMVASDMHNLGPRPPFMKDAYEIVKKNYGSKRAKNLFIENPKTLLE 239
ST MTC330         RKKRVRFFLEKNLVHMVASDMHNLGPRPPFMKDAYEIVKKNYGSKRAKNLFIENPKTLLE 239
ST CNRZ1066       RKKRVRFFLEKNLVHMVASDMHNLGPRPPFMKDAYEIVKKNYGSKRAKNLFIENPKTLLE 239
SO SK610          MKKRAQYFLERDLVHVIASDMHNLDHRPPHMEEAYDIIAQKYSEDKAKELFKDNPRKIIM 239
SSan ATCC49296    MKKRAQYFLERDLVHVIASDMHNLDHRPPHMEEAYDIIAKKYSGDKAKELFKDNPRKIIM 239
SM ACA-DC198      FKKRARYFLEKNLVHCVASDMHNLGPRPPFMDKAREIVTKDFGPNRAYALFEENPQTLLE 239
SIni 9066         FKKRAKFFLDENLVHCIASDMHNLKKRPPFMQEAYQHVTKHYGRKRARELFITNPQTLIE 239
SB ATCC700338     FKKRARYFLEKNLVHCVASDMHNLGPRPPFMDEAREIITKDFGTKRADALFEGNPQTLLE 239
SSal CCHSS3       RKKRVRFFLEKNLVHMVASDMHNLGLRPPFMKDAYEIVKKNYGPKRAKNLFIENPKTLLE 239
SInf SK1302       MKKRARYFLERDLVHIVASDMHNLDGRPPYMREAYEIVSKRYGRDKARELFVENPEKVIK 239
SA A909           FKKRARYFLEENLVHFVASDMHNLDVRPPFLAEAYKIICRDFGKERANQLFIENAQSILK 239
SG CH1            MKKRAQFFLEKDLVHFVASDMHNLDPRPPYMQEAYQIISKKYGEPHAEQLFRKNQELLLR 239
SAur 5C           IRKLAIQMIENNLTHFIGSDAHNTEIRPFLMKDLFNDKKLRDYYE-DMNGFISNAKLVVD 233
LL JRF1           IQKLSFKMIENHLTHFVASDAHNVTSRAFKMKEAFEIIEDSYGSG-VSRMLQNNADSVIL 233
LR ATCC9595       VQQFSEDIIDAGLAHVFASDAHHLPGRSYEMSAAFKRLTRKRGEK-KARIFEENARALVN 234
BS 168            LKAFSLRLVEANLIHFVASDAHNVKTRNFHTQEALYVLEKEFGSE-LPYMLTENAELLLR 233
                   :     :::  * : ..** *:   *                        :  *   ::
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SP TIGR4          DQLI------------------ 243
SP D39            DQLI------------------ 243
SP CDC1873-00     DQLI------------------ 243
ST MR1C           NQYL------------------ 243
ST MR2C           NQYL------------------ 243
ST MTC360         NQYL------------------ 243
ST MTC330         NQYL------------------ 243
ST CNRZ1066       NQYL------------------ 243
SO SK610          DQLI------------------ 243
SSan ATCC49296    DQLI------------------ 243
SM ACA-DC198      NKDL------------------ 243
SIni 9066         NDYL------------------ 243
SB ATCC700338     NKDL------------------ 243
SSal CCHSS3       NQYL------------------ 243
SInf SK1302       DQII------------------ 243
SA A909           NHYI------------------ 243
SG CH1            SEYI------------------ 243
SAur 5C           DKKIPKRMPQQDYKQKRWFGL- 254
LL JRF1           NESFYQEEPIK-IKTKKFLGLF 254
LR ATCC9595       GDPLVRFNERK-VEKR-LLSRY 254
BS 168            NQTIFRQPPQP-VKRRKLFGFF 254
                  .. :
```

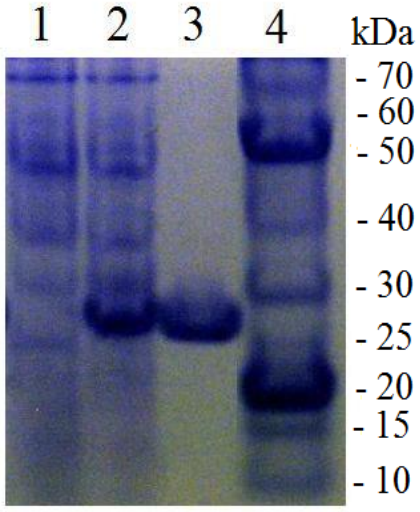

Fig. 3.3. SDS-PAGE of purified histidine-tagged *Streptococcus thermophilus* MR-1C Wzh protein. Wzh was purified using the PrepEase® histidine-tagged protein purification mini kit-high specificity (USB, Santa Clara, California, USA). Lane 1: Uninduced culture lysate of *E. coli* BL21 cells containing the pET-22b/*wzh* vector; Lane 2: IPTG-induced culture lysate of *E. coli* BL21 cells containing the pET-22b/*wzh* vector; Lane 3: Fraction 2 from the elution step containing purified Wzh; Lane 4: BenchMark Protein Ladder (Invitogen, Carlsbad, California, USA).

released from phosphotyrosine peptide-1 and 2345.7 ± 135.2 pmol PO_4 min^{-1} μg^{-1} being released from phosphotyrosine peptide-2 (Fig. 3.4). When 1 mM of the PTP inhibitor sodium vanadate was added to reactions the ability of Wzh to release phosphate from both phosphopeptides was reduced by 45-50% (1135.9 ± 132.2 and 1249.3 ± 87.5 pmol PO_4 min^{-1} μg^{-1} for phosphotyrosine peptide-1 and 2, respectively) when compared to reactions that contained purified Wzh alone (Fig. 3.4). To further investigate the effect of sodium vanadate on Wzh, it was added to reactions in concentrations of 5 and 10 mM. In the presence of 5 mM sodium vanadate the ability of Wzh to release phosphate from both phosphotyrosine peptides was reduced by 78-84% (497.0 ± 68.2 and 378.9 ± 78.8 pmol PO_4 min^{-1} μg^{-1} for phosphotyrosine peptide-1 and 2, respectively) (Fig. 3.4). When 10 mM of sodium vanadate was added to reactions of Wzh and either phosphotyrosine peptide only about 12% (282.5 ± 59.3 and 259.4 ± 62.3 pmol PO_4 min^{-1} μg^{-1} for phosphotyrosine peptide-1 and 2, respectively) of the original activity of the enzyme remained (Fig. 3.4). Reactions that contained 2 μg purified Wzh and the synthetic phosphoserine/threonine peptide that had an OD_{600} that was comparable to that seen in

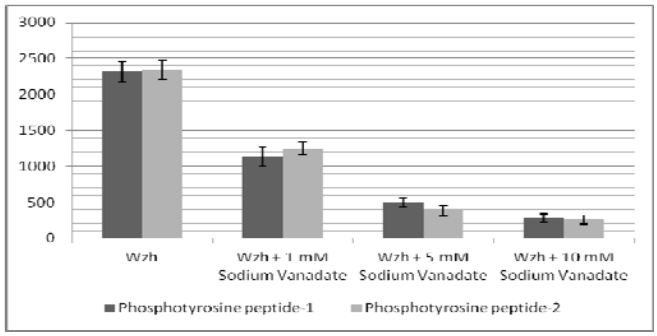

Fig. 3.4. Graphic representation of the PTP activity of *Streptococcus thermophilus* MR-1C Wzh alone and in the presence of the PTP inhibitor sodium vanadate.

reactions containing only the dye additive. These results indicated that Wzh has no detectable activity against the synthetic phosphoserine/threonine peptide.

4. Discussion

The *S. thermophilus* MR-1C Wzh protein was established to have phosphotyrosine phosphatase activity. The PTP inhibitor sodium vanadate had a dramatic effect on purified Wzh, reducing the enzymatic activity by around 45-50% at 1 mM, 74-84% at 5 mM, and at least 88% at 10 mM when compared to the activity of purified Wzh alone. Using similar methodologies, LaPointe et al. (2008) observed a 55% decrease in the activity of the PTP from *Lb. rhamnosus* when in the presence of 1 mM sodium vanadate. Purified Wzh had no detectable activity against the phosphoserine/threonine peptide in reactions that contained 20 times the concentration of Wzh and were incubated for three times as long as the phosphotyrosine phosphatase reactions. This indicates that this enzyme is unlikely to be responsible for removing phosphate groups from serine and threonine. Recently, phosphorylation on serine and threonine residues was observed in the PTK from *Klebisella pneumoniae* but the role of phosphorylation of these sites is not clear (Lin et al. 2009). It is unknown if the *S. thermophilus* MR-1C PTK would also be phosphorylated at serine or threonine residues but if it does occur a separate phosphatase would be necessary to remove phosphate from these residues.

Results from protein sequence comparison of the *S. thermophilus* MR-1C Wzh protein to the well studied PTP Cps4B from *S. pneumoniae* suggests that Wzh is likely to use a comparable mechanism to remove phosphate groups from phosphotyrosine

containing substrates. The mutation of amino acids in the active site or those interacting with metal ligands in the PTPs from *S. pneumoniae* and *Lb. rhamnosus* decreased the activity by at least 80%, reaffirming that these residues are important for the function of the enzyme and the stability of the metal cluster (Morona et al. 2002; Mijakovic et al. 2005; LaPointe et al. 2008; Hagelueken et al. 2009). For example, Cps4B mutants in which Arg139 was changed to alanine had only 5% of the wild type enzymatic activity, identifying Arg139 as a catalytic key player in Cps4B activity (Hagelueken et al. 2009). Replacement of the histidine residues at positions 5, 7, or 42 by alanine in the *Lb. rhamnosus* PTP lead to drastic reductions of up to 99% of the enzymatic activity (LaPointe et al. 2008). All of these amino acids are highly conserved in all the Gram-positive PTPs compared, including that from *S. thermophilus* MR-1C.

Active site loop I plays a role in controlling the access of phosphotyrosine containing substrates to the catalytic site (Kim et al. 2011). The sequence of this region is identical in all streptococcal PTPs and the [(G/P)x(Y/F)] motif is present in all the PTPs compared. The amino acid sequence of loop II is more variable between the species of PTPs compared. This sequence is highly similar when comparing the species of streptococci to each other but different when comparing the species of streptococci to *S. aureus*, *L. lactis* subsp. *cremoris*, *Lb. rhamnosus*, and *B. subtilis*. The sequence motif [(G/P)x1-2FGx0-1(K/R)] is not strictly conserved, although it is present in all strains of *S. thermophilus*. The phenylalanine residue located in the middle of this motif is conserved in all species except *Staph. aureus* 5C. It has been suggested that this phenylalanine residue functions as a gatekeeper to control the access of phosphotyrosine

into the active site while conserved glycine or proline residues on both sides of phenylalanine allow sharp turns in loop II and confer conformational flexibility. Many of the amino acids in Loop I and II are positively charged and hydrophobic residues that line a deep active-site pocket that can accommodate the bulky aromatic ring of phosphotyrosine (Kim et al. 2011).

It is interesting to note that the overall structure of the PTPs is conserved but the PTPs fall into 2 clear structural groups consisting of the streptococci proteins in one group and the PTPs from *Staph. aureus* 5C, *L. lactis* subsp. *cremoris* JRF1, *Lb. rhamnosus* ATCC 9595, and *B. subtilis* subsp. *subtilis* str. 168 in the other group. The groups have sequence differences within the conserved motifs and the carboxyl termini of the proteins. However, all the amino acids identified by Hagelueken et al. (2009) necessary for the coordination of metal and phosphate ions are conserved in *S. thermophilus* MR-1C Wzh indicating that the active site is similar in arrangement to that of *S. pneumoniae* Cps4B. The amino acids that are thought to be important for catalysis in loop I and II as identified by Kim et al. (2011) in Cps4B from *S. pneumoniae* are also well conserved in *S. thermophilus* MR-1C Wzh.

Taken together these results prove that the *S. thermophilus* MR-1C Wzh protein functions as a PTP that could be responsible for removing phosphate from the PTK Wze. However, the ability of *S. thermophilus* Wzh to remove the phosphate groups from Wze still needs to be directly confirmed by other research methods.

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CHAPTER 4

PROTEIN-PROTEIN INTERACTIONS AMONG THE COMPONENTS OF THE BIOSYNTHETIC MACHINERY RESPONSIBLE FOR EXOPOLYSACCHARIDE PRODUCTION IN *STREPTOCOCCUS THERMOPHILUS* MR-1C[1]

Abstract

Aim: Identify protein-protein interactions among the biosynthetic machinery responsible for exopolysaccharide production in *Streptococcus thermophilus* MR-1C.

Methods and Results: Protein-protein interactions were investigated using the yeast two-hybrid system. A strong protein-protein interaction was detected between the transmembrane activation protein Wzd and the protein tyrosine kinase Wze. Weaker protein-protein interactions were detected between two duplicate Wze proteins and between Wze and the phosphotyrosine phosphatase Wzh. Protein-protein interactions involving a Wzd/Wze fusion protein and Wzd and Wze may indicate that these proteins form multi-protein complexes. All combinations of the Wzh, Wzd, Wze, Wzg (regulation), CpsE (glycosyl-1-phosphate transferase), CpsS (polymerization), CpsL (unknown), CpsW (regulation), and CpsU (membrane translocation) proteins were analyzed for protein-protein interactions but no additional interactions were discovered using the yeast two-hybrid system.

Conclusions: Interactions among the phosphotyrosine phosphatase, tyrosine kinase, and transmembrane activation protein are important in the regulation of capsule biosynthesis in *Strep. thermophilus* MR-1C.

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[1] Coauthored by Angela D. Cefalo, Jeffery R. Broadbent, and Dennis L. Welker (2011) J. Appl. Microbiol. **110** (3): 801-812.

Significance and Impact of Study: This study provides some valuable insight into the organization and interactions between the many proteins involved in exopolysaccharide production. A better understanding of this process may facilitate the genetic manipulation of capsule production to impart desirable properties to dairy starter cultures.

1. Introduction

The exopolysaccharide (EPS) producing lactic acid bacteria have been used in fermented dairy products, such as yogurt and cheese, to improve rheological properties and to replace stabilizers such as gelatin, modified starch, or carrageenan (Perry *et al*. 1997; Low *et al*. 1998; Petersen *et al*. 2000; Hassan *et al*. 2003; Ramchandran and Shah 2009). The EPS from these organisms may have beneficial effects on human health including preventing chronic gastritis, reducing the symptoms of colitis, lowering cholesterol, preventing ulcers, acting as anticarcinogens, preventing tumor formation, and stimulating the immune system (Forsén *et al*. 1987; Kitazawa *et al*. 1991, 1992, 1998; Nakajima *et al*. 1992, 1995; Nagaoka *et al*. 1994; Sreekumar and Hosono 1998; Zubillaga *et al*. 2001; Şengül *et al*. 2005; Rodríguez *et al*. 2009). The beneficial properties of these organisms both in dairy foods and as probiotics could be enhanced if a thorough understanding of the molecular processes involved in EPS production could be gained.

In the mechanism for intracellular assembly of EPS, repeating sugar units are assembled on the inner face of the cytoplasmic membrane by the sequential transfer of sugar nucleotide diphospho-precursors to a carrier lipid, which may be undecaprenyl phosphate, and then exported (Johnson and Wilson 1977; De Vuyst *et al.* 2001; Cartee *et al*. 2005; Whitfield 2006). The first step in this process is catalyzed by a glycosyl-1-

phosphate transferase that transfers a sugar-1-phosphate to the lipid carrier. In *Streptococcus thermophilus* MR-1C, the glycosyl-1-phosphate transferase is encoded by the fifth gene in the cluster, *cpsE*. The *cpsF, cpsN, cpsP, cpsQ, cpsR, cpsT,* and *cpsV* genes are likely to encode the remaining glycosyltransferases that sequentially transfer the remaining sugar residues to the repeat unit (Stingele *et al*. 1996, 1999; Almiron-Roig *et al*. 2000; De Vuyst *et al.* 2001; Jolly and Stingele 2001; Broadbent *et al*. 2003; Cartee *et al*. 2005).

The *Strep. thermophilus* MR-1C *cpsU* and *cpsS* genes are homologous to *Escherichia coli wzx* and *wzy*, respectively (Broadbent *et al*. 2003). In the synthesis of *E. coli* colanic acid (CA), group 1, and group 4 capsules, Wzx is thought to be the flippase which translocates the repeating sugar units through the membrane and Wzy is believed to be a polymerase involved in production of long chain polymers by successive transfer of the growing lipid-linked chain to the reducing end of a single repeat unit (Liu *et al*. 1996; Drummelsmith and Whitfield 1999; Feldman *et al*. 1999; Valvano 2003; Whitfield 2006).

The first four genes in the *Strep. thermophilus* MR-1C EPS gene cluster, *wzg*, *wzh*, *wzd*, and *wze*, are highly conserved among different species of EPS producing streptococci (Broadbent *et al.* 2003). The *Streptococcus pneumoniae* Wzg homologue, Cps2A, functions in the regulation of capsule production as an activator of gene transcription. The deletion of *cps2A* not only reduced encapsulation but also caused a reduction in the phosphorylation of Cps2D (Wze), suggesting that Cps2A could have

functions other than or in addition to transcriptional control (Cieslewicz *et al*. 2001; Bender *et al*. 2003).

