INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road. Ann Arbor, MI 48106-1346 USA
800-521-0600

Catabolite repression and virulence gene expression in *Listeria monocytogenes* 10403S

by

Stefanie Evans Gilbreth

A DISSERTATION

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Food Science and Technology

Under the Supervision of Professor Robert W. Hutkins

Lincoln, Nebraska

May, 2003

UMI Number: 3078608

UMI®

UMI Microform 3078608

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

DISSERTATION TITLE

Catabolite Repression and Virulence Gene Expression in Listeria monocytogenes

BY

Stefanie Evans Gilbreth

SUPERVISORY COMMITTEE:

	Approved	Date
Signature	Robert Hutkins	2-14-03
Typed Name	Robert Hutkins	
Signature	Andrew K Benson	2/14/03
Typed Name	Andrew Benson	
Signature	Kenneth W Nickerson	2/14/03
Typed Name	Ken Nickerson	
Signature	Paul Blum	2/14/03
Typed Name	Paul Blum	
Signature		
Typed Name		
Signature		
Typed Name		

University of Nebraska | Graduate College

Catabolite repression and virulence gene expression in *Listeria monocytogenes* 10403S

Stefanie Evans Gilbreth, Ph.D.

University of Nebraska, 2003

Advisor: Robert W. Hutkins

Previous studies have suggested that the specific type and amount of carbohydrate available for growth affects the expression of virulence genes in *Listeria monocytogenes*. Which carbohydrates influence virulence gene expression and how carbohydrates mediate expression, however, is not clear. The goal of this project, therefore, was to determine how carbohydrates affect virulence gene expression in *L. monocytogenes* 10403S. Growth studies were initially conducted in a defined medium containing combinations of glucose and various sugars. Metabolism of arbutin, arabitol, cellobiose, mannose, maltose, trehalose and salicin were repressed in the presence of glucose, and only when glucose had been consumed were these sugars fermented, indicating that catabolite repression by glucose had occurred. To determine if virulence gene expression was also influenced by catabolite repression conditions, primer extension experiments were performed using primers for *hly*, which encodes for a hemolysin, and *prfA*, which encodes the regulator protein PrfA. In the

presence of cellobiose and arbutin, transcription of hemolysin was markedly reduced, compared to transcription levels in all of the other sugars tested. When cells were grown in the presence of both glucose and cellobiose, there was no change in *hly* expression compared to cells grown on glucose only. However, none of the sugars tested affected transcription of *prfA* in *L. monocytogenes*. On the other hand, *prfA* could be targeted by carbohydrates at a level other than transcription such as activity. Activity of hemolysin when grown with the above mentioned sugars was also examined along with global gene expression using an expression array. These results demonstrate that catabolite repression occurs in *L. monocytogenes* and suggests that, at least in strain 10403S, cellobiose and arbutin repress expression of hemolysin.

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge my advisor, Dr. Hutkins, for his support and guidance. I would also like to acknowledge the members that served on my committee, Dr. Benson, Dr. Blum and Dr. Nickerson, for their guidance and time. I would also like to thank Chao & Min Zhang for their help with the microarray experiments. Todd Swanson and Dojin Ryu for their help with the HPLC work. Jun Goh, Jaehyoung Kim, Meena Oommen and Lisa Durso for help with a variety of things in the lab and for camaraderie in the lab.

I would like to thank and acknowledge my parents for providing me with support and guidance throughout my life, especially during graduate school. Thanks to my sister for always listening and supporting me. And many thanks to my husband, Jason for his patience, understanding and commitment to help me with school and support me in any way. Many thanks also goes out to everyone else who has supported me during this time.

Table of Contents

page

Acknowledgements.....i

Table of Contents.....ii

List of Tables.....iv

List of Figures.....v

Literature Review: *Listeria monocytogenes*

The effect of carbohydrates on virulence gene expression in *L. monocytogenes* and other pathogens.....1