The *wzh*, *wzd*, and *wze* genes of *Strep. thermophilus* MR-1C are homologous to genes that encode a tyrosine kinase/phosphatase regulatory system in other bacteria (Grangeasse *et al*. 1998, 2002; Vincent *et al*. 1999; Morona *et al*. 2000, 2002; Bender and Yother 2001; Wugeditsch *et al*. 2001; Preneta *et al*. 2002; Broadbent *et al*. 2003; Soulat *et al*. 2006; Ferreira *et al*. 2007). In most Gram-positive bacteria, the transmembrane activation domain and the nucleotide binding domain that contains the tyrosine phosphorylation sites are separate proteins as opposed to being contained in a single protein in the typical Gram-negative system (Vincent *et al*. 1999; Morona *et al*. 2000; Cieslewicz *et al*. 2001; Doublet *et al*. 2002; Broadbent *et al*. 2003; Olivares-Illana *et al*. 2008). In the proposed mechanism for *Strep. pneumoniae* (Morona *et al*. 2000), the dephosphorylated form of the protein tyrosine kinase (PTK) CpsD (Wze) interacts with the transmembrane activation domain CpsC (Wzd) and ATP, possibly causing a conformational change in CpsC that would promote interactions with other proteins allowing capsular polysaccharide (CPS) polymerization and biosynthesis to proceed at the maximal level. The autophosphorylation of tyrosine residues on CpsD promotes transfer of the polymer to the undefined cell wall-CPS ligase (Morona *et al*. 2000, 2003, 2004; Bender *et al*. 2003). CpsD is dephosphorylated by the phosphotyrosine phosphatase (PTP), CpsB (Wzh) (Bender and Yother 2001; Morona *et al*. 2002). Results obtained by Morona *et al*. (2003) imply that the switching between phosphorylated and

non-phosphorylated forms of CpsD is needed for efficient, high molecular weight polysaccharide synthesis.

Olivares-Illana *et al*. (2008) proposed a general model for the regulation of polysaccharide synthesis based on experimental results from *Staphylococcus aureus*. In this model, the unphosphorylated tyrosine kinase domains associate into an octameric ring structure that would extend to the transmembrane domains and constrain their action. Each tyrosine tail would interact with the active site of the neighboring subunit. Upon phosphorylation, the kinase domains would disassociate releasing the constraint on the transmembrane domains causing a switch in conformation that may affect interactions with other proteins or alter the affinity of the machinery to the polysaccharide. The kinase domains may then be free to phosphorylate other substrates. Cycling between phosphorylated and non-phosphorylated forms regulates the switch between polymerization and export of polysaccharides.

Recent research suggests that these proteins may have more than one functional role in exopolysaccharide synthesis. In both Gram-negative and Gram-positive systems, there is evidence that PTKs can phosphorylate other proteins. Wzc_{ca} from *E. coli* K-12 and YwqD from *Bacillus subtilis* seem to activate by phosphorylation the UDP-glucose dehydrogenases that are needed to synthesize building blocks for EPS synthesis (Grangeasse *et al*. 2003; Mijakovic *et al*. 2003; Lacour *et al*. 2008). In Gram-negative organisms, Wzc_{ca} may be involved in the phosphorylation of glycosyltransferases, including WcaJ. In support of this, a tyrosine residue that undergoes phosphorylation and seems to be important for proper function has been identified in WcaJ from *Klebsiella*

pneumoniae (Obadia *et al*. 2007; Lin *et al*. 2009). Similarly, the *Strep. thermophilus* CNRZ1066 PTK EpsD (Wze) in conjunction with EpsC (Wzd) may regulate the glycosyltransferase EpsE by the phosphorylation of one of its tyrosine residues (Minic *et al*. 2007). Wzc from *E. coli* has been demonstrated to have ATPase activity. It is possible that the energy produced from the hydrolysis of ATP by Wzc_{ca} could be used for CA synthesis (Obadia *et al*. 2007; Soulat *et al*. 2007). Wzc is involved in a protein-protein interaction with the outer membrane lipoprotein Wza; therefore Wzc could have functions interacting with biosynthetic machinery and coupling to the export pathway in *E. coli* (Nesper *et al*. 2003; Reid and Whitfield 2005; Collins *et al*. 2007). CpsC (Wzd) from *Strep. pneumoniae* seems to be involved in the cell wall attachment of CPS, possibly by interacting with other proteins such as the cell wall capsular polysaccharide ligase (Morona *et al*. 2006). The Gram-negative PTP Wzb has been shown to dephosphorylate substrates other than Wzc. Wzb may function in the dephosphorylation of enzymes involved in nucleotide sugar precursor synthesis or glycosyltransferases (Bugert and Geider 1997; Grangeasse *et al*. 1998; Vincent *et al.* 1999; Preneta *et al*. 2002; Nakar and Gutnick 2003).

Relatively little is known about how the proteins involved in EPS biosynthesis assemble and interact with one another. This study used the yeast two-hybrid system to detect protein-protein interactions among the *Strep. thermophilus* MR-1C Wzg, Wzh, Wze, Wzd, CpsE, CpsP, CpsQ, CpsS, CpsL, CpsU, and CpsW proteins that are involved in EPS production in this organism. The yeast two-hybrid system has never been used to study protein interactions in EPS biosynthesis and this is the first study to analyze the

direct interaction of some of these proteins. Minic *et al.* (2007) isolated protein complexes from *Strep. thermophilus* CNRZ1066 that had a molecular size that suggested protein interactions between the transmembrane activation domain, the PTK, and the glycosyl-1-phosphate transferase. Protein interactions between the transmembrane activation domain and the PTK were also suggested by the analysis of the crystalline structure of a chimeric protein containing these two domains in *Staph. aureus* (Olivares-Illana *et al*. 2008). This study demonstrates the direct protein-protein interaction between the putative PTK Wze (CpsD) and the transmembrane activator protein Wzd (CpsC). It also demonstrates that these proteins have the ability to form multi-protein complexes. Protein-protein interactions were also detected between two identical PTK Wze proteins and between the PTK Wze and the PTP Wzh. No other protein interactions were detected among Wzg, Wzh, Wze, Wzd, CpsE, CpsP, CpsQ, CpsS, CpsL, and CpsW.

2. Materials and methods

2.1. Construction of plasmids

The *wzg, wzh, wzd, wze, cpsE, cpsP, cpsQ, cpsS, cpsL, cpsW,* and *cpsU* genes that are involved in EPS production in *S. thermophilus* MR-1C (GenBank accession number AF448249.1) were amplified using PCR and sequence specific primers (Table 4.1). The PCR products were ligated into the *E. coli* vectors pGEM4Z, pGEM5Z, or pGEM7Z, *E. coli* DH5α cells were transformed with the constructs, and sequenced to identify exact matches to *S. thermophilus* MR-1C genes. The gene inserts were recovered from the vectors using restriction enzymes to create *Bam*HI, *Eco*RI or *Sal*I sites on the 5' end of the insert and *Sal*I or *Pst*I sites on the 3' end of the insert. The inserts were then cloned

into the yeast vectors pGAD424 and pGBT9 using the above restriction sites allowing synthesis of corresponding fusion proteins with the Gal4 activation domain (AD) and the Gal4 DNA binding domain (BD), respectively (Fields and Song 1989; Chien *et al*. 1991). The resulting yeast vectors with gene inserts were checked for sequence accuracy and proper frame alignment of the start codon before yeast cells were transformed with the constructs (Table 4.1).

Table 4.1. *Streptococcus thermophilus* MR-1C, pGAD424, and pGBT9 oligonucleotides used in this study.

Gene or Vector	Primer	Sequence 5' to 3'	Location	RS	Use
wzg	864	AAAAGGATCCGTCGACAAGGAGC AATTTATATGAGTTCGCGT	6395-6419	*Bam*HI	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	865	AAAACTGCAGCTTATTTTTCCTCC ATCAATTTTTTG	7863-7838	*Pst*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	AR	AATACCTGCGTGGGTTAA	7286-7269	None	PCR confirmation of yeast clones
wzh	425	CCCGGGTCTAGAGAATTCGTGATT GACGTTCACTCAC	7863-7881	*Eco*RI	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	426	CCCGGGGTCGACGGATCCTCTTGA TTCATAATATCTCC	8613-8594	*Sal*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
wzd	394	CCCGGGTCTAGAGAATTCATAGGA GATATTATGAATCAAG	8591-8612	*Eco*RI	Gene insert amplification; construction of Wzd/Wze fusion; PCR confirmation of yeast clones; sequencing
	396	CCCGGGTCGACGGATCCAAAGGC ATTTCTTCTCCTT	9318-9300	*Sal*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	506	CCCGGGATCCGGTCTCAAATTTTA TCTGTATCCGGAA	9299-9279	*Eco*31I	Construction of Wzd/Wze fusion; PCR confirmation of yeast clones; sequencing
wze	395	CCCGGGTCTAGAGAATTCGAAGGA GAAGAAATGCCTTTATT	9300-9321	*Eco*RI	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	397	CCCGGGTCGACGGATCCTTAAAAC ACACTAACGCGTT	10079-10059	*Sal*I	Gene insert amplification; construction of Wzd/Wze fusion; PCR confirmation of yeast clones; sequencing
	500	AAAACACACTAACGCGTT	10076-10059	None	PCR confirmation of yeast clones; sequencing
	507	CCCGGGAATTCGGTCTCCAATTAT GCCTTTATTAAAGTTAGTT	9311-9331	*Eco*31I	Construction of Wzd/Wze fusion; PCR confirmation of yeast clones; sequencing
cpsE	492	GGGGGTCGACCAAGTGGAGGAAA TGAGATG	10097-10116	*Sal*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	493	CCCCTGCAGTAATTTTTGTTTCATC AG	10817-10800	*Pst*I	Gene insert amplification; PCR confirmation of yeast clones;

					sequencing
cpsP	571	CCCCGTCGACGGATAAGGAATAATCGATGAAG	14051-14072	*Sal*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	572	CCCGGGATCCGGTCTCCTGCAGCTATTCCACTCAGT	14918-14905	*Pst*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
cpsQ	563	CCCCGTCGACTGGAGGATATATTACACATGG	14989-15009	*Sal*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	564	CCCCCTGCAGAATCATTCACCAACCTTTCCC	15847-15827	*Pst*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
cpsS	22	CTCCTGCATCTCGTAATACCAATCAC	16666-16641	None	PCR confirmation of yeast clones
	23	GATCTTCTTAAGCAAGGTTCAC	16523-16544	None	PCR confirmation of yeast clones
	826	GGGGGATCCGTCGACAAGGTTGGGGGAATGATTATGGTTGCATTAG	15840-15860	*Bam*HI	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	827	GGGCTGCAGATCATCTCCTTCCTATCAC	17129-17111	*Pst*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
cpsL	828	GGGGGATCCGTCGACATATTTTGGAGGAAAAA	22714-22730	*Bam*HI	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	829	GGGCTGCAGTTTATTTCATCACAATATAATC	23622-23601	*Pst*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
cpsW	830	GGGGGATCCGTCGACATGTATATGGAGATACC	24565-24549	*Bam*HI	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	831b	AAAACTGCAGTTTATTTTAGAGATTTTTCAAAAAGGG	23645-23671	*Pst*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
cpsU	832	GGGGGATCCGTCGACTTTCGGGAGGGTTATTA	31992-31976	*Bam*HI	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	833b	AAAACTGCAGTCTATTTATTCATAATAAACTTTAGCTTATT	30559-30589	*Pst*I	Gene insert amplification; PCR confirmation of yeast clones; sequencing
	UR	AGGGTCATTGGATTCTGT	31292-31275	None	PCR confirmation of yeast clones
pGAD424	496	ATTCGATGATGAAGATAC	790-807	None	PCR confirmation of yeast clones; sequencing
	497	TTTCAGTATCTACGATTC	886-869	None	PCR confirmation of yeast clones; sequencing
pGBT9	883	GAGACAGCATAGAATAAG	799-816	None	PCR confirmation of yeast clones; sequencing
	498	GTCAAAGACAGTTGACTG	852-869	None	PCR confirmation of yeast clones; sequencing
	499	AAATTCGCCCGGAATTAG	911-928	None	PCR confirmation of yeast clones; sequencing

Oligonucleotide primers were derived from the genome sequence of *Streptococcus thermophilus* MR-1C (GenBank accession number AF448249.1) and from the vector sequences of pGAD424 (GenBank accession number U07647.1) and pGBT9 (GenBank accession number U07646.1). In some cases additional nucleotides were added to create the restriction sites (RS) used for cloning gene inserts. Parts of nucleotide sequences that have been altered or that are not included in the original gene or vector sequence are underlined.

2.2. Construction of a Wzd/Wze fusion protein

A fusion protein containing the membrane spanning protein Wzd and the putative PTK Wze was created in an attempt to mimic the organization of Gram-negative PTKs. The *wzd* gene was amplified by PCR using forward primer 394 and reverse primer 506. The *wze* gene was amplified by PCR using forward primer 507 and reverse primer 397. The *wzd* and *wze* fragments were then cloned separately into pGEMT and sequenced to identify exact matches to *Strep. thermophilus* MR-1C genes. Using the restriction endonucleases *Sph*I (pGEMT) and *Eco*31I (506 primer) the *wzd* insert was cut from the pGEMT vector. The *wze* insert was cut out of pGEMT using the restriction endonucleases *Eco*31I (507 primer) and *Apa*I (pGEMT). The inserts were then cloned together by ligating the two insert fragments into pGEM5Z that had been cut at its *Sph*I and *Apa*I sites. After the sequence was checked for accuracy, the *wzd/wze* fusion was then excised from this vector using restriction endonucleases *Eco*RI (394 primer) and *Sal*I (397 primer) and cloned into the yeast vectors pGAD424 and pGBT9 (Table 4.1). The yeast vectors were checked for sequence accuracy and proper frame alignment of the start codon before yeast cells were transformed with the constructs. The fusion protein was constructed so that the last codon upstream of the stop codon of *wzd* is immediately before the start codon of *wze* (sequence of junction is ATTATG).

2.3. DNA sequencing

DNA sequencing reactions were carried out by the Center for Integrated BioSystems at Utah State University using an ABI prism 3730 DNA analyzer (Applied Biosystems, Carlsbad, CA) and Taq FS terminator chemistry. The universal primers T7

and SP6 as well as gene specific primers (Table 4.1) were used to obtain the sequence of inserts in pGEM4Z, pGEM5Z, and pGEM7Z. Sequencing of inserts in pGAD424 was accomplished using the vector primers 496 and 497 (Table 4.1). Sequencing of inserts in pGBT9 was accomplished using vector primers 883, 498, and 499 (Table 4.1). The use of vector primers allowed the sequences to be analyzed for proper alignment of the start codon. Sequences were analyzed for exact matches to the *Strep. thermophilus* MR-1C EPS gene cluster by using the BLASTN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Yeast transformation

Saccharomyces cerevisiae strain HF7C that contains the reporter genes *lacZ* and *HIS3* was used for expression of the yeast/*Strep. thermophilus* MR-1C fusion proteins. The pGAD424 and pGBT9 plasmids containing the *Strep. thermophilus* MR-1C genes were cotransformed into *Saccharomyces cerevisiae* HF7C according to procedures described in the Matchmaker two-hybrid protocols (Clontech, Mountain View, CA). The transformants were plated on SD minimal medium supplemented with the appropriate dropout solution and selected using the markers *LEU2* (pGAD424) and *TRP1* (pGBT9). The transformed cells were maintained on SD minimal agar or frozen in SD minimal broth without tryptophan and leucine to ensure that the plasmids were retained. Three clones from at least two separate transformations were selected for each of the gene combinations and tested for protein-protein interactions. Yeast clones that contain the same combination of genes but in different plasmids were also tested. Yeast clones that contain the same gene in the two different plasmids were analyzed for same protein dimerization. Yeast clones that contained pGAD424 with a gene insert and an empty

pGBT9 and vice versa were tested to verify that the genes do not activate transcription alone.

2.5. Yeast clone PCR

Selected yeast clones were verified to contain the appropriate *Strep. thermophilus* MR-1C genes using PCR. Yeast cells were prepared by suspending a 2-3 day old colony in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) in a microcentrifuge tube. The tubes were then submerged in liquid nitrogen until the cells were completely frozen and then allowed to thaw. This freeze/thaw cycle was repeated three times. The PCR reactions consisted of 2-3 µl of the prepared cells, 1-2 µl of each gene specific primer or vector primer (100 µM stocks) (Table 4.1), 8 µl 2.5x master PCR mix (5 Prime), 1-2 µl $MgCl_2$ (25 mM), and enough distilled H_2O to make a total volume of 20 µl.

2.6. Yeast two-hybrid β-galactosidase filter assay

Protein-protein interactions were detected by the filter assay method using X-gal as a substrate to test for β-galactosidase production. Three day old colonies grown on SD minimal agar without tryptophan and leucine were transferred to a sterile Whatman #5 filter. The filter was then transferred to a pool of liquid nitrogen and completely submerged for 10 seconds to permeabilize the cells. After thawing, the filters were placed on top a Whatman #5 filter that had been pre-soaked with 3 ml of a Z-buffer/X-gal solution that contains 100 ml Z-buffer (16.1 g of $Na_2HPO_4 \cdot 7H_20$, 5.50 g of $NaH_2PO4 \cdot H_2O$, 0.75 g of KCl, and 0.246 g of $MgSO_4.7H_2O$ per L), 0.27 ml β-mercaptoethanol, and 1.67 ml X-gal stock solution (20 mg/ml in N,N-

dimethylformamide) in a petri-dish. The filters were then incubated at 30 ^{o}C and checked periodically over an 8 hour time period for the appearance of blue colonies indicating the production of β-galactosidase.

2.7. Yeast two-hybrid histidine prototrophy

The transformants were also tested for protein-protein interactions using the reporter gene *HIS3*. In this method, if there is a protein-protein interaction the yeast becomes prototrophic for histidine. The transformants were plated on SD minimal media lacking tryptophan, leucine, and histidine. This media also contained 25 mM 3-aminotriazole to inhibit the low level of residual *HIS3* expression in the *GAL1-HIS3* fusion and to ensure histidine auxotrophy without interaction of the binding domain and activation domain (Durfee *et al*. 1993). The plates were incubated at 30 ^{o}C and growth of the transformants was observed over an 8 day period.