Introduction.....2

Listeria and Listeriosis.....3

Physiological characteristics of *L. monocytogenes*.....4

Ecology of *L. monocytogenes*.....5

Phosphotransferase system and catabolite repression.....6

Pathogenesis of *L. monocytogenes*.....13

Virulence gene regulation and expression.....15

Environmental conditions that affect virulence gene expression.....20

Effect of utilizable sugars on virulence gene expression.....21

Other mechanisms of regulation.....24

Regulation of virulence genes by carbohydrates in other pathogens.....29

Conclusions.....33

Materials and Methods.....35

Bacterial strains.....36

Variations in sugar utilization with a collection of strains.....36

Catabolite repression studies.....37

Primer extension studies on virulence gene expression.....39

The effect of growth medium on expression of hemolysin.....42

Global gene expression using arrays.....43

Results.....47

Carbon source utilization by *L. monocytogenes*.....48

Catabolite repression studies.....50

Primer extension studies.....57

The effect of growth medium on the expression of hemolysin.....61

Global gene expression using microarrays.....67

Discussion.....69

Conclusions.....80

Literature Cited.....83

List of Tables

page

Figure

1. Environmental conditions that affect virulence gene expression.....22

2. Hemolysin Activities in *L. monocytogenes* grown on Biolog plates...65

3. Hemolysin Activities in *L. monccytogenes* grown in DM.....66

4. Hemolysin Activities in *L. monocytogenes* grown in BHI.....67

5. Microarray results.....70

6. Genes induced during growth in various carbon sources.....71

List of Figures

page

Figure

1. The dual role of HPr in the phosphotransferase system (PTS) and catabolite repression.....7

2. *L. monocytogenes* pathogenesis.....14

3. Organization and regulation of the PrfA-dependent virulence genes.....16

4. Putative role of HPr and the *bvr* locus in regulating virulence gene expression.....26

5. Sugar utilization.....50

6-13. Growth of *L. monocytogenes* on various carbohydrates to examine catabolite repression.....54-57

14-15. Primer extension analysis of the expression of the *prfA* and *hly* genes in *L. monocytogenes*.....60-61

The effect of carbohydrates on virulence gene expression in *L. monocytogenes* and other pathogens: a review

Introduction

Listeria monocytogenes is a ubiquitous food-borne pathogen that can cause serious illness in immunocompromised people, children and pregnant women. *L. monocytogenes* can grow in many extreme environments, such as high salt and low temperatures, making it a problem for the food industry. *L. monocytogenes* is normally found in the environment growing in decaying vegetation. Thus it easily finds its way into processing facilities where, due to its ability to adapt to extreme conditions it is able to survive and grow in food products that are eventually consumed by humans. When *Listeria monocytogenes* encounters a suitable host, an entire set of virulence genes are expressed and the infection process begins. What triggers the expression of virulence genes and how Listeria is able to discriminate between being in a host and being in its normal environment is now the subject of much research interest.

In previous work, the availability of carbohydrates has been shown to affect the expression of virulence genes in *Listeria monocytogenes*. There are conflicting reports however, on what carbohydrates contribute to this regulation and the precise means by which regulation occurs. Initial reports showed that cellobiose, a plant derived carbohydrate, repressed virulence gene expression and that repression could be seen at concentrations as low as 1mM. The repression of virulence genes was believed to be a signal sensing response and not a general effect caused by substrate utilization (Park et.al., 1993).

However, more recent reports claim that any utilizable sugar represses virulence gene expression, which would indicate that this phenomenon might be due to a more general mechanism of catabolite repression (Milenbachs et.al., 1997). Recently, Brehm et. al. (1999) characterized the *bvrABC* locus, which is responsible for sensing β-glucosides, and showed that both of these hypotheses could be correct (Brehm et. al., 1999).

This review will detail past and present work on the role of carbohydrates in the regulation of virulence genes. The PEP-dependent phosphotransferase system and the physiology of *L. monocytogenes* will also be covered along with the pathogenicity of *L. monocytogenes* and the infectious process.

Listeria and Listeriosis

L. monocytogenes is the causative agent of listeriosis, a disease usually causing symptoms very similar to the flu. For the average individual, listeriosis is not dangerous, however certain populations are very susceptible to the disease. In immunocompromised individuals, the elderly, and children the disease can cause meningitis and septicemia and in pregnant woman it can cause spontaneous abortion (Schuchat et. al., 1991). The mortality rate associated with listeriosis is much higher than that associated with illness caused by enteric pathogens, making the need to control *L. monocytogenes* critical. Approximately 28% of all food-borne pathogen related deaths in the U.S. are attributed to *L. monocytogenes* (Mead et. al., 1999). Interestingly, of the 12 *L. monocytogenes* serotypes, the 4b serotype causes over 50% of the

global listeriosis cases, whereas most of the *L. monocytogenes* strains found in food belong to the 1/2 serotype (Vazquez-Boland 2001).