3. Results

3.1. Protein-protein interactions involving Wzg, Wzh, Wzd, Wze, CpsE, CpsL, CpsS (Wzy), CpsU (Wzx), and CpsW

Table 4.2 lists the putative functions of the *Strep. thermophilus* MR-1C proteins encoded by the *eps* gene cluster. The Wzh (CpsB), Wzd (CpsC), and Wze (CpsD) proteins of *Strep. thermophilus* MR-1C that are predicted to function as a PTP, transmembrane activation protein, and a PTK respectively, were tested for protein-protein interactions among different components of the system and for dimerization of the same protein. The results of these experiments indicate a strong interaction between the transmembrane activation protein Wzd and the PTK Wze, as evidenced by the formation

Table 4.2. *Streptococcus thermophilus* MR-1C postulated protein functions and properties for the *eps* gene cluster.

Gene	Location (NT)	Putative Protein Functions	Size (AA)	Mass (kDa)	pI
wzg	6408-7862	Regulation	484	53.2	8.87
wzh	7863-8594	Phosphotyrosine phosphatase	243	28.0	8.42
wzd	8603-9301	Transmembrane activation protein, chain length determination	232	25.8	5.38
wze	9311-10060	Protein tyrosine kinase, membrane translocation	249	27.3	9.37
cpsE	10114-10797	Phosphogalactosyltransferase	227	25.8	9.20
cpsF	10800-11921	Branching galactosyltransferase	373	42.6	8.67
cpsN	12556-13440	Glycosyltransferase	294	33.9	9.03
cpsP	14067-14909	Rhamnosyltransferase	280	32.6	8.36
cpsQ	15006-15845	Glycosyltransferase	279	32.0	6.61
cpsS	15848-17128	Polymerization	426	50.2	9.40
cpsR	17135-18277	Glycosyltransferase	380	42.8	8.25
cpsT	18277-19209	Glycosyltransferase	310	37.3	7.94
cpsL	22731-23621	Unknown	296	32.7	8.40
cpsW	23646-24548	Regulation	300	33.9	9.56
orf 14.9	26655-27224	Unknown	570	49.1	5.07
cpsU	30560-31975	Membrane translocation	471	53.5	9.60
cpsV	32872-33837	Glycosyltransferase	321	37.8	9.36
cpsX	38688-39824	Membrane translocation	378	42.0	9.25

Gene locations are based on the Genbank file for *Streptococcus thermophilus* MR-1C (accession number AF448249.1). Protein functions are predicated from homologous proteins found in other bacteria (Broadbent *et al*., 2003).

of dark blue colonies indicating β-galactosidase production (Appendix A) and quick (within 2 days), abundant growth on plates lacking histidine by yeast clones that contained vectors with these genes (Table 4.3). Yeast clones that contained identical *wze* genes in both vectors yielded light blue colonies in the β-galactosidase assay (Appendix A) indicating a weaker protein-protein interaction between two PTKs. Consistent with this result, growth of these yeast clones in the absence of histidine was slight and slow to develop (Table 4.3). Light blue colonies after the β-galactosidase assay (Appendix A)

Table 4.3. Protein-protein interactions detected between Wzh, Wzd, and Wze.

Gene Insert in pGAD424	Gene Insert in pGBT9	Interaction Tested	β-Galactosidase Assay	Growth on Plates Lacking Histidine
wzd	*wze*	Transmembrane activation protein/PTK	Dark blue	+++
wze	*wzd*	PTK/transmembrane activation protein	Dark blue	+++
wze	*wze*	PTK/PTK	Light blue	+
wzh	*wze*	PTP/PTK	Light blue	+
wze	*wzh*	PTK/PTP	Light blue	+

PTP, phosphotyrosine phosphatase. PTK, protein tyrosine kinase. β-Galactosidase production is indicated by the formation of blue colonies. On plates lacking histidine, + indicates a slight amount of growth, ++ indicates moderate growth, and +++ indicates abundant growth.

and slow, weak growth on plates lacking histidine were also detected for yeast clones containing combinations of *wzh* with *wze* (Table 4.3), indicating a protein-protein interaction between the PTP and the PTK. No protein-protein interactions were detected between two identical PTPs, two identical transmembrane activation proteins, or the PTP and the transmembrane activation protein (data not shown).

The Wzh, Wzd, and Wze proteins may be involved in regulatory interactions with other proteins involved in EPS production. To determine if such protein-protein interactions take place Wzh, Wzd, and Wze were tested in combination with the proteins Wzg (regulation), CpsE (glycosyl-1-phosphate transferase), CpsL (unknown), CpsS (polymerization), CpsU (membrane translocation), and CpsW (regulation). All yeast clones containing *wzh*, *wzd*, or *wze* in combination with *wzg*, *cpsE*, *cpsL*, *cpsS*, *cpsU*, or *cpsW* did not produce β-galactosidase as was shown by a white colony color during the assay and were unable to grow on plates lacking histidine. Therefore, no additional protein-protein interactions were detected involving Wzh, Wzd, or Wze (data not shown).

All possible combinations of Wzg, CpsE, CpsL, CpsS, CpsU, and CpsW were tested for protein-protein interactions. None of the yeast clones containing any

combination of *wzg*, *cpsE*, *cpsL*, *cpsS*, *cpsU*, and *cpsW* produced β-galactosidase or were able to grow on plates lacking histidine, therefore no interactions were detected among these proteins (data not shown).

Controls that contained one vector with a *wzg*, *wzh*, *wzd*, *wze, cpsE, cpsL, cpsS, cpsU,* or *cpsW* insert and the corresponding vector without inserts did not produce β-galactosidase or grow on plates lacking histidine indicating that none of these genes alone can activate transcription of the reporter genes (data not shown).

3.2. Protein-protein interactions between the glycosyltransferases CpsE, CpsP, and CpsQ

Protein-Protein interactions among the *Strep. thermophilus* MR-1C glycosyltransferases were tested for by creating yeast clones that contained combinations of the priming glycosyl-1-phosphate transferase, CpsE, and those that are thought to function in the addition of the next sugar residues to the repeat unit, CpsP and CpsQ. Yeast clones that contained any combination of these genes did not produce β-galactosidase or grow on plates lacking histidine; therefore, no protein-protein interactions between CpsE, CpsP, and CpsQ were detected (data not shown). Controls that contained one vector with a *cpsP* or *cpsE* insert and the corresponding vector without any inserts did not produce β-galactosidase or grow on plates lacking histidine ensuring that these genes do not activate reporter gene transcription alone (data not show). A faint blue color was detected in the control clones containing one vector with a *cpsQ* insert and the corresponding vector without any inserts, indicating that CpsQ has the ability to activate reporter gene transcription alone. This was considered the background level for

this set of experiments and any yeast colonies that contained *cpsQ* and did not produce a level of β-galactosidase greater than this were scored as negative for protein interactions. The *cpsQ* control clone did not grow on plates lacking histidine (data not shown).

3.3. Protein-protein interactions between a Wzd/Wze fusion protein and Wzh, Wzd, Wze, and CpsE

A strong interaction was detected between the *Strep. thermophilus* MR-1C Wzd and Wze proteins and this interaction may facilitate protein-protein interactions involving Wzh, Wzd, Wze, and CpsE. In other species, homologous proteins have been shown to interact to form multi-protein complexes (Doublet *et al*. 2002; Collins *et al*. 2006; Olivares-Illana *et al*. 2008). To test for the formation of multi-protein complexes, a Wzd/Wze fusion protein was tested for further interactions with Wzd and Wze. Yeast colonies that contained the *wzd/wze* fusion in pGAD424 and either *wzd* or *wze* in pGBT9 were able grow on plates lacking histidine, however these clones had a white colony color in the β-galactosidase assay. In the reverse orientation with either *wzd* or *wze* in pGAD424 and the *wzd/wze* fusion in pGBT9, no growth was detected on plates lacking histidine and again no β-galactosidase was produced. Yeast clones that contained the *wzd/wze* fusion in both plasmids were also negative for β-galactosidase production and growth on plates lacking histidine (Table 4.4). These results indicate there is likely a problem with the protein conformation in some constructs but still suggest the formation of multi-protein complexes involving Wze and Wzd. The Wzd/Wze fusion protein was also tested for interactions with the PTP Wzh and the glycosyl-1-phosphate transferase CpsE. The yeast clones that contained these combination of genes were unable to grow

Table 4.4. Protein-protein interactions detected between the Wzd/Wze fusion protein and Wzd and Wze.

Gene Insert in pGAD424	Gene Insert in pGBT9	Interaction Tested	β-Galactosidase Assay	Growth on Plates Lacking Histidine
wzd:wze	*wzd*	Transmembrane activation PTK fusion protein /transmembrane activation protein	White	++
wzd:wze	*wze*	Transmembrane activation PTK fusion protein /PTK	White	++

β-Galactosidase production is indicated by the formation of blue colonies. On plates lacking histidine, + indicates a slight amount of growth, ++ indicates moderate growth, and +++ indicates abundant growth.

on plates lacking histidine or produce β-galactosidase (data not shown). The *wzd/wze* fusion controls that contained one plasmid with the insert and one plasmid without an insert did not produce β-galactosidase or grow on plates lacking histidine. Control experiments for the other gene vector combinations again showed that none of these genes alone can activate transcription of the reporter genes (data not shown).

4. Discussion

This study demonstrated that the MR-1C transmembrane activation protein Wzd and the PTK Wze directly interact with one other and may form multi-protein complexes to regulate capsule synthesis in *Strep. thermophilus*. Research done by Olivares-Illana *et al*. (2008) suggests that multi-protein complexes are also formed by the transmembrane activator protein (CapA) and the PTK (CapB) in *Staph. aureus* and work done with Wzc in *E. coli* establishes its ability to form protein complexes in the inner membrane of the cell (Doublet *et al*. 2002; Collins *et al*. 2006).

This study also detected an interaction between two identical *Strep. thermophilus* MR-1C Wze proteins that is consistent with this protein having transphosphorylation abilities. In the model for *E. coli*, an interaction between the cytoplasmic PTK domains

in different Wzc subunits has been predicted by this protein's transphosphorylation ability, but has not been observed to date (Collins *et al*. 2006).

The present study detected no protein-protein interactions between the *Strep. thermophilus* MR-1C PTK Wze and the Wzg, CpsE, CpsL, CpsS, CpsW, or CpsU proteins. However, evidence suggests that bacterial PTKs may induce phosphorylation of other proteins such as UDP-glucose dehydrogenases or glycosyltransferases (Grangeasse *et al*. 2003; Mijakovic *et al*. 2003; Obadia *et al*. 2007; Lacour *et al*. 2008; Lin *et al*. 2009). The sizes of protein complexes identified by Minic *et al*. (2007) suggest the possibility of protein-protein interactions between the transmembrane activation domain (EpsC/Wzd), the PTK (EpsD/Wze), and the glycosyl-1-phosphate transferase (EpsE) in *Strep. thermophilus* CNRZ1066. A tyrosine residue in EpsE has been shown to be necessary for the glycosyltransferase activity of this protein. When combined, these data suggest that EpsE may be regulated by phosphorylation of this tyrosine residue, possibly due to the action of EpsD in conjunction with EpsC. The contrasting results of this study and those acquired by Minic *et al.* (2007) could be the result of different methodologies and may imply that the interaction between the PTK and the glycosyl-1-phosphate transferase is not strong enough to be detected by the yeast two-hybrid system.

In *E. coli*, the Wzc protein that contains both the transmembrane activation domain and the PTK has been shown to be involved in a protein-protein interaction with the outer membrane lipoprotein, Wza. The interaction of Wza and Wzc is needed for synthesis of CA. Therefore, Wzc may not only interact with the biosynthetic machinery but also have functions coupled to the export pathway (Nesper *et al*. 2003; Reid and

Whitfield 2005; Collins *et al*. 2007). In this study, no protein-protein interactions were detected between the *Strep. thermophilus* MR-1C transmembrane activation protein (Wzd) or the PTK (Wze) and a protein thought to be involved in membrane translocation (CpsU).

Although a direct interaction has not been observed to date, it has been postulated that the *E. coli* PTK Wzc could affect the way that the polymerase (Wzy) functions; thereby influencing the amount and size of the polymer produced (Whitfield and Larue 2008). In this study, no protein-protein interactions were detected between the *Strep. thermophilus* MR-1C transmembrane activation protein (Wzd) or the PTK (Wze) and the putative polymerase (CpsS).

It has been suggested that the *E. coli* PTP Wzb could be involved in the dephosphorylation of other proteins involved in EPS synthesis such as glycosyltransferases (Bugert and Geider 1997; Grangeasse *et al*. 1998; Vincent *et al*. 1999; Preneta *et al*. 2002; Nakar and Gutnick 2003). Other than the interaction with Wze, this study detected no protein-protein interactions between the *Strep. thermophilus* MR-1C PTP Wzh and other EPS proteins. It is possible that the phosphorylation state of the glycosyltransferases and other proteins would be important in interactions with Wzh or that the dephosphorylation of other proteins by Wzh may not require a close interaction.

A Wzd/Wze (transmembrane activation domain/PTK) fusion protein was created to investigate the possibility that these two proteins in combination would promote protein-protein interactions with other *Strep. thermophilus* MR-1C proteins involved in

EPS biosynthesis. Some of the yeast clones that contained the Wzd/Wze fusion protein in combination with either Wzd or Wze had the ability to grow abundantly on media lacking histidine but did not produce β-galactosidase. These results suggest the formation of multi-protein complexes of Wzd and Wze in *Strep. thermophilus* MR-1C. It was thought that the presence of Wzd in the Wzd/Wze fusion protein might affect the phosphorylation state of Wze, thereby strengthening the protein-protein interaction with the PTP Wzh or the glycosyl-1-phosphate transferase CpsE. However, no interaction was detected between the Wzd/Wze fusion protein and Wzh or CpsE.

The yeast two-hybrid system did not detect any protein-protein interactions between the *Strep. thermophilus* MR-1C glycosyl-1-phosphate transferase (CpsE) and the glycosyltransferases CpsP and CpsQ that are thought to add the next sugar precursors to the carrier lipid. The model for the intracellular assembly of EPS by multiple glycosyltransferases suggests that interactions between these proteins are a possibility (Stingele *et al*. 1996, 1999; De Vuyst *et al*. 2001; Jolly and Stingele 2001; Broadbent *et al*. 2003). The phosphorylation state of CpsE or the binding of sugar polymers to the protein may be important in the interaction with other glycosyltransferases. Alternately, the glycosyltransferases may interact only with the phosphorylated form of Wze in complex with Wzd and not directly with each other.

The yeast two-hybrid system failed to detect any other protein interactions between the *Strep. thermophilus* MR-1C Wze, Wzd, Wzh, Wzg, CpsE, CpsL, CpsS, CpsU, or CpsW proteins in this study. The phosphorylation state of proteins such as Wze or CpsE is unknown in the yeast system and may affect the interactions between these

proteins and others involved in EPS biosynthesis. Other protein-protein interactions may have to take place or other protein components may be necessary for the interaction of these proteins. It is also possible that no direct interactions between some of the proteins required for the synthesis, polymerization, membrane translocation, and regulation of EPS exist.

Manipulation of EPS producing genes could greatly benefit many industrial processes. A more detailed understanding of how the different EPS proteins interact and function will aid construction of strains with enhanced properties. This work provides new insights into the protein-protein interactions that take place in the biosynthetic machinery involved in EPS production in *Strep. thermophilus* MR-1C. Further studies will be required to determine how the proteins of this complex system assemble and function in EPS synthesis.

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CHAPTER 5

INTRASPECIFIC AND INTERSPECIFIC INTERACTIONS AMONG PROTEINS REGULATING EXOPOLYSACCHARIDE SYNTHESIS IN *STREPTOCOCCUS THERMOPHILUS*, *STREPTOCOCCUS INIAE*, AND *LACTOCOCCUS LACTIS* SUBSP. *CREMORIS* AND THE ASSESSMENT OF POTENTIAL LATERAL GENE TRANSFER[2]

Abstract

Using the yeast two-hybrid system intraspecific protein interactions were detected in *Streptococcus iniae* and *Lactococcus lactis* subsp. *cremoris* between the transmembrane activation protein (CpsC and EpsA, respectively) and the protein tyrosine kinase (CpsD and EpsB, respectively), between two protein tyrosine kinases, and between the protein tyrosine kinase and the phosphotyrosine phosphatase (CpsB and EpsC, respectively). For each of these intraspecific interactions, interspecific interactions were also detected when one protein was from *S. iniae* and the other was from *Streptococcus thermophilus*. Interactions were also observed between two protein tyrosine kinases when one protein was from either of the *Streptococcus* species and the other from *L. lactis* subsp. *cremoris*. The results and sequence comparisons performed in this study support the conclusion that interactions among the components of the tyrosine kinase/phosphatase regulatory system are conserved in the order Lactobacillales and interspecific genetic exchanges of the genes that encode these proteins have the potential

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to form functional recombinants. A better understanding of intraspecific and interspecific protein interactions involved in regulating exopolysaccharide biosynthesis may facilitate construction of improved strains for industrial uses as well as identification of factors needed to form functional regulatory complexes in naturally occurring recombinants.

1. Introduction

Bacterial exopolysaccharide (EPS) or capsular polysaccharide (CPS) biosynthesis is typically controlled by a protein tyrosine kinase (PTK)/phosphotyrosine phosphatase (PTP) regulatory system. In Gram-negative bacteria the PTK consists of a transmembrane activation domain that functions as a polysaccharide co-polymerase that determines polymer chain length and a nucleotide-binding domain that contains the phosphorylation sites (Vincent et al. 1999; Wugeditsch et al. 2001; Doublet et al. 2002; Grangeasse et al. 2002; Preneta et al. 2002; Bender et al. 2003; Obadia et al. 2007; Tocilj et al. 2008). In most Gram-positive systems, the PTK and the transmembrane activation domain are separate proteins that function together with the PTP to regulate EPS/CPS production (Morona et al. 2000; Bender and Yother 2001; Cieslewicz et al. 2001; Bender et al. 2003; Broadbent et al. 2003; Morona et al. 2003, 2004; Soulat et al. 2006; Olivares-Illana et al. 2008). The PTP reverses tyrosine phosphorylation allowing the switching between phosphorylated and non-phosphorylated forms of the PTK that is required for efficient, high molecular weight polysaccharide synthesis (Grangeasse et al. 1998; Vincent et al. 1999; Bender and Yother 2001; Morona et al. 2002, 2003; Preneta et al. 2002; LaPointe et al. 2008; Bechet et al. 2009). Based on their work on the Gram-positive bacterium *Staphylococcus aureus*, Olivares-Illana et al. (2008) suggested that

multiple unphosphorylated PTK subunits associate such that their active sites can efficiently transphosphorylate neighboring subunits. Upon phosphorylation, dissociation of the PTKs may allow them to phosphorylate other endogenous substrates, such as the polysaccharide unit polymerase, the flippase, or the lipid sugar transferase. On phosphorylation the affinity of these proteins to the polysaccharide units could be altered and the polymerization and export of EPS controlled (Olivares-Illana et al. 2008).