Physiological characteristics of *Listeria monocytogenes*

L. monocytogenes is a unique foodborne organism because it can survive under conditions that other pathogens cannot. In its natural habitat, (i.e. plants, soil, vegetation), it is subject to a wide range of temperatures and environmental conditions. Not surprisingly, therefore, in food processing environments, it is able to survive low pH, high salt (10-20%), low moisture conditions and can grow at temperatures ranging from 4-45°C (Farber et. al., 1990). Inside a host *L. monocytogenes* encounters many other hurdles such as low pH in the stomach (2.0), and oxidative stress from the host immune system. Remarkably, *L. monocytogenes* is able to survive in all of these stressful environments. The ability of this pathogen to discriminate which environment it is in and then switch on and off the appropriate genes that are needed may be key to its survival. One of the ways in which it may discriminate differences in the environment may be by sensing the presence or availability of carbohydrates. Understanding how this pathogen advances to the infection process by sensing carbohydrate availability may provide a basis for development of effective control strategies.

Ecology of *Listeria monocytogenes*

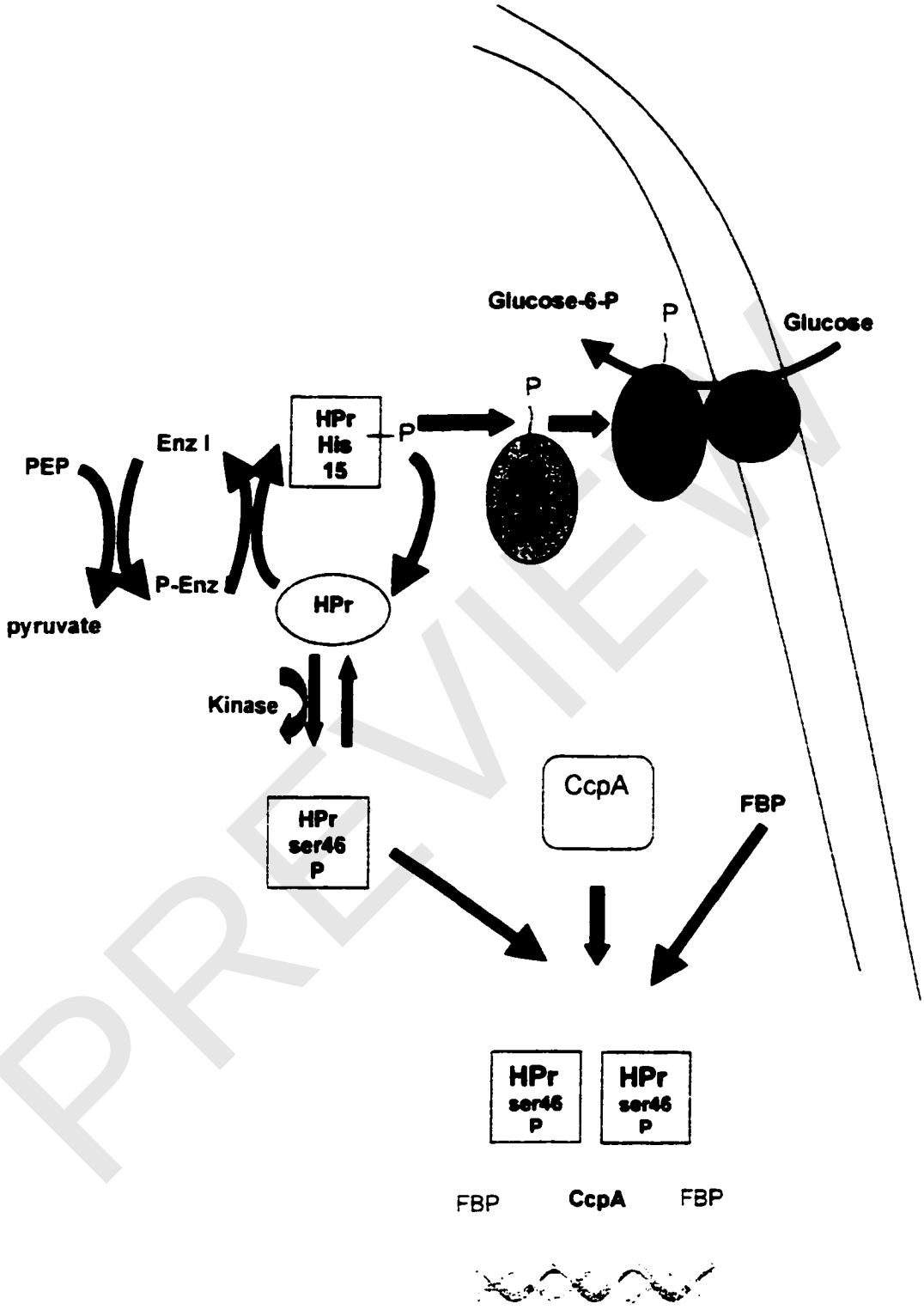
Listeria monocytogenes can be isolated from soil, water, effluents, silage, a large variety of foods, and the feces of humans and animals. The natural habitat of this organism is decomposing plant matter making it a saprophytic bacteria (Welshimeri et.al., 1971). It is widely disseminated in rural environments. Domesticated ruminants may play a key role in maintenance of the organism in the rural environment via a continuous fecal-oral enrichment cycle (Weis et.al., 1975).