In a prior study, the yeast two-hybrid system was utilized to study protein interactions among the transmembrane activation protein, the PTK, the PTP, and the glycosyl-1-phosphate transferase of *Streptococcus thermophilus* MR-1C (see chapter 4). Protein-protein interactions were identified between the transmembrane activation protein and the PTK, two identical PTKs, and between the PTK and the PTP but not between the glycosyl-1-phosphate transferase and the PTK or the PTP. To determine the generality and specificity of these protein-protein interactions, the transmembrane activation protein , the PTK, the PTP and the glycosyl-1-phosphate transferase from *S. thermophilus* MR-1C (Wzd, Wze, Wzh, and CpsE, respectively), *Streptococcus iniae* 9066 (CpsC, CpsD, CpsB, and CpsE, respectively), and *Lactococcus lactis* subsp. *cremoris* JRF1(EpsA, EpsB, EpsC, and EpsD, respectively) were tested for their ability to interact with proteins from the same or the other two bacterial systems. This is the first study to investigate whether these regulatory proteins require their cognately encoded counterparts in order to form protein-protein interactions or whether they can form interspecific protein-protein interactions. The *eps/cps* gene clusters of several pathogenic as well as non-pathogenic bacteria including *S. thermophilus* have been shown to have a chimeric structure that

results from horizontal gene transfer events (Bourgoin et al. 1996, 1999; Coffey et al. 1998; Pluvinet et al. 2004; Hols et al. 2005; Tyvaert et al. 2006; Delorme et al. 2007; Rasmussen et al. 2008; Liu et al. 2009; Eng et al. 2011). It is thought that the evolution of the *S. thermophilus eps* gene cluster involved DNA exchanges with other species of *Streptococcus* as well as with lactic acid bacteria, such as *L. lactis*, during co-culture in milk (Guédon et al. 1995; Bourgoin et al. 1996, 1999; Pluvinet et al. 2004; Tyvaert et al. 2006; Rasmussen et al. 2008; Liu et al. 2009; Eng et al. 2011). Therefore, it is important to investigate the ability of proteins from different species to interact to form functional regulatory complexes since such interactions are expected to be required in naturally occurring and in genetically engineered recombinants.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

S. thermophilus MR-1C is a capsule producing, Gram-positive, lactic acid bacterium with properties useful in the dairy industry as a cheese starter culture (Perry et al. 1997; Low et al. 1998). *L. lactis* subsp. *cremoris* JRF1 is a dairy fermentation strain that produces ropy EPS and was obtained from Dr. Ashraf N. Hassan (Dairy Science Department, South Dakota State University, Brookings, South Dakota). It was originally isolated from retail cultured buttermilk (Hassan et al. 2003). *S. iniae* 9066 is a commensal strain of a fish pathogen that was isolated from swabbing a healthy fish (Fuller et al. 2001; Lowe et al. 2007). *S. iniae* 9066 was obtained from Dr. Melody N. Neely (Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, Michigan).

S. thermophilus MR-1C and *L. lactis* subsp. *cremoris* JRF1 were cultured in M17 broth containing 0.5% lactose (Terzaghi and Sandine 1975) and incubated at 37 ^{o}C without aeration. *S. iniae* 9066 was cultured in Todd-Hewitt medium supplemented with 2% yeast extract and 2% proteose peptone and incubated at 37 ^{o}C without aeration.

Escherichia coli DH5α (Invitrogen, Carlsbad, California, USA) was grown at 37 ^{o}C in Luria-Bertani broth with shaking or on Luria-Bertani agar plates containing ampicillin at a concentration of 50 µg/ml for plasmid selection. *Saccharomyces cerevisiae* HF7C (Clontech, Mountain View, California, USA) was grown at 30 ^{o}C on Yeast Peptone Dextrose (YPD) agar plates or YPD broth with shaking. SD minimal medium containing the needed dropout solution (Clontech) was used to select for *S. cerevisiae* HF7C transformants containing the appropriate plasmids.

2.2. *Construction of plasmids*

The *wzh*, *wzd*, *wze*, and *cpsE* genes of *S. thermophilus* MR-1C (GenBank accession number AF448249.1) were cloned into the yeast two hybrid vectors pGAD424 and pGBT9 as described previously (see chapter 4). The *cpsB*, *cpsC*, *cpsD*, and *cpsE* genes of *S. iniae* 9066 (GenBank accession number HQ698911) and the *epsA*, *epsB*, *epsC*, and *epsD* genes of *L. lactis* subsp. *cremoris* JRF1 (GenBank accession number HQ665557) were amplified using PCR and sequence specific primers that contained appropriate restriction sites for cloning (Table 5.1). The *S. iniae* 9066 *cpsB*, *cpsC*, *cpsD*, and *cpsE* genes and the *L. lactis* subsp. *cremoris* JRF1 *epsA*, *epsC*, and *epsD* genes were ligated into pGEM4Z (Promega, Madison, Wisconsin, USA) using the restriction sites *Bam*HI (5') and *Pst*I (3'). The *L. lactis* subsp. *cremoris* JRF1 *epsB* gene was ligated into

Table 5.1. *Streptococcus iniae* 9066, *Lactococcus lactis* subsp. *cremoris* JRF1, pGAD424, and pGBT9 oligonucleotides used in this study.

Bacterial Strain or Vector	Primer	Location	RS	Sequence (5'-3')
S. iniae 9066	836*	*cpsB* 5'	*Bam*HI	GGGGGATCCGTCGACTGGGAGGAAAGAAAATGATTGACATCCATTCC
	837*	*cps*B 3'	*Pst*I	GGGCTGCAGCCTATAAATAATCATTTTC
	838*	*cpsC* 5'	*Bam*HI	GGGGGATCCGTCGACTTATAGGAGAAAAGATGAAC
	839*	*cpsC* 3'	*Pst*I	GGGCTGCAGCTTACATTTTATTTGTGTTTGG
	840*	*cpsD* 5'	*Bam*HI	GGGGGATCCGTCGACGTAAGAGGTTAAGGATGTCAC
	841*	*cpsD* 3'	*Pst*I	GGGCTGCAGTTCACTTTCTGGAATGTTTTTTAC
	842*	*cpsE* 5'	*Bam*HI	GGGGGATCCCTCGAGTGAAAGAAAAAAAGATGAAAAGAAGTCAA
	843*	*cpsE* 3'	*Pst*I	GGGCTGCAGCTTACTCCTGTTTAGCGTC
L. lactis subsp. *cremoris* JFR1	856*	*epsA* 5'	*Bam*HI	AAAAGGATCCGTCGACTAAATATTGGAGAAGAAATGCAG
	857*	*epsA* 3'	*Pst*I	AAAACTGCAGCTATCTTTTATTCCTTTTTGATGATCTATT
	858*	*epsB* 5'	*Bam*HI	AAAAGGATCCGTCGACAAAGAGGGGAGTTCAGGATGGCTAAA
	859*	*epsB* 3'	*Hind*III	AAAAAAGCTTAGATCTTTACTCTACTCCGTAGTAGTAATAC
	860*	*epsC* 5'	*Bam*HI	AAAAGGATCCGTCGACGTAGAAGAGGAGAGCAAATG
	861*	*epsC* 3'	*Pst*I	AAAACTGCAGTTAAAATAATCCCAAAAATTTCTTTG
	862*	*epsD* 5'	*Bam*HI	AAAAGGATCCGTCGACAAATGGAGGGGATAATGGAAGTTTTTGAGGCATC
	863*	*epsD* 3'	*Pst*I	AAAACTGCAGTTAATAAGCATCCGATCCATGTAG
pGAD424	496†	Cloning site 5'	None	ATTCGATGATGAAGATAC
	497†	Cloning site 3'	None	TTTCAGTATCTACGATTC
pGBT9	883†	Cloning site 5'	None	GAGACAGCATAGAATAAG
	498†	Cloning site 5'	None	GTCAAAGACAGTTGACTG
	499†	Cloning site 3'	None	AAATTCGCCCGGAATTAG

Note: Oligonucleotide primers were derived from the genome sequences of *Streptococcus iniae* 9066 (GenBank accession number HQ698911) and *Lactococcus lactis* subsp. *cremoris* JFR1 (GenBank accession number HQ665557) and from the vector sequence of pGAD424 (GenBank accession number U07647.1) and pGBT9 (GenBank accession number U07646.1). Similar oligonucleotide primers for cloning the *Streptococcus thermophilus* MR-1C genes are described in Cefalo et al. 2011.

* Primer used for gene insert amplification; PCR confirmation of yeast clones, and sequencing.

† Primer used for PCR confirmation of yeast clones and sequencing.

pGEM7Z (Promega) using the restriction sites *Bam*HI (5') and *Hind*III (3'). The pGEM4Z and pGEM7Z vectors with gene inserts were electroporated (2500 V, 200 Ω, 25 μF) into *E. coli* DH5α cells using a Gene Pulser® II Electroporation System (Bio Rad, Hercules, California, USA) and sequenced to identify exact matches to the *S. iniae* 9066 and *L. lactis* subsp. *cremoris* JRF1 genes. The gene inserts were removed from the pGEM4Z vector by restriction digests and then cloned into the yeast vectors pGAD424 and pGBT9 (Clontech) using the same restriction sites. The *epsB* gene of *L. lactis* subsp. *cremoris* JRF1 was cut out of the pGEM7Z vector using the restriction enzymes *Bam*HI (5') and *Xho*I (3') to create ends that would be compatible for ligation into the yeast vectors pGAD424 and pGBT9 via *Bam*HI and *Sal*I restriction sites. The resulting yeast vectors with gene inserts were transformed into *E. coli* DH5α cells, analyzed for sequence accuracy and proper frame alignment of the start codon, and then electroporated into *S. cerevisiae* HF7C (Matchmaker two-hybrid protocols, Clontech).

2.3. DNA sequencing

All DNA sequencing reactions were done using an ABI prism 3730 DNA analyzer and Taq FS terminator chemistry at the Utah State University Center for Integrated BioSystems. Nucleotide sequences of *S. iniae* 9066 and *L. lactis* subsp. *cremoris* JRF1 genes were determined by the primer walking technique using gene specific primers based on the sequence of related strains. To sequence inserts in pGEM4Z and pGEM7Z the universal primers T7 and SP6 as well as gene specific primers (Table 5.1) were used. Gene inserts in pGAD424 and pGBT9 were sequenced using vector primers located on the 5' and 3' end of the cloning site to allow sequences to

be analyzed for accuracy of the insert genes and proper frame alignment of the start codon.

2.4. Yeast transformation

The cloning of genes into the pGAD424 and pGBT9 vectors allowed the synthesis of corresponding fusion proteins with the Gal4 activation domain (AD) and the Gal4 DNA binding domain (BD), respectively (Fields and Song 1989; Chien et al. 1991). For the expression of the yeast/*S. thermophilus* MR-1C, *S. iniae* 9066, or *L. lactis* subsp. *cremoris* JRF1 fusion proteins the *S. cerevisiae* strain HF7C (*LacZ and HIS3*) was used. The pGAD424 and pGBT9 plasmids containing the *S. thermophilus* MR-1C, *S. iniae* 9066, and *L. lactis* subsp. *cremoris* JRF1 genes were cotransformed into *S. cerevisiae* HF7C according to procedures described in the Matchmaker two-hybrid protocols (Clontech). Electroporated cells were plated on SD minimal medium (Matchmaker two-hybrid protocols, Clontech) and transformants were selected using the markers *LEU2* and *TRP1* in the pGAD424 and pGBT9 vectors, respectively. To ensure plasmid retention, yeast clones were maintained on SD minimal agar or frozen in SD minimal broth without tryptophan and leucine. For each gene combination, three clones from at least two separate transformation experiments were tested for protein-protein interactions. To test for protein dimerization, yeast clones were created that had an identical copy of the same gene in both pGAD424 and pGTB9. To test that the genes do not activate transcription alone, yeast clones were created that contained pGAD424 with a gene insert plus pGBT9 alone and vice versa.

2.5. Yeast clone PCR

Yeast clones were verified to contain the appropriate *S. thermophilus* MR-1C, *S. iniae* 9066, and *L. lactis* subsp. *cremoris* JRF1 genes using PCR. For each yeast clone, a 2-3 day old colony was suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) in a microcentrifuge tube. The cell suspensions were then frozen in liquid nitrogen a total of three times allowing the cells to completely thaw between each of the freezing cycles. Each 20 µl PCR reaction contained 2-3 µl of the cellular suspension, 1-2 µl of each gene specific primer or vector primer (100 µM stocks) (Table 5.1), 8 µl 2.5X master PCR mix (5 Prime), 1-2 µl $MgCl_2$ (25 mM), and 3-7 µl distilled H_2O. After an initialization step of 95 ^oC for 5 minutes, PCR consisted of 50 cycles at a denaturation temperature of 95 ^oC for 45 seconds, an annealing temperature that was optimized for the individual primers for 45 seconds, and then an extension temperature of 72 ^oC for 1-3 minutes.

2.6. Yeast two-hybrid β-galactosidase filter assay

In the yeast two-hybrid system protein-protein interactions are indicated by the production of β-galactosidase in the yeast clones. β-galactosidase production was detected using the filter assay method with bromo-chloro-indolyl-galactopyranoside (X-gal) as the enzymatic substrate. For each combination of genes, three yeast clones from at least two separate transformation experiments were inoculated onto SD minimal agar without tryptophan and leucine and then grown at 30 ^oC for 3 days. The colonies were then transferred to a sterile Whatman #5 filter and submerged in a pool of liquid nitrogen for 10 seconds to permeabilize the cells. The filters were allowed to thaw and then placed on top a Whatman #5 filter in a Petri dish that had been pre-soaked with 3 ml of a

Z-buffer/X-gal solution containing 100 ml Z-buffer (16.1 g of $Na_2HPO_4 \cdot 7H_20$, 5.50 g of $NaH_2PO4 \cdot H_2O$, 0.75 g of KCl, and 0.246 g of $MgSO_4.7H_2O$ per L), 0.27 ml β-mercaptoethanol, and 1.67 ml X-gal stock solution (20 mg/ml in N,N-dimethylformamide). The Petri dishes containing the filters were incubated at 30 ^oC and checked periodically during an 8 hour time period for the appearance of blue colonies indicating β-galactosidase production.

2.7. Yeast two-hybrid histidine prototrophy

Protein-protein interactions in the yeast two-hybrid system are also indicated by histidine prototrophy due to the reporter gene *HIS3*. To test for histidine prototrophy three yeast clones from at least two separate transformation experiments for each combination of genes were plated on SD minimal media that did not contain tryptophan, leucine, or histidine. To inhibit low level *HIS3* expression in the GAL1-*HIS3* fusion, and to ensure histidine auxotrophy without interaction of the binding domain and activation domain, 25 mM 3-aminotriazole was added to the SD minimal agar plates (Durfee et al. 1993). Agar plates were incubated at 30 ^oC and observed for growth of the yeast clones over an 8 day period.

2.8. Computational analysis of protein and nucleotide sequences

Alignments were performed using the program ClustalW2 (Larkin et al. 2007) located at http://www.ebi.ac.uk/Tools/clustalw2/index.html. Gene sequence information from *S. thermophilus* MR-1C (accession number AF448249.1), *S. iniae* 9066 (accession number HQ698911), *L. lactis* subsp. *cremoris* JRF1 (accession number HQ665557),

Streptococcus oralis ATCC 35037 (accession number ADMV01000012.1), *Streptococcus gordonii* str. *Challis* substr. CH1 (accession number CP000725.1), *Streptococcus pneumoniae* D39 (accession number CP000410.1), *Staph. aureus* 5C (accession number U81973.1) and *Lactobacillus rhamnosus* ATCC 9595 (accession number AY659976), was translated into protein sequences using the translate tool on the Swiss Institute of Bioinformatics website (http://expasy.org/tools). Blast searches on the genes were conducted to identify potential horizontal gene transfer events and protein and nucleotide identities and similarities (positives) were calculated using the default settings for the Needleman-Wunsch Global Sequence Alignment Tool at http://blast.ncbi.nlm.nih.gov/. The *Lb. rhamnosus* ATCC 9595 data was included since its phosphatase has been recently studied (LaPointe et al. 2008) and this species is also used in dairy fermentations.

3. Results

3.1. Analysis of intraspecific protein-protein interactions between the PTP, the trans-membrane activation protein, the PTK, and the glycosyl-1-phosphate transferase

Proteins proposed to function as a PTP, a transmembrane activation protein, a PTK, and a glycosyl-1-phosphate transferase were tested in combination with each other to identify interactions between proteins from *S. iniae* 9066 (CpsB, CpsC, CpsD, and CpsE, respectively) and from *L. lactis subsp. cremoris* JRF1 (EpsC, EpsA, EpsB, and EpsD, respectively), as was described previously for the *S. thermophilus* MR-1C proteins (Wzh, Wzd, Wze, and CpsE, respectively) by Cefalo et al. (see chapter 4). Strong intraspecific protein-protein interactions were detected between the transmembrane

activation protein and the PTK in all three bacterial systems. Yeast clones containing the transmembrane activation protein in combination with the PTK produced β-galactosidase (Appendix A) and had the ability to grow abundantly on plates lacking histidine (Table 5.2). A weak interaction was detected between two identical PTKs in all three bacterial systems, as yeast clones that contained the gene encoding the PTK in both plasmids were light blue in the β-galactosidase assay (Appendix A) and were able to grow weakly on plates lacking histidine after an extended incubation (6 days) (Table 5.2). Another weak interaction was detected between the PTP and the PTK in all three bacterial systems (Appendix A). No protein-protein interactions were detected between the PTP and the

Table 5.2. Intraspecific protein-protein interactions detected between the PTP, the transmembrane activation protein, the PTK, and the glycosyl-1-phosphate transferase.

Bacterial Strain	Gene Insert in pGAD424	Gene Insert in pGBT9	Interaction Tested	Colony Color after β-Gal Assay	Growth on Plates Lacking Histidine
Streptococcus iniae 9066*	*cpsC*	*cpsD*	Transmembrane activation protein/PTK	Dark blue	+++
	cpsD	*cpsC*	PTK/transmembrane activation protein	Dark blue	+++
	cpsD	*cpsD*	PTK/PTK	Light blue	+
	cpsB	*cpsD*	PTP/PTK	Light blue	+
	cpsD	*cpsB*	PTK/PTP	Light blue	+
Lactococcus lactis subsp. cremoris JRF1	*epsA*	*epsB*	Transmembrane activation protein/PTK	Blue	++
	epsB	*epsA*	PTK/transmembrane activation protein	Dark blue	+++
	epsB	*epsB*	PTK/PTK	Light blue	+
	epsC	*epsB*	PTP/PTK	Light blue	+
	epsB	*epsC*	PTK/PTP	Light blue	+

β-Galactosidase (β-Gal) production is indicated by the formation of blue colonies. On plates lacking histidine, + indicates a slight amount of growth, ++ indicates moderate growth, and +++ indicates abundant growth. Protein-protein interactions were not detected between the glucosyl-1-phosphate transferase and the PTK, PTP, or transmembrane activation protein in either *Streptococcus iniae* 9066 or *Lactococcus lactis subsp. cremoris JRF1*. Yeast clones that contained any of the genes above inserted into pGAD424 and pGBT9 with no gene inserts and vice versa did not produce β-galactosidase or grow on plates lacking histidine assuring that no gene could activate transcription of the reporter genes alone.

* Results seen for the interactions of *Streptococcus thermophilus* MR-1C proteins (see chapter 4) were equivalent to those seen for the interactions of the homologous proteins from *S. iniae* 9066.

transmembrane activation protein, the PTP and the glycosyl-1-phosphate transferase, the transmembrane activation protein and the glycosyl-1-phosphate transferase, or the PTK and the glycosyl-1-phosphate transferase in any of the three bacterial systems. Yeast clones that contained these combinations of proteins produced no detectable β-galactosidase and were unable to grow on plates lacking histidine (data not shown). Yeast clones that contained two identical inserts of the PTP, the transmembrane activation protein, or the glycosyl-1-phosphate transferase in both plasmids did not produce β-galactosidase or grow on plates lacking histidine; therefore no dimerization of these proteins was detected by the yeast-two hybrid system for any of the bacterial systems tested (data not shown).

3.2. Interspecific protein-protein interactions between the transmembrane activation protein and the PTK, two PTKs, and the PTK and PTP from different bacterial species

To investigate if the transmembrane activation proteins were restricted to recognize only their own cognately-encoded PTK or were more functionally flexible, the transmembrane activation protein from each species was tested for the ability to interact with the PTK from the two other species. Yeast clones that contained the *S. thermophilus* MR-1C transmembrane activation protein (Wzd) in combination with the *S. iniae* 9066 PTK (EpsD) produced β-galactosidase (Appendix A) and were able to grow abundantly on plates lacking histidine (Table 5.3). Yeast clones that contained the *S. iniae* 9066 transmembrane activation protein (CpsC) in combination with the *S. thermophilus* MR-1C PTK (Wze) also produced β-galactosidase (Appendix A) and were able to grow abundantly on plates lacking histidine (Table 5.3). These results indicate that the *S.*

thermophilus MR-1C transmembrane activation protein has the ability to interact with the *S. iniae* 9066 PTK and vice versa. Yeast clones that contained the transmembrane activation protein from either *S. thermophilus* MR-1C (Wzd) or *S. iniae* 9066 (CpsC) and the PTK from *L. lactis* subsp. *cremoris* JRF1 (EpsB) did not produce β-galactosidase or grow on plates lacking histidine (Table 5.3). Yeast clones that contained the *L. lactis* subsp. *cremoris* JRF1 transmembrane activation protein (EpsA) and the PTK from either *S. thermophilus* MR-1C (Wze) or *S. iniae* 9066 (CpsD) did not produce β-galactosidase or grow on plates lacking histidine (Table 5.3). These results indicate that the *L. lactis* subsp. *cremoris* JRF1 transmembrane activation protein or PTK cannot interact with the PTK or transmembrane activation protein, respectively, from *S. thermophilus* MR-1C or *S. iniae* 9066.

This study (Table 5.2) and Cefalo et al. (2011) identified a weak intraspecific protein-protein interaction between two identical PTKs in *S. thermophilus* MR-1C, *S. iniae* 9066, and *L. lactis* subsp. *cremoris* JRF1. To investigate the specificity of this interaction, the PTK from each species was tested for protein interactions with the PTKs of the other two species. Yeast clones that contained any combination of *S. thermophilus* MR-1C Wze, *S. iniae* 9066 CpsD, or *L. lactis* subsp. *cremoris* JRF1 EpsB produced β-galactosidase (Appendix A) and were able to grow weakly on plates lacking histidine after a prolonged incubation (6 days) (Table 5.3). These results indicate that the PTK from one species has the ability to interact with the PTKs from either of the other two species.

Similarly, a weak intraspecific protein-protein interaction between the PTP and the PTK in *S. thermophilus* MR-1C, *S. iniae* 9066, and *L. lactis* subsp. *cremoris* JRF1 was indicated by results from this study (Table 5.2) and our prior work (Cefalo et al. 2011). The PTP from each species was therefore tested for protein interactions with the PTKs of the other two species. All yeast clones that contained the *S. thermophilus* MR-1C PTK (Wze) in combination with the *S. iniae* 9066 PTP (CpsB) or the *S. iniae* 9066 PTK (CpsD) in combination with the *S. thermophilus* MR-1C PTP (Wzh) produced β-galactosidase (Appendix A) and were able to weakly grow on plates lacking histidine after a prolonged incubation (6 days) (Table 5.3). These results indicate that the *S. thermophilus* MR-1C PTK can interact with the *S. iniae* 9066 PTP and vice versa. In contrast, yeast clones that contained the *L. lactis* subsp. *cremoris* JRF1 PTK (EpsB) in combination with either the *S. iniae* 9066 PTP (CpsB) or the *S. thermophilus* MR-1C PTP (Wzh) did not produce β-galactosidase or grow on plates lacking histidine (Table 5.3). All yeast clones that contained the *L. lactis* subsp. *cremoris* JRF1 PTP (EpsC) in combination with either the *S. iniae* 9066 PTK (CpsD) or the *S. thermophilus* MR-1C PTK (Wze) did not produce β-galactosidase or grow on plates lacking histidine (Table 5.3). Thus, the PTP or PTK from *L. lactis* subsp. *cremoris* JRF1 is apparently not able to interact with the PTK or PTP, respectively from either *S. thermophilus* MR-1C or *S. iniae* 9066.

3.3. Computational analysis of protein sequences

Positive or negative interspecific interactions among these proteins might be related to the similarity and divergence of these proteins. To investigate this relationship

Table 5.3. Interspecific protein-protein interactions between the transmembrane activation protein and the PTK, two PTKs, and the PTK and PTP from different bacterial species.

Interaction	Gene Insert in pGAD424 and Protein Function	Gene Insert in pGBT9 and Protein Function	Colony Color after β-Gal Assay*	Growth on Plates Lacking Histidine†
transmembrane activation protein/ PTK	MR-1C *wzd*; transmembrane activation protein	9066 *epsD*; PTK	Dark blue	+++
	MR-1C *wzd*; transmembrane activation protein	JRF1 *epsB*; PTK	White	No growth
	MR-1C *wze*; PTK	9066 *epsC*, transmembrane activation protein	Dark blue	+++
	MR-1C *wze*; PTK	JRF1 *epsA*; transmembrane activation protein	White	No growth
	9066 *epsC*; transmembrane activation protein	MR-1C *wze*; PTK	Dark blue	+++
	9066 *epsC*; transmembrane activation protein	JRF1 *epsB*; PTK	White	No growth
	9066 *epsD*; PTK	MR-1C *wzd*; transmembrane activation protein	Dark blue	+++
	9066 *epsD*; PTK	JRF1 *epsA*; transmembrane activation protein	White	No growth
	JRF1 *epsA*; transmembrane activation protein	MR-1C *wze*; PTK	White	no growth
	JRF1 *epsA*; transmembrane activation protein	9066 *epsD*; PTK	White	No growth
	JRF1 *epsB*; PTK	MR-1C *wzd*; transmembrane activation protein	White	no growth
	JRF1 *epsB*; PTK	9066 *epsC*; transmembrane activation protein	White	No growth
PTK/PTK	MR-1C *wze*; PTK	9066 *cpsD*; PTK	Light blue	+
	MR-1C *wze*; PTK	JRF1 *epsB*; PTK	Light blue	+
	9066 *cpsD*; PTK	MR-1C *wze*; PTK	Light blue	+
	9066 *cpsD*; PTK	JRF1 *epsB*; PTK	Light blue	+
	JRF1 *epsB*; PTK	MR-1C *wze*; PTK	Light blue	+
	JRF1 *epsB*; PTK	9066 *cpsD*; PTK	Light blue	+
PTK/PTP	MR-1C *wze*; PTK	9066 *epsB*; PTP	Light blue	+
	MR-1C *wze*; PTK	JRF1 *epsC*; PTP	White	No growth
	MR-1C *wzh*; PTP	9066 *epsD*; PTK	Light blue	+
	MR-1C *wzh*; PTP	JRF1 *epsB*; PTK	White	No growth
	9066 *cpsD*; PTK	JRF1 *epsC*; PTP	White	No growth

9066 *epsB*; PTP	MR-1C *wze*; PTK	Light blue	+
9066 *epsB*; PTP	JRF1 *epsB*; PTK	White	No growth
JRF1 *epsB*; PTK	MR-1C *wzh*; PTP	White	No growth
JRF1 *epsB*; PTK	9066 *epsB*; PTP	White	No growth
JRF1 *epsC*; PTP	MR-1C *wze*; PTK	White	No growth
JRF1 *epsC*; PTP	9066 *cpsD*; PTK	White	No growth

MR-1C indicates *Streptococcus thermophilus* strain MR-1C, 9066 indicates *Streptococcus iniae* strain 9066, and JRF1 indicates *Lactococcus lactis* subsp. *cremoris* JRF1.
[*] β-Galactosidase (β-Gal) production is indicated by the formation of blue colonies.
[†] On plates lacking histidine, + indicates a slight amount of growth, ++ indicates moderate growth, and +++ indicates abundant growth.
Yeast clones that contained any of the genes above inserted into pGAD424 and pGBT9 with no gene inserts and vice versa did not produce β-galactosidase or grow on plates lacking histidine assuring that no gene could activate transcription of the reporter genes alone.

the predicted PTPs from *S. thermophilus* MR-1C (Wzh), *S. iniae* 9066 (CpsB), and *L. lactis* subsp. *cremoris* JRF1 (EpsC) were aligned along with those from two other well-studied pathogenic species *S. pneumoniae* D39 (CpsB) and *Staph. aureus* 5C (Cap5C) and another lactic acid bacteria used in dairy fermentations *Lb. rhamnosus* ATCC 9595 (Wzb) (Fig. 5.1). *S. oralis* ATCC 35037 (CpsB) and *S. gordonii* str. *Challis* substr. CH1 (Wzh) were included in the alignment because of the nucleotide homology of the PTP genes to those of *S. iniae* and *S. thermophilus,* respectively. The four PTP motifs containing conserved histidine, aspartate, and glutamate residues that are believed to be important in binding divalent cations and in coordination of the catalytic site are labeled (Aravind and Koonin 1998; Shi 2004; LaPointe et al. 2008). As shown in Fig. 5.1, the overall structure of the eight proteins is conserved but the PTPs fall into two clear structural groups composed, respectively, of the five *Streptococcus* proteins and of the three other proteins, with each group being composed of proteins from both pathogenic and dairy fermentation species. These groups contain sequence differences within the conserved motifs and in the carboxyl termini of the proteins. At the amino acid level, *S.*

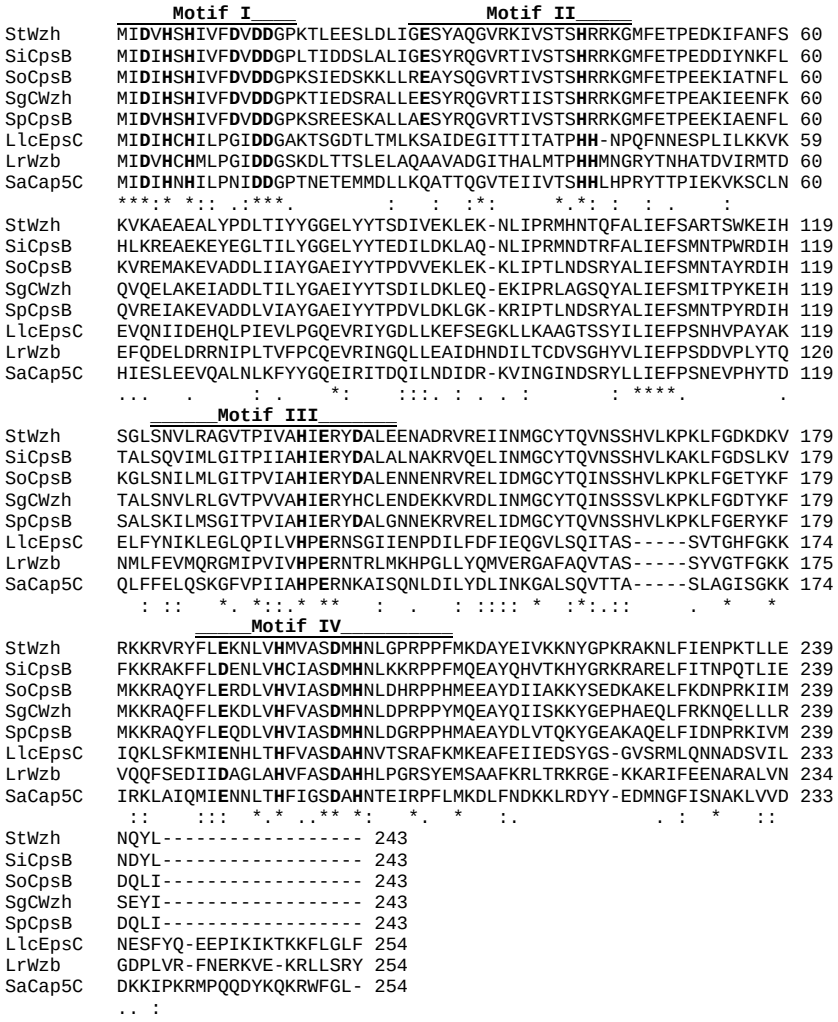

Fig. 5.1. Alignment of the PTPs from *Streptococcus thermophilus* MR-1C (Wzh, StWzh), *Streptococcus iniae* 9066 (CpsB, SiCpsB), *Streptococcus oralis* ATCC 35037 (CpsB, SoCpsB), *Streptococcus gordonii* str. *Challis* substr. CH1 (Wzh, SgCWzh), *Streptococcus pneumoniae* D39 (CpsB, SpCpsB), *Lactococcus lactis* subsp. *cremoris* JRF1 (EpsC, LlcEpsC), *Lactobacillus rhamnosus* ATCC 9595 (Wzb, LrWzb), and *Staphylococcus aureus* (Cap5C, SaCap5C). The four PTP motifs are labeled with the conserved histidine, aspartate, and glutamate residues indicated in bold type. Amino acids that are identical in all sequences of the alignment are indicted by an asterisk, conserved substitutions are indicated by two dots, and semi-conserved substitutions are indicated by one dot.

thermophilus MR-1C Wzh and *S. iniae* 9066 CpsB are 69% identical and 83% similar. *L. lactis* subsp. *cremoris* JRF1 EpsC is 25% identical and 44% similar to *S. thermophilus* MR-1C Wzh and 24% identical and 43% similar to *S. iniae* 9066 CpsB (Table 5.4).

The transmembrane activation proteins from *S. thermophilus* MR-1C (Wzd), *S. iniae* 9066 (CpsC), and *L. lactis* subsp. *cremoris* JRF1 (EpsA) along with those from *S. pneumoniae* D39 (CpsC), *Staph. aureus* 5C (Cap5A), *S. oralis* ATCC 35037 (CpsC), *S. gordonii* str. *Challis* substr. CH1 (Wzd), and *Lb. rhamnosus* ATCC 9595 (Wzd) were also aligned (Fig. 5.2). The predicted positions of two motifs that are found in polysaccharide co-polymerase proteins involved in chain length determination are indicated (Becker et al. 1995; Tocilj et al. 2008). At the amino acid level, *S. thermophilus* MR-1C Wzd and *S. iniae* 9066 CpsC are 56% identical and 78% similar. *L.*

Table 5.4. Amino acid identities and similarities between the PTPs, transmembrane activation domains, and PTKs of *Streptococcus thermophilus* MR-1C, *Streptococcus iniae* 9066, and *Lactococcus lactis* subsp. *cremoris* JRF1.