Because of the wide dissemination of *L. monocytogenes*, it easily finds its way into food processing plants via workers' clothing, transport equipment, the contaminated hides or surfaces of animals, raw plant tissue, raw food of animal origin and possibly healthy human carriers. *L. monocytogenes* is especially found in the processing environment in moist areas such as floor drains, condensed and stagnant water, floors, residues and processing equipment (Food Microbiology, 1997). It is the changes in food processing and distribution that has caused increase incidence of Listeriosis (Schlech III et. al., 2000). Many different foods have been implicated in Listeriosis outbreaks, including, beef, pork, ham, sausages, milk, soft cheeses, pates, salads and industrially produced refrigerated ready-to-eat products (Food Microbiology, 1997).

Phosphotransferase system and Catabolite repression

Listeria monocytogenes requires carbohydrates as an energy and carbon source. Glucose is the preferred carbohydrate source, and the utilization of other carbohydrates varies among the different strains (Bergey's manual, 1986). Christensen and Hutkins reported that *L. monocytogenes* has at least two systems for transporting glucose. The first system identified was a proton motive force-mediated system (PMF) and the second system consisted of a glucose specific phosphotransferase system (PTS) (Christensen et. al., 1994). The former was described as a low affinity PMF-driven system and the latter a high affinity PTS. The PMF-driven system has a Km of 2.9 mM, whereas the PTS system has a Km value of 0.11 mM (Parker et. al., 1997). The PTS is a cascade of proteins that ultimately transport a phosphate onto the substrate that is entering the cell. For example, when one molecule of glucose from outside the cell is transported in by this system, it is phosphorylated and becomes one molecule of glucose-6-phosphate inside the cell. The PTS consists of 2 cytoplasmic proteins, HPr and enzyme I (EI) and depending on the substrate 2-3 other proteins or domains that are membrane associated and sugar specific (Figure 1). HPr and Enzyme I are expressed constitutively in the cytoplasm (Parker et.al., 1997). Whereas the Enzyme II complex is inducible when the substrate is present (Mitchell et. al., 1993). The PTS system derives the initial phosphate used in the cascade from phosphoenolpyruvate (PEP) thereby making it PEP dependent (Parker et. al., 1997). In the PTS, the phosphate that originates with phosphoenolpyruvate is passed to Enzyme I and

Fig. 1. The dual role of HPr in the phosphotransferase system (PTS) and catabolite repression. When HPr is involved with the PTS it is phosphorylated at the His 15 residue by transport from enzyme I. The original phosphate is derived from phosphoenolpyruvate. After HPr receives its phosphate from enzyme I, it is transported from HPr to the Enzyme II complex which ultimately transports the sugar into the cell and phosphorylates it at the same time. When HPr is phosphorylated by an ATP-dependent kinase at Ser46, it is involved with catabolite repression by binding with CcpA in the presence of fructose-1-6-bisphosphate. This structure then binds the promoters of genes to prevent transcription.



then from Enzyme I onto HPr, a regulatory protein. HPr then passes this phosphate onto the Enzyme II complex. The enzyme II complex is sugar specific and can contain up to three proteins or protein domains, termed IIA, IIB, and IIC. In this case the enzyme IIC domain is the portion of EII that is embedded in the membrane and catalyzes the transport of the substrate (Figure 1). The phosphorylated Enzyme II complex finally transfers the phosphate onto glucose (or other PTS sugars) as it enters the cytoplasm (Saier et. al., 1995). Evidence for the presence of a fructose PTS system in *L. monocytogenes* was provided by Mitchell et. al. in 1993. This was further confirmed by Christensen et. al. in 1998 by cloning and sequencing of the HPr and Enzyme I proteins.

In gram positive bacteria, the key protein in this cascade is HPr or histidine containing protein. HPr is a 90 residue protein of which the His 15 and Ser 46 residues are the most significant. Importantly, whether HPr is phosphorylated at Ser 46 or His 15 has a major influence on regulation of sugar utilization and perhaps other activities in *L. monocytogenes*. When HPr is involved with sugar transport it is phosphorylated at His 15 by transfer of a phosphate from Enzyme I. Mutational analysis of HPr showed that phosphorylation at His 15 is essential for PTS function (Christensen et. al., 1999). When HPr is involved with catabolite repression, it is phosphorylated at Ser 46 by an ATP-dependent membrane associated kinase (Reizer et. al., 1998). In this way, HPr provides a regulatory mechanism in this cascade that controls the expression of metabolic genes (Christensen et. al., 1998).