Putative Protein Function	Organism and Protein Name	Organism and Protein Name	Identity	Similarity
PTP	*Streptococcus thermophilus* MR-1C Wzh	*Streptococcus iniae* 9066 CpsB	69%	83%
	Streptococcus iniae 9066 CpsB	*Lactococcus lactis* subsp. *cremoris* JRF1 EpsC	24%	43%
	Lactococcus lactis subsp. *cremoris* JRF1 EpsC	*Streptococcus thermophilus* MR-1C Wzh	25%	44%
Transmembrane activation protein	*Streptococcus thermophilus* MR-1C Wzd	*Streptococcus iniae* 9066 CpsC	56%	78%
	Streptococcus iniae 9066 CpsC	*Lactococcus lactis* subsp. *cremoris* JRF1 EpsA	24%	45%
	Lactococcus lactis subsp. *cremoris* JRF1 EpsA	*Streptococcus thermophilus* MR-1C Wzd	24%	44%
PTK	*Streptococcus thermophilus* MR-1C Wze	*Streptococcus iniae* 9066 CpsD	59%	75%
	Streptococcus iniae 9066 CpsD	*Lactococcus lactis* subsp. *cremoris* JRF1 EpsB	33%	50%
	Lactococcus lactis subsp. *cremoris* JRF1 EpsB	*Streptococcus thermophilus* MR-1C Wze	31%	47%

lactis subsp. *cremoris* JRF1 EpsA is 24% identical and 44% similar to *S. thermophilus* MR-1C Wzh and 24% identical and 45% similar to *S. iniae* 9066 CpsB (Table 5.4). The amino acid sequences in Motif I of *S. thermophilus* MR-1C and *S. iniae* 9066 are highly similar to each other, while the *L. lactis* subsp. *cremoris* JRF1 sequences was very divergent.

The PTKs from *S. thermophilus* MR-1C (Wze), *S. iniae* 9066 (CpsD), and *L. lactis* subsp. *cremoris* JRF1 (EpsB) along with those from *S. pneumoniae* D39 (CpsD), *Staph. aureus* 5C (Cap5B), *S. oralis* ATCC 35037 (CpsD), *S. gordonii* str. *Challis* substr. CH1 (Wze), and *Lb. rhamnosus* ATCC 9595 (Wze) were aligned (Fig. 5.3). These proteins contain amino acid sequences that resemble the conserved Walker A, A' and B motifs found in other bacterial PTKs (Walker et al. 1982; Soulat et al. 2007; Jadeau et al. 2008; Bechet et al. 2009). The C-terminal end of bacterial PTKs contain up to seven tyrosine residues that are all potentially phosphorylatable (Grangeasse et al. 2002; Paiment et al. 2002; Morona et al. 2003; Jadeau et al. 2008). At the amino acid level, *S. thermophilus* MR-1C Wze and *S. iniae* 9066 CpsD are 59% identical and 75% similar. *L. lactis* subsp. *cremoris* JRF1 EpsB is 33% identical and 50% similar to *S. iniae* 9066 CpsD and 31% identical and 47% similar to *S. thermophilus* MR-1C Wze (Table 5.4). The important amino acids in the Walker A, A' and B motifs are conserved in all of these proteins but the positioning of the tyrosine residues was only shared between the *Streptococcus* species.

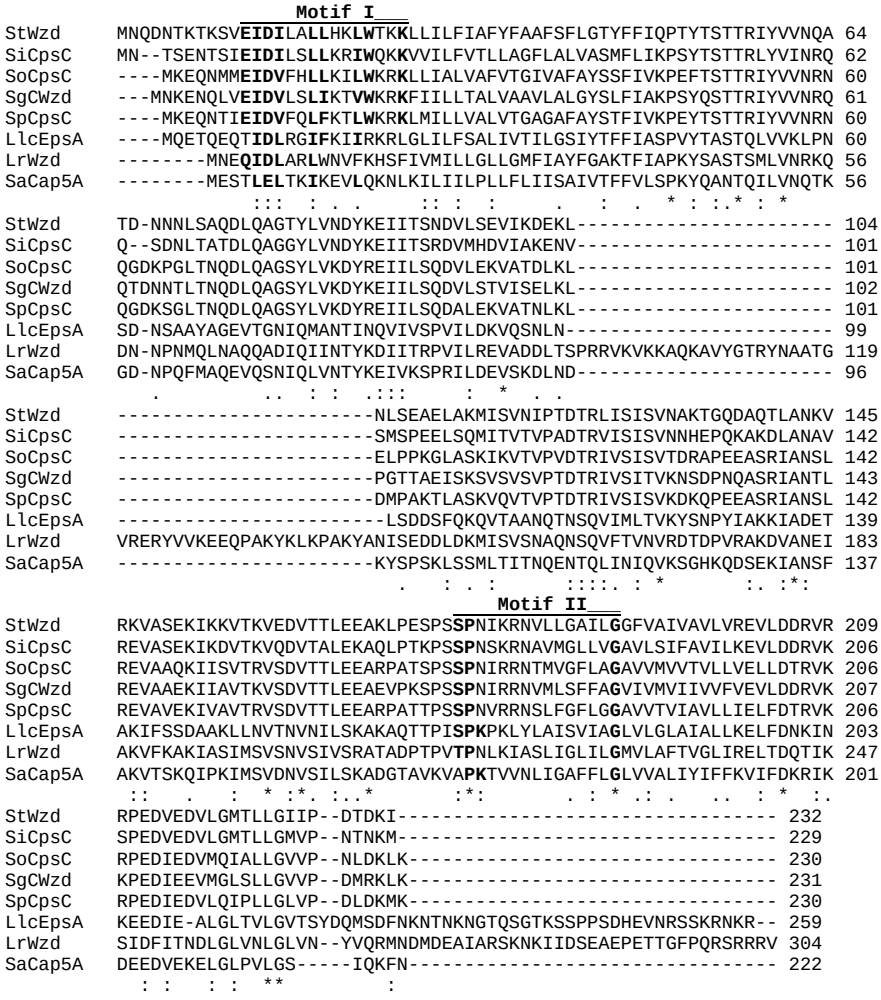

Fig. 5.2. Alignment of the transmembrane activation proteins from *Streptococcus thermophilus* MR-1C (Wzd, StWzd), *Streptococcus iniae* 9066 (CpsC, SiCpsC), *Streptococcus oralis* ATCC 35037 (CpsC, SoCpsC), *Streptococcus gordonii* str. *Challis* substr. CH1 (Wzd, SgCWzd), *Streptococcus pneumoniae* D39 (CpsC, SpCpsC), *Lactococcus lactis* subsp. *cremoris* JRF1 (EpsA, LlcEpsA), *Lactobacillus rhamnosus* ATCC 9595 (Wzd, LrWzd), and *Staphylococcus aureus* (Cap5A, SaCap5A). Two motifs are labeled which are similar to the consensus sequences [E/Q][I/L]D[L/I] X_3L[I/F]XXLWX[A/G]K and SPKX_{11}GX_3G (with X being any amino acid and alternative residues enclosed in brackets) that are found in transmembrane activation proteins in *Escherichia coli, Salmonella enterica, and Rhizobium meliloti* (Becker et al. 1995; Tocilj et al. 2008). Conserved amino acids similar to those in these consensus sequences are labeled in bold type. Amino acids that are identical in all sequences of the alignment are indicted by an asterisk, conserved substitutions are indicated by two dots, and semi-conserved substitutions are indicated by one dot.

```
StWze     ------------------MPLLKLVKSKVDFAKKTEEYYNTIRTNIQFS---GAQMKVIA 39
SiCpsD    ------------------MSQLNLVRSKREHYQHAEEYYNSIRTNIQFS---GRDYKAIV 39
SoCpsD    ------------------MPTLEIAQKKLDLARKAEEYYNALRTNIQLS---GNNLKVIA 39
SgCWze    ------------------MPILELSNNKKQSIQKTEEYYNALRTNIQLS---GEDIKIVA 39
SpCpsD    ------------------MPTLEISQAKLDFVKKAEEYYNSLCTNLQLS---GDGLKVFS 39
LlcEpsB   -----------MAKNKRSIDNNHYIITSVNPQSPISEQYRTIRTTIDFK-MADQGIKSFL 48
LrWze     MNFSLEKILHRHKEDQETQKNGVMLVTFAEPKHVVSEQFRTVRTNIEFAGAALDQCQVVM 60
SaCap5B   -----------MSKKENTTTT---LFVYEKPKSTISEKFRGIRSNIMFS-KANGEVKRLL 45
                                  .    .* :. : :.: :        : .
             Walker A                  Walker A'
StWze     ISSVEAREGKSTTSVNLAISFASVGLRTLLIDADTRNSVLSGTFKSNEPYKGLSNFLSGN 99
SiCpsD    LTSVQPGEGKSTTSINLAISFAKAGFKTLLIDADVRNSVMSGAFKSDDRYEGLSSYLSGN 99
SoCpsD    ITSVKPGEGKSTTSTNIAWAFARAGYKTLLIDADIRNSVMSGVFKSREKITGLTEFLSGT 99
SgCWze    VTSVQPNEGKSTTSTNLAIAFARAGYTTLLIDADIRNSIMSGVFKSKEKITGLTDYLVGK 99
SpCpsD    ITSVKLGEGKSTTSTNIAWAFARAGYKTLLIDGDIRNSVMLGVFKARDKITGLTEFLSGT 99
LlcEpsB   VTSSETDEGKTTVSANIAVAFAQQGKKVLLIDGDLRKPTVNITFKVQN-RVGLTNILMHQ 107
LrWze     FTSSAMSEGKSTVSANVAVTWAQAGKKVLLIDADLRRPTVHATFRTLN-LDGVTTVLTGK 119
SaCap5B   VTSEKPGAGKSTVVSNVAITYAQAGYKTLVIDGDMRKPTQNYIFNEQN-NNGLSSLIIGR 104
          .:*     **:*.  *:* ::*  *  .*:**.* *..     *.  :   *::  :
                                                          Walker B
StWze     ADLNETICQT-DISGLDVIASGPIPPNPTSLLQNDNFRHLMEVARSRYDYVIIDTPPIGM 158
SiCpsD    AELSSVISRT-DVPNLMLIPSGQVPPNPTTLLQNSNFNFMIDTVKELFDYIIIDTPPIGL 158
SoCpsD    TDLSQGLCET-NVENLFVIQAGSVSPNPTALLQSENFATMIDTLRKYFDYIVVDTAPIGV 158
SgCWze    NDLSQGLCET-DVPNLFVIESGQSSPNPTALLQSKNFDEMMNILRRHYDYIIVDTPPIGL 158
SpCpsD    TDLSQGLCDT-NIENLFVIQAGSVSPNPTALLQRKNFSTMLETLRKYFDYIIVDTAPVGV 158
LlcEpsB   SSIEDAIQGTRLSENLTIITSGPIPPNPSELLASSAMKNLIDSVSDFFDVVLIDIPPLSA 167
LrWze     EKPDDVVEET-FVDNMSIITSGPVPPNPSELLNSKRMAGLLEWAREKYDIIVLDAPPVLA 178
SaCap5B   TTMSEAITST-EIENLDLLTAGPVPPNPSELIGSERFKELVDLFNKRYDIIIVDTPPVNT 163
             .. :  *    .: :: :*  .***: *:  . :  :::     :* :::* .*:
StWze     FIDAAIIAHQADASLLVTAAGKIKRRFVTKAVEQLEQSGSQFLGVVLNKVDMTVDKYGSY 218
SiCpsD    VIDSAIIAQKADATILVTEAGSIKRRFVQKAKEQMEQSGAQFLGVILNKVDQ---QLGSY 215
SoCpsD    VIDAAIITQKCDASVLVTAAGETNRRDVQKAKEQLEQTGKPFLGIVLNKLNTSVEKYGSY 218
SgCWze    VIDAAIISQKCDASILVAEAGSVKRKALQKSKEQLEQTGTPFLGVVLNKYDVSGDRYGVY 218
SpCpsD    VIDAAIITRKCDASILVTEAGEINRRDIQKAKEQLEHTGKPFLGVVLNKFDTSVDKYGSY 218
LlcEpsB   VTDAQILSSYVGGVVLVVRAYETKKESLAKTKKKLEQVNANILGVVLHGVDSSDSPSYYY 227
LrWze     VSDVQVLVPKTDGVVVVANMGKTLKGDLKRTVEVLKLANAKILGSVERVKAKHGDRGYGY 238
SaCap5B   VTDAQLYARAIKDSLLVIDSEKNDKNEVKKAKALMEKAGSNILGVILNKTKVDKSSSYYH 223
          . *  :        ::*    .  :  : ::   ::  .  :** : .           :
StWze     GSYGSYGEYGKKSNQKEGHSRAHRRRKVGWN 249
SiCpsD    GAYGSYGDYGKT--KKSSKSKKHSRK----- 239
SoCpsD    GAYGNYGNYGKK------------------- 230
SgCWze    GAYGNYGAYGKKH------------------ 231
SpCpsD    GDYGKN----KK------------------- 226
LlcEpsB   -----YGVE---------------------- 231
LrWze     GYGYGYGNESNK------------------- 250
SaCap5B   ----YYGDE---------------------- 228
```

Fig. 5.3. Alignment of the PTKs from *Streptococcus thermophilus* MR-1C (Wze, StWze), *Streptococcus iniae* 9066 (CpsD, SiCpsD), *Streptococcus oralis* ATCC 35037 (CpsD, SoCpsD), *Streptococcus gordonii* str. *Challis* substr. CH1 (Wze, SgCWze), *Streptococcus pneumoniae* D39 (CpsD, SpCpsD), *Lactococcus lactis* subsp. *cremoris* JRF1 (EpsB, LlcEpsB), *Lactobacillus rhamnosus* ATCC 9595 (Wze, LrWze), and *Staphylococcus aureus* (Cap5B, SaCap5B). The typical Walker A motif is GXXGXGK[T/S] but only GK[S/T] is well conserved in bacterial PTKs, the Walker A' motif has the consensus sequence ILVFM(3)DXDXR, and the Walker B motif of bacterial PTKs is extended to ILVFM (3)DXXP (with (3) in the Walker A' and B motifs indicating that the consensus sequence contains any 3 of the previous amino acids) (Walker et al. 1982; Jadeau et al. 2008; Bechet et al. 2009). The residues in the Walker A, A' and B motifs and the tyrosine residues in the carboxyl terminal end are in bold. Amino acids that are identical in all sequences of the alignment are indicted by an asterisk, conserved substitutions are indicated by two dots, and semi-conserved substitutions are indicated by one dot.

3.4. *Computational analysis of nucleotide sequences to identify potential horizontal gene transfer events*

Potential horizontal gene transfer events specifically involving the sequences found in the MR-1C *wzh*, *wzd* and *wze* genes were investigated using blast searches. We found that the first 165 nucleotides encoding the N-terminal 55 amino acids of the Wzh/CpsB proteins are 79% identical in *S. thermophilus* MR-1C and *Streptococcus gordonii* str. *Challis substr*. CH1. However, blast alignments of the remaining portion of the *wzh* genes and of the *wzd* and *wze* genes from these two species showed no significant similarities (data not shown). Similarly, the 605 nucleotides encoding the N-terminal end of the Wzh/CpsB protein are 71% identical in *S. iniae* 9066 and *Streptococcus oralis* ATCC 35037, but no significant similarity was found between the *cpsC* and *cpsD* genes of these species (data not shown).

Investigation of potential horizontal gene transfer events involving the genes encoding the priming glycosyltransferase revealed that 370 nucleotides encoding amino acids near the C-terminal end of the *L. lactis* subsp. *cremoris* JRF1 EpsD protein are 90% identical to the glucose-1-phosphate transferase gene found in *S. thermophilus* ND03. However, blast alignments of the *epsC*, *epsA*, and *epsB* genes from *L. lactis* subsp. *cremoris* JRF1, respectively, with the *wzh*, *wzd* and *wze* genes from *S. thermophilus* ND03 revealed no significant similarities (data not shown). The *L. lactis* subsp. *cremoris* JRF1 *eps* gene cluster also contains 261 base pairs that are 85% identical to a pseudogene in the *eps* gene cluster of *S. thermophilus* MTC310, but blast alignments of the *epsC*, *epsA*, and *epsB* genes from *L. lactis* subsp. *cremoris* JRF1, respectively, with the *wzh*,

wzd and *wze* genes from *S. thermophilus* MTC310 also revealed no significant similarities (data not shown).

4. Discussion

The results of this study suggest that intraspecific interactions between the transmembrane activator protein and the PTK, between two PTK subunits, and between the PTK and PTP are conserved in the order Lactobacillales. The results of interspecific protein interaction experiments and the nucleotide sequence comparisons contained in this work support previous studies on the evolution of *S. thermophilus eps/cps* gene clusters by lateral gene transfer events involving either transfer of complete genes (Pluvinet et al. 2004; Hols et al. 2005; Rasmussen et al. 2008; Eng et al. 2011) or of gene fragments (Guédon et al. 1995; Bourgoin et al. 1996, 1999; Pluvinet et al. 2004; Tyvaert et al. 2006).

Interactions of the Wzh (CpsB), Wzd (CpsC) and Wze (CpsD) proteins play critical roles in the regulation of capsule synthesis by Gram-positive organisms. A strong intraspecific protein-protein interaction takes place between the transmembrane activation protein and the PTK of *S. iniae* 9066 and of *L. lactis* subsp. *cremoris* JRF1. These interactions are similar to that previously observed between the transmembrane activation protein and the PTK of *S. thermophilus* MR-1C using the yeast two- hybrid system (see chapter 4) and are also consistent with results from *S. thermophilus* CNRZ1066 and *Staph. aureus* serotype 5 that were derived using alternative research methodologies (Minic et al. 2007; Olivares-Illana et al. 2008). An intraspecific protein-protein interaction between two identical PTKs was observed in *S. iniae* 9066 and in *L.*

lactis subsp. *cremoris* JRF1 that was again similar to that seen with the PTK from *S. thermophilus* MR-1C (see chapter 4). These results are consistent with the PTKs from these organisms having transphosphorylation abilities similar to that of the PTK Wzc of *E. coli* (Collins et al. 2006). In addition, an intraspecific protein-protein interaction was detected between the PTK and the PTP from *S. iniae* 9066 and from *L. lactis* subsp. *cremoris* JRF1 which is consistent with the interaction between these proteins seen in *S. thermophilus* MR-1C (see chapter 4) and with the results of Bender and Yother (2001) who used ELISA's to demonstrate that the PTP (CpsB) was able to interact with both the phosphorylated and dephosphorylated forms of the PTK (CpsD) in *S. pneumoniae*.

One potential additional target for tyrosine phosphorylation is the glycosyltransferase that starts synthesis of the polysaccharide units used in EPS biosynthesis (Minic et al. 2007; Obadia et al. 2007; Lin et al. 2009). In particular, the size of protein complexes isolated by Minic et al. (2007) suggest that protein interactions occur between the transmembrane activation protein (EpsC), the PTK (EpsD), and a glycosyltransferase (EpsE) from *S. thermophilus* CNRZ1066. In support of this, the glycosyltransferase activity of EpsE required EpsC and EpsD and a tyrosine residue that is necessary for the glycosyltransferase activity of EpsE was identified. The glycosyltransferase activity of EpsE is negatively affected by the PTP EpsB suggesting that this protein might be responsible for the dephosphorylation of EpsE (Minic et al. 2007). However, as was found by Cefalo et al. (see chapter 4) for the *S. thermophilus* MR-1C proteins, no protein interactions were detected between the glycosyl-1-phosphate transferase and the transmembrane activator domain, the PTK, or the PTP in *S. iniae*

9066, or *L. lactis* subsp. *cremoris* JRF1 using the yeast two-hybrid system. It may be that the transmembrane activation protein and the PTK must first form multi-protein complexes, that other protein components are needed, or that the glycosyl-1-phosphate transferase must first bind sugar polymers in order for these protein interactions to occur. The phosphorylation state of the glycosyl-1-phosphate transferase and the PTK is unknown in the yeast system and may play a role in some interactions involving these proteins.

In this study, the transmembrane activation protein of *S. thermophilus* MR-1C was able to interact with the PTK of *S. iniae* 9066 and vice versa. The transmembrane activation protein and the PTK from these two species are homologous having 56% identity and 78% similarity and 59% identity and 75% similarity, respectively. The *L. lactis* subsp. *cremoris* JRF1 transmembrane activation protein was unable to interact with the PTK from either *S. thermophilus* MR-1C or *S. iniae* 9066. The *L. lactis* subsp. *cremoris* JRF1 PTK also could not interact with the transmembrane activation protein from either *S. thermophilus* MR-1C or *S. iniae* 9066. The *L. lactis* subsp. *cremoris* JRF1 transmembrane activation protein has 24% identity and less than 45% similarity and the PTK has less than 33% identity and less than 50% similarity with the corresponding protein in either *S. thermophilus* MR-1C or *S. iniae* 9066. This divergence may explain the inability to form interspecific interactions in these studies. In particular, it has been postulated that a conserved motif (labeled Motif I in Fig. 5.2) found in the N-terminal cytoplasmic region of polysaccharide co-polymerases from *E. coli* and *Salmonella enterica* could be involved in the interaction of this protein with other proteins of the

system (Tocilj et al. 2008). The amino acid sequence of this region is highly similar when comparing *S. thermophilus* MR-1C and *S. iniae* 9066 and greatly dissimilar when comparing *L. lactis* subsp. *cremoris* JRF1 and *S. thermophilus* MR-1C or *S. iniae* 9066 (Fig. 5.2).

The PTKs from *S. thermophilus* MR-1C, *S. iniae* 9066, and *L. lactis* subsp. *cremoris* JRF1 also had the ability to form interspecific protein-protein interactions with each other. The PTK of *S. thermophilus* is 59% identical and 75% similar to the PTK of *S. iniae* 9066, but the PTK of *L. lactis* subsp. *cremoris* JRF1 is 33% or less identical and 50% or less similar to the PTKs of either *S. thermophilus* MR-1C or *S. iniae* 9066 (Fig. 5.3). However, the important amino acids within the Walker A, A' and B motifs were highly conserved in these proteins. These results suggest that the ability of the PTKs to form protein complexes may be dependent on relatively few conserved amino acids that may also be important in facilitating phosphorylation of other proteins by the PTK.

It has been suggested that the PTP involved in EPS production may recognize a cognate PTK, but little research exists to support this viewpoint (Bender and Yother 2001; Morona et al. 2002; LaPointe et al. 2008). In this study, the PTP/PTK protein interaction was preserved if one protein was from *S. thermophilus* MR-1C and the other was from *S. iniae* 9066. This could be due to the degree of homology between the PTPs (69% identical and 83% similar) and the PTKs (59% identical and 75% similar) from these two species. This finding is consistent with the work of Preneta et al. (2002) who demonstrated that the PTP from *Klebsiella pneumoniae* was able to dephosphorylate the PTK from *E. coli*, suggesting that the action of the PTP is not specific to its cognately-

encoded kinase. The PTKs and PTPs of *E. coli* and *K. pneumoniae* are approximately 51% identical and 71% similar and 54% identical and 69% similar, respectively. The PTK or PTP from *L. lactis* subsp. *cremoris* JRF1 was unable to interact with the PTP or the PTK, respectively, from either *S. thermophilus* MR-1C or *S. iniae* 9066. The *L. lactis* subsp. *cremoris* JRF1 PTP is less than 25% identical and less than 44% similar and the PTK is less than 33% identical and less than 50% similar to the corresponding proteins of either *S. thermophilus* MR-1C or *S. iniae* 9066. The *S. thermophilus* MR-1C and *S. iniae* 9066 PTPs are similar to one another within the four conserved PTP motifs as well as in the sequence of the carboxyl termini but different in these features from the PTP of *L. lactis* subsp. *cremoris* JRF1 (Fig. 5.1). PTKs can vary greatly in the arrangement and position of phosphorylated tyrosine residues, suggesting that the substrate recognition site of the PTP must vary accordingly (LaPointe et al. 2008). The arrangement of the phosphorylated tyrosine residues is highly similar in the *S. thermophilus* MR-1C and *S. iniae* 9066 PTKs and less conserved in the PTK of *L. lactis* subsp. *cremoris* JRF1. Together these differences in the structures of the PTPs and PTKs may explain the inability of the *L. lactis* subsp. *cremoris* JRF1 PTK or PTP to interact with the opposing protein from either *S. thermophilus* MR-1C or *S. iniae* 9066.

All three bacterial gene transfer mechanisms (i.e. conjugation, transduction, and natural competence) have been shown to be active in *S. thermophilus* resulting in gene content that is 20% variable with 8% likely to be derived from recent horizontal gene transfers (Burrus et al. 2002; Bolotin et al. 2004; Hols et al. 2005; Blomqvist et al. 2006; Ammann et al. 2008; Rasmussen et al. 2008; Fontaine et al. 2010; Eng et al. 2011).

Eps/cps gene clusters are prone to horizontal gene transfers in many bacterial species, causing these clusters to have a mosaic structure. Different studies have found evidence suggesting lateral transfer of gene-sized (Pluvinet et al. 2004; Hols et al. 2005; Rasmussen et al. 2008; Eng et al. 2011) or sub-gene regions (Guédon et al. 1995; Bourgoin et al. 1996, 1999; Pluvinet et al. 2004; Tyvaert et al. 2006) of the *eps* gene cluster in *S. thermophilus*.

The 79% identical match between the first 165 nucleotides of the PTP genes from *S. thermophilus* MR-1C and *Streptococcus gordonii* str. *Challis* substr. CH1 overlaps the conserved Motifs I and II (Fig. 5.1). However, blast alignments of the *wzd* and *wze* genes from these two species showed no significant similarities. This suggests lateral gene transfer of a functional sub-gene region between *S. thermophilus* and *S. gordonii* or to both these species from a third species.

The 71% identical nucleotide match between the PTP genes of *S. iniae* 9066 and *Streptococcus oralis* ATCC 35037 covers the first 201 amino acids in the proteins and includes the conserved Motifs I, II and III (Fig. 5.1) but no significant similarity was found between the *cpsC* and *cpsD* genes of these strains. Interestingly, the *cpsB*, *cpsC*, or *cpsD* genes of *S. iniae* 9066 also share no significant nucleotide similarity as determined by the Needleman-Wunsch Global Sequence Alignment Tool with the *wzh*, *wzd*, or *wze* genes respectively, of *S. thermophilus* MR-1C, although as noted above the proteins share a high degree of similarity.

Insertion sequence elements (IS*981*, IS*1191*, and IS*S1*) are a prominent feature of *Streptococcus eps*/*cps* gene clusters including those of *S. thermophilus* and *S. iniae* and

could facilitate genetic exchange of genes or partial genes between species (Guédon et al. 1995; Bourgoin et al. 1996, 1999; Pluvinet et al. 2004; Hols et al. 2005; Tyvaert et al. 2006; Lowe et al. 2007; Rasmussen et al. 2008; Eng et al. 2011). The high degree of identity (at least 98%) of these insertion sequence elements that are found in both the genomes of *S. thermophilus* and *L. lactis* suggests that horizontal transfer has recently occurred between these lactic acid bacteria during coculture in milk. The identified horizontal transfers of *eps/cps* genes between *Lactococcus* and *Streptococcus* involve genes or sub-gene regions for glycosyltransferases whose function may not rely on protein-protein interactions rather than of the *wzh*, *wzd* or *wze* genes (Guédon et al. 1995; Bourgoin et al. 1996, 1999; Broadbent et al. 2003; Pluvinet et al. 2004; Tyvaert et al. 2006; Rasmussen et al. 2008; Liu et al. 2009; Eng et al. 2011). For example, this study found evidence supporting the horizontal gene transfer of part of the glucose-1-phosphate transferase gene between *L. lactis* subsp. *cremoris* JRF1 and *S. thermophilus* ND03 and a pseudogene between *L. lactis* subsp. *cremoris* JRF1 and *S. thermophilus* MTC310. The glucose-1-phosphate gene in *S. thermophilus* ND03 and the pseudogene in *S. thermophilus* MTC310 are flanked by genes associated with various IS elements that could facilitate horizontal gene transfer between species. In this study with the exception of the Wze/EpsB interactions, the proteins from the two *Streptococcus* species were unable to interact with their counterparts from *L. lactis* subsp. *cremoris* JRF1. We note that the gene order of the *wzh*, *wzd* and *wze* genes in the *eps* gene cluster is different in *L. lactis* than in the streptococcal species and that the greater divergence of these genes between *L. lactis* and the streptococcal species may decrease the likelihood of successful

lateral transfer of functional genes or sub-gene regions, such as we identified with the *S. thermophilus* MR-1C and *S. iniae* 9066 *wzh/cpsB* genes.

A better understanding of the protein-protein interactions involved in EPS production will benefit many industrial processes by aiding in the construction of strains with enhanced properties. This study suggests that functional regulatory complexes can be formed in naturally occurring or genetically engineered recombinant strains. A better understanding of the protein interactions that take place in EPS/CPS production could also lead to new treatment strategies for microbial pathogens in which capsule production is important for virulence. This study provides insight into the protein-protein interactions involved in EPS production and their conservation among Gram-positive bacteria in the order Lactobacillale. Further research should include the confirmation of protein-protein interactions by a different method and the determination of the biological significance of interspecific interactions by gene replacement and its effects on capsule synthesis. Investigation of the specific regions responsible for the interactions between the proteins would also enhance understanding of the mechanisms underlying regulation of EPS biosynthesis.

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CHAPTER 6

CONCLUSIONS

Exopolysaccharide (EPS) biosynthesis is a complex process that involves many protein components that must function together to form high molecular weight polysaccharides. Present day research has greatly added to our understanding but much still remains unknown about how the proteins involved in EPS biosynthesis function and interact. The research contained in this dissertation contributes new information by establishing the function of one of the proteins involved in regulating EPS synthesis and identifying protein-protein interactions among regulatory proteins that take place during EPS biosynthesis in Gram-positive organisms.

Due to its homology to known phosphotyrosine phosphatases (PTPs), it had been hypothesized that *Streptococcus thermophilus* Wzh/EpsB functioned to remove phosphate groups from the PTK and other proteins involved in EPS biosynthesis such as the priming glycosyltransferase. However, the phosphatase activity of *S. thermophilus* Wzh/CpsB had never been directly demonstrated. The research in this dissertation was designed to establish the PTP activity of *S. thermophilus* Wzh by testing the purified protein's ability to release phosphate from synthetic phosphotyrosine peptides and by sequence comparisons to known PTPs. The *S. thermophilus* MR-1C Wzh protein contained all the conserved aspartate, glutamate, histidine, and arginine resides that were identified as being important for metal binding and catalysis in the well studied PTP Cps4B *Streptococcus pneumoniae* TIGR4 (Fig. 6.1). Two loops thought to function in

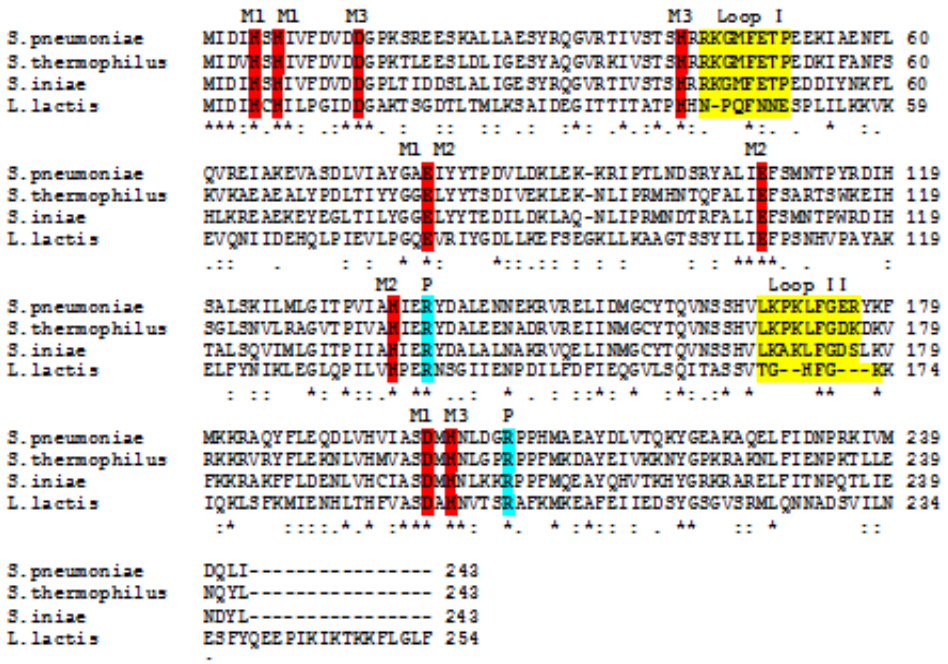


Fig. 6.1. Protein sequence comparison of the PTPs from *Streptococcus pneumoniae* TIGR4 (Cps4B), *Streptococcus thermophilus* MR-1C (Wzh), *Streptococcus iniae* 9066 (CpsB), and *Lactococcus lactis* subsp. *cremoris* (EpsC). Amino acid residues important in binding metal ions (M1, M2, and M3) are in red and those important in binding phosphate ions (P) are in blue. The two conserved loops important in controlling access of phosphotyrosine contains substrates to the active site are indicated in yellow. Amino acids that are identical in all sequences of the alignment are indicted by an asterisk, conserved substitutions are indicated by two dots, and semi-conserved substitutions are indicated by one dot.

controlling access of phosphotyrosine containing substrates to the active site in *S. pneumoniae* Cps4B were also conserved in *S. thermophilus* Wzh (Fig. 6.1). Purified *S. thermophilus* MR-1C Wzh was able to release phosphate from both phosphotyrosine peptides tested and the activity of Wzh was dramatically decreased by the presence of 1, 5, and 10 mM of the PTP inhibitor sodium vanadate. Purified Wzh was also tested for activity against a synthetic phosphoserine/threonine peptide but no phosphatase activity was detected. These results confirm that Wzh functions as a PTP

that could remove phosphate groups from the protein tyrosine kinase (PTK), priming glycosyltransferase, or other proteins involved in EPS biosynthesis. The role for Wzh as the PTP that removes phosphate from the PTK Wze is supported by research contained in this dissertation that identifies that a protein-protein interaction does take place between Wzh and Wze that would allow phosphate removal to proceed by the mechanism described in *S. pneumoniae*. However, the ability of *S. thermophilus* Wzh to remove phosphate from Wze still needs to be directly demonstrated in order to more conclusively establish its role in the regulation of EPS biosynthesis.

The research in this dissertation is the first to investigate the direct interaction of many of the proteins that are involved in Gram-positive EPS biosynthesis and the first to employ the yeast two-hybrid system to do so. It was determined that a strong protein-protein interaction takes place between the PTK and the transmembrane activation protein in the organisms *S. thermophilus* MR-1C, *Streptococcus iniae* 9066, and *Lactococcus lactis* subsp. *cremoris* JRF1. A weaker protein-protein interaction was detected between two identical PTKs in *S. thermophilus* MR-1C, *S. iniae* 9066, and *L. lactis* subsp. *cremoris* JRF1 that would be consistent with these PTKs having transphosphorylation ability as was seen for the PTK of *Escherichia coli*. A weaker protein-protein interaction was also detected between the PTK and the PTP in *S. thermophilus* MR-1C, *S. iniae* 9066, and *L. lactis* subsp. *cremoris* JRF1. The protein-protein interactions above were present in all three species tested and may represent a conserved organization for the EPS biosynthetic machinery in the family

Streptococcaceae. Further research is required to investigate the possibility of these protein interactions being conserved in other Gram-positive bacteria.

A *S. thermophilus* MR-1C PTK/transmembrane activation fusion protein was created and interactions were detected between the fusion protein and the PTK or transmembrane activation protein. This suggests that the PTK and transmembrane activation protein of *S. thermophilus* may form multi-protein complexes as has been found for these proteins in other organisms. It was thought that the presence of the PTK in the PTK/transmembrane activation fusion protein might affect the phosphorylation state of the PTK, thereby strengthening the protein-protein interaction with the PTP or the glycosyl-1-phosphate transferase. However, no interaction was detected between the PTK/transmembrane activation fusion protein and the PTP or priming glycosyltransferase. The results did indicate that there is likely a problem with the protein conformation in some constructs and therefore, the formation of multi-protein complexes and the lack of interaction with the PTP or priming glycosyltransferase need to be confirmed by other research methodologies.

Research has suggested that an interaction between the PTK and the priming glycosyltransferase, between the PTP and priming glycosyltransferase, between one glycosyltransferase and the next, between the PTK and the membrane translocation protein, or between the PTK and the polymerase could take place. All combinations of the *S. thermophilus* MR-1C Wzh (PTP), Wzd (transmembrane activation protein), Wze (PTK), Wzg (regulation), CpsE (glycosyl-1-phosphate transferase), CpsS (polymerization), CpsL (unknown), CpsW (regulation), and CpsU (membrane

translocation) proteins were analyzed for protein-protein interactions but no additional interactions were discovered using the yeast two-hybrid system. No protein-protein interactions were detected when the *S. iniae* 9066 or *L. lactis* subsp. *cremoris* JRF1 glycosyl-1-phosphate transferase was combined with the transmembrane activator domain, the PTK, or the PTP using the yeast two-hybrid system. The priming glycosyltransferase of *S. thermophilus* MR-1C was tested for protein-protein interactions with the glycosyltransferases (CpsP and CpsQ) thought to add the next sugar precursors to the carrier lipid and no interactions were detected. It is possible that the PTK and the transmembrane activation protein could form multi-protein complexes that would provide scaffolding for other protein-protein interactions to take place or that other undiscovered protein interactions must first take place before these interactions can occur. The phosphorylation states of protein such as the PTK or priming glycosyltransferase are also unknown in the yeast system and may play an important role in the interactions of these proteins. The priming glycosyltransferase may require the binding of sugar polymers to the protein in order to interact with other glycosyltransferases or may only interact with the phosphorylated form of Wze in complex with Wzd and not directly with each other. It is also possible that no direct interactions between some of the proteins required for the synthesis, polymerization, membrane translocation, and regulation of EPS exist. Further research is needed to clarify the presence or absence of these protein-protein interactions.

To determine the generality and specificity of the protein-protein interactions identified by the yeast two-hybrid system the PTK, transmembrane activation protein, PTP, and the glycosyl-1-phosphate transferase from *S. thermophilus* MR-1C, *S. iniae*

9066, and *L. lactis* subsp. *cremoris* JRF1 were tested for their ability to interact with the other two systems. The research contained in this dissertation is the first to investigate whether these regulatory proteins require their cognately encoded counterparts in order to form protein-protein interactions or whether they can form interspecific interactions that would be required in naturally occurring and in genetically engineered recombinants. Using the yeast two-hybrid system, it was determined that the transmembrane activation protein of *S. thermophilus* MR-1C was able to interact with the PTK of *S. iniae* 9066 and vice versa, probably due to the fact that transmembrane activation protein and the PTK from these two species are highly homologous. However, the *L. lactis* subsp. *cremoris* JRF1 transmembrane activation protein was unable to interact with the PTKs from either *S. thermophilus* MR-1C or *S. iniae* 9066. The *L. lactis* subsp. *cremoris* JRF1 PTK also could not interact with the transmembrane activation protein from either *S. thermophilus* MR-1C or *S. iniae* 9066. This is probably due to the lower degree of homology shared between the PTK and transmembrane domain of *L. lactis* subsp. *cremoris* JRF1and the corresponding protein in either of the *Streptococcus* species. In particular, it is thought that a conserved motif in the N-terminal cytoplasmic region of the transmembrane activation protein is important in interactions with other proteins in the system. The sequence in this motif is highly similar when comparing the species of streptococci but quite different when comparing *L. lactis* subsp. *cremoris* to either species of streptococci (Fig. 6.2).

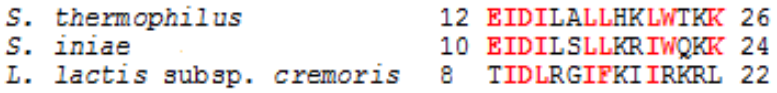


Fig 6.2. Protein sequence comparison of a motif in the N-terminal cytoplasmic region of the transmembrane activation domain in *Streptococcus thermophilus* MR-1C, *Streptococcus iniae* 9066, and *Lactococcus lactis* subsp. *cremoris* JRF1. This motif is thought to be important in the interactions of the transmembrane activation domain with other proteins of the system. The conserved amino acids of the motif are indicated in red.

The PTKs from *S. thermophilus* MR-1C, *S. iniae* 9066, and *L. lactis* subsp. *cremoris* JRF1 had the ability to form interspecific protein-protein interactions with each other. The PTK from *L. lactis* subsp. *cremoris* JRF1is not highly homologous to the PTKs of *S. thermophilus* MR-1C or *S. iniae* 9066 and this suggests that the ability of the PTKs to form protein complexes may be dependent on relatively few conserved amino acids that may also be important in facilitating phosphorylation of other proteins by the PTK. In support of this the amino acids within the Walker A, A' and B motifs that are important in the binding and catalysis of ATP were highly conserved in these proteins (Fig. 6.3).

It has been suggested that the PTP involved in EPS production may recognize a cognate PTK, but little research exists to support this viewpoint. The PTK from *S. thermophilus* MR-1C was able to interact with the PTP from *S. iniae* 9066 and vice versa which is consistent with the viewpoint that the action of the PTP is not specific to its cognately-encoded kinase. However, the PTPs and the PTKs of the two species of streptococci are highly homologous and the PTP/PTK interaction is not conserved when the more dissimilar *L. lactis* subsp. *cremoris* proteins and the *S. thermophilus* MR-1C or the *S. iniae* 9066 proteins were combined. The PTPs from *S. thermophilus* MR-1C, *S.*

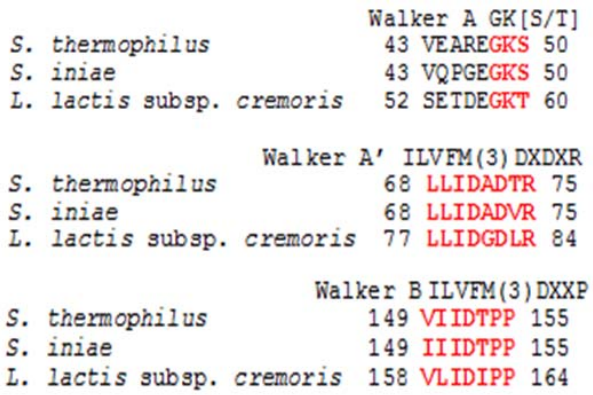


Fig. 6.3. Protein sequence comparisons of the Walker A, A' and B motifs of *Streptococcus thermophilus* MR1C, *Streptococcus iniae* 9066, and *Lactococcus lactis* subsp. *cremoris*. These motifs are important in the binding and catalysis of ATP. The conserved amino acids of the motifs are indicated in red.

iniae 9066, and *L. lactis* subsp. *cremoris* JRF1 all contained the aspartate, glutamate, histidine, and arginine residues that were identified to be important in the binding of metal and phosphate ions in *S. pneumoniae* TIGR4 Cps4B. However, the sequence of the *L. lactis* subsp. *cremoris* PTP differed greatly from either of the species of streptococcal PTPs in loops I and II (Fig. 6.1). These loops are involved in controlling the access of phosphotyrosine containing substrates to the active site and therefore would be important in protein interactions of the PTP with its substrates, such as the PTK. The carboxyl terminus of the PTP from *L. lactis* subsp. *cremoris* also had a different arrangement from the streptococcal PTPs and could affect the ability of the *L. lactis* subsp. *cremoris* PTP to interact with the PTK from the streptococcal species (Fig. 6.1.). The arrangement of the phosphorylated tyrosine residues is highly similar in the *S. thermophilus* MR-1C and *S. iniae* 9066 PTKs and less conserved in the PTK of *L. lactis* subsp. *cremoris* JRF1 (Fig 6.4). The differences in the arrangement of phosphorylated tyrosine residues may require

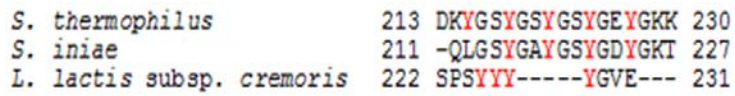


Fig. 6.4. Protein sequence comparison of the arrangement of phosphorylated tyrosine residues in the PTKs of *Streptococcus thermophilus* MR-1C (Wze), *Streptococcus iniae* 9066 (CpsD), and *Lactococcus lactis* subsp. *cremoris* (EpsB). The phosphorylated tyrosine residues are indicated in red.

the active sites of the PTP to differ accordingly. This could alter the ability of the *L. lactis* subsp. *cremoris* PTP to interact with the streptococcal PTKs and vice versa. More research is needed to verify the importance of these structural differences in the interaction of the PTK and PTP and in the catalytic mechanism of the PTP. These results suggest that functional regulatory complexes can be formed in naturally occurring or genetically engineered recombinant strains but the determination of the biological significance of interspecific interactions by gene replacement and its effects on capsule synthesis needs to be analyzed.

Eps/cps gene clusters are prone to horizontal gene transfers in many bacterial species, causing these clusters to have a mosaic structure. The research contained in this dissertation investigated the possible transfer of gene or subgene regions in *S. thermophilus* MR-1C, *S. iniae* 9066, and *L. lactis* subsp. *cremoris* JRF1. The first 165 nucleotides of the PTP genes from *S. thermophilus* MR-1C and *Streptococcus gordonii* str. *Challis* substr. CH1, which overlap the conserved Motifs I and II, are highly homologous but the PTK and transmembrane activation protein of these two species showed no significant similarities. Similarly, the nucleotides in the PTP genes of *S. iniae* 9066 and *Streptococcus oralis* ATCC 35037 which cover the first 201 amino acids in the

protein and include the conserved Motifs I, II and III are highly homologous but the PTK and transmembrane activation protein of these two species showed no significant similarities. Evidence was also found that supports the horizontal gene transfer of part of the glucose-1-phosphate transferase gene between *L. lactis* subsp. *cremoris* JRF1 and *S. thermophilus* ND03 and a pseudogene between *L. lactis* subsp. *cremoris* JRF1 and *S. thermophilus* MTC310. The glucose-1-phosphate gene in *S. thermophilus* ND03 and the pseudogene in *S. thermophilus* MTC310 are flanked by genes associated with various IS elements that could facilitate horizontal gene transfer between these species. The identified horizontal transfers of *eps/cps* genes between *Lactococcus* and *Streptococcus* involve genes or sub-gene regions for glycosyltransferases whose function may not rely on protein-protein interactions rather than of the *wzh*, *wzd* or *wze* genes. The gene order of the *wzh*, *wzd* and *wze* genes in the *eps* gene cluster is different in *L. lactis* than in the streptococcal species and the greater divergence of these genes between *L. lactis* and the streptococcal species may decrease the likelihood of successful lateral transfer of functional genes or sub-gene regions, such as was identified with the *S. thermophilus* MR-1C and *S. iniae* 9066 *wzh/cpsB* genes. These results support the horizontal transfer of genes between species of streptococci and between Streptococci and lactococci.

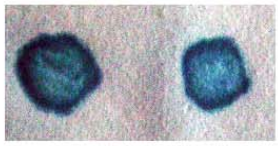
A better understanding of the functions and interactions of the proteins involved in EPS production will benefit many industrial processes by aiding in the construction of strains with enhanced properties and could also lead to new treatment strategies for microbial pathogens in which capsule production is important for virulence. The research in this dissertation has established that *S. thermophilus* Wzh acts as a PTP as

well as providing insight into the protein-protein interactions involved in EPS production and their conservation among Gram-positive bacteria in the family Streptococcaceae. It provides evidence for lateral gene transfer between streptococci species and streptococci and lactococci and suggests that functional regulatory complexes can be formed in naturally occurring or genetically engineered recombinant strains. Further research should include the confirmation of protein-protein interactions by a different method, determination of the biological significance of interspecific interactions by gene replacement and analyzing its effects on capsule synthesis, investigation of the specific regions responsible for the interactions between the proteins, and the confirmation of the hypothetical function of EPS proteins.

APPENDICES

APPENDIX A

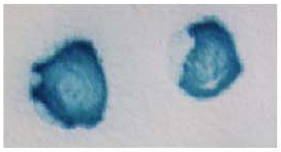
PHOTOGRAPHS OF POSITIVE β-GALACTOSIDASE FILTERS



S. thermophilus MR-1C transmembrane activation protein (Wzd) and protein tyrosine kinase (Wze).

S. thermophilus MR-1C protein tyrosine kinase (Wze) dimerization.

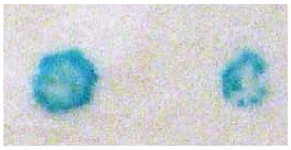
S. thermophilus MR-1C protein tyrosine kinase (Wze) and phosphotyrosine phosphatase (Wzh).



S. iniae 9066 transmembrane activation protein (CpsC) and protein tyrosine kinase (CpsD).

S. iniae 9066 protein tyrosine kinase (CpsD) dimerization.

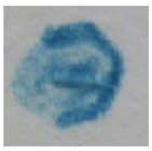
S. iniae 9066 protein tyrosine kinase (CpsD) and phosphotyrosine phosphatase (CpsB).



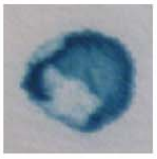
L. lactis subsp. *cremoris* JRF1 transmembrane activation protein (EpsA) and protein tryrosine kinase (EpsB).

L. lactis subsp. *cremoris* JRF1 protein tyrosine kinase (EpsB) dimerization.

L. lactis subsp. *cremoris* JRF1 protein tyrosine kinase (EpsB) and phosphotyrosine phosphatase (Eps C).



S. thermophilus MR-1C transmembrane activation protein (Wzd) and *S. iniae* 9066 protein tyrosine kinase (CpsD).



S. iniae 9066 transmembrane activation protein (CpsC) and *S. thermophilus* MR-1C protein tyrosine kinase (Wze).

S. thermophilus MR-1C protein tyrosine kinase (Wze) and *S. iniae* 9066 protein tyrosine kinase (CpsD).

S. thermophilus MR-1C protein tyrosine kinase (Wze) and *L. lactis* subsp. *cremoris* JRF1 protein tyrosine kinase (EpsB).

S. iniae 9066 protein tyrosine kinase (CpsD) and *L. lactis* subsp. *cremoris* JRF1 protein tyrosine kinase (EpsB).

S. thermophilus MR-1C protein tyrosine kinase (Wze) and *S. iniae* 9066 phosphotyrosine phosphatase (CpsB).

S. iniae 9066 protein tyrosine kinase (CpsD) and *S. thermophilus* MR-1C phosphotyrosine phosphatase (Wzh).

APPENDIX B

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CURRICULUM VITAE

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Education:

- 2001-2012: Ph.D. in Biology, Utah State University, Logan, UT.
- 1997-2001: B.S. in Microbiology, Minor in Chemistry, Weber State University, Ogden, UT.

Research Experience:

- 2001-2012: Regulation of exopolysaccharide biosynthesis in commercial dairy strains of *Streptococcus thermophilus* and *Lactococcus lactis* subsp. *cremoris*, and in the fish pathogen *Streptococcus iniae* at Utah State University under Dr. Dennis L. Welker.
- 1999-2001: Antimicrobial resistance at Weber State University under Dr. Mohammad Sondossi.

Teaching Experience:

- 2011: Lecturer for Human Physiology (BIOL 2420) at Utah State University Logan Campus.
- 2007: Lecturer for Elementary Microbiology (BIOL 1110) at Utah State University Brigham City Campus.
- 2001-2012: Laboratory Teaching Assistant for the following courses: General Microbiology (BIOL 3300), Microbial Physiology (BIOL 5300), Biology and the Citizen (BIOL 1010), Human Physiology (BIOL 2420), and Human Anatomy (BIOL 2320) at Utah State University Logan Campus.

Publications and Posters:

- Cefalo, A.D., Broadbent, J.R., and Welker, D.L. 2011. Intraspecific and interspecific interactions among proteins regulating exopolysaccharide synthesis in *Streptococcus thermophilus*, *Streptococcus iniae*, and *Lactococcus lactis* subsp. *cremoris* and the assessment of potential lateral gene transfer. Can. J. Microbiol. 57(12): 1002-1015.
- Cefalo, A.D., Broadbent, J.R., and Welker, D.L. 2011. Protein-protein interactions among the components of the biosynthetic machinery responsible for exopolysaccharide production in *Streptococcus thermophilus* MR-1C. J. Appl. Microbiol. 110(3): 801-812.

- D.L. Welker, J.R. Broadbent, A.D. Cefalo, D.J. McMahon, and C.J. Oberg. Mechanism(s) for the loss of exopolysaccharide synthesis phenotype in strains of *Streptococcus thermophilus*. Seventh Symposium on Lactic Acid Bacteria, September 2002.
- D.L. Welker, C.J. Oberg, A.D. Cefalo, D.J. McMahon, and J.R. Broadbent. Insights on the mechanism(s) for the loss of exopolysaccharide synthesis phenotype in *Streptococcus thermophilus*. American Society for Microbiology Annual Meeting, May 2002.

Awards and Activities:

- 2001-2012: Departmental Teaching Assistantship
- 2001-2006: Hosted High School Students in the laboratory for the Biotechnology Summer Academy at Utah State University.
- 2001: Graduated *magna cum laude* from Weber State University
- 2001: Microbiology Student of the Year at Weber State University

Additional Training:

- 2005: Radiation Safety Training at Utah State University
- 2003: Microbial Fermentation: Development and Scale-Up at Utah State University
- 2002 and 2003: Laboratory Safety Training at Utah State University