When bacteria are growing on two carbon sources, one of the sources will be used preferentially until depleted. For example, if *L. monocytogenes* is grown with glucose and one other sugar, such as cellobiose, the glucose will be utilized first and the cellobiose second. This phenomenon is termed diauxic growth. When glucose is present, it prevents transcription of genes and therefore utilization of other carbon sources that might be present. After the preferred carbon source (ex. glucose) is gone, the genes to utilize the second carbon source present are then upregulated and expressed allowing utilization of the second carbon source (Stulke et. al., 1998). In summary, diauxic growth, also known as catabolite repression allows the cell to control the expression of genes that are not needed when an easily metabolized carbon source is present. Diauxic growth or catabolite repression is accomplished by phosphorylation at Ser 46 of the HPr protein. Ser 46 is phosphorylated by an ATP dependent kinase that is allosterically activated by key metabolites such as fructose 1,6-bisphosphate, an intermediate of glucose metabolism (Saier et. al., 1995). Thus, the phosphorylation at ser 46 inhibits phosphorylation at his 15, which in turn down regulates the PTS (Christensen et. al., 1999). Once HPr is phosphorylated at ser 46 it then binds with another protein, CcpA or catabolite control protein A. The binding of CcpA and HPr occurs in the presence of the glycolytic intermediate fructose 1,6 bisphosphate. This complex binds to DNA regions called catabolite repression elements (CRE's). CRE's are *cis*-acting palindromic elements and can be found upstream of the promoter region, within the coding sequence, or within the promoter region of genes. The binding of

this complex to CRE's mediates repression by preventing binding of the RNA polymerase at the transcriptional start site, causing a transcriptional roadblock, or interfering with interaction of RNA polymerase with its activator, thus preventing transcription of genes that are not needed (Ramseier et.al., 1995; Fujita et. al., 1995; Martin-Verstraete et. al., 1995). The HPr-CcpA complex was initially considered as a possible regulator of virulence genes, however CcpA mutant analysis revealed that it was not involved with repression of virulence genes (Behari et. al., 1998).

In gram negative bacteria, catabolite repression is controlled in an entirely different manner. The cAMP receptor protein or CRP is responsible for catabolite repression and is dependent on cyclic AMP or cAMP. The levels of cAMP in the cytoplasm of the cell vary depending on growth rate and the carbon source present. When cAMP is present, it binds to CRP and causes a conformational change in the protein. This complex, cAMP-CRP, then binds to promoters to recruit RNA polymerase and promote the transcription of target genes. cAMP is made by the biosynthetic enzyme, adenylate cyclase. Adenylate cyclase is stimulated by the glucose-specific enzyme II ($EIIA^{Glc}$) of the PTS. When $EIIA^{Glc}$ is phosphorylated it allosterically activates adenylate cyclase to make cAMP, which in turn stimulates CRP (Saier et. al., 1997). In addition, stimulation of adenylate cyclase by cAMP requires the presence of HPr (Peterofsky et al., 1995). This occurs when glucose is not present and thus promotes the transcription of genes needed to utilize other substrates that are present. In the presence of glucose, $EIIA^{Glc}$ loses its phosphate and then

inhibits catabolic enzymes and responsive permeases, such as the lactose permease, which transports lactose into the cell. Additionally, it cannot stimulate adenylate cyclase. This mechanism is called inducer exclusion (Stulke et. al., 1999). In summary, the cAMP-CRP complex binds to the promoter regions to induce transcription of target genes. Thus, catabolite repression in gram negative bacteria is under positive regulation. In contrast, in gram positive bacteria the HPr-CcpA complex binds to the promoter region to prevent transcription of target genes, thus catabolite repression is under negative regulation (Saier, et. al., 1995).

Recently a protein similar to CcpA has been found in *Bacillus* and named CcpB. CcpB has been shown to be involved with carbon catabolism in *Bacillus* (Chavaux et. al., 1998). Its presence is suspected in *L. monocytogenes* as well, where it could potentially serve a regulatory role (Behari et. al., 1998). Other modes of carbohydrate mediated virulence gene regulation could be occurring, including modification of activity of the PrfA protein, the regulator of virulence gene expression.

Another mode of regulation for the phosphotransferase system involves phosphorylation of transcriptional regulators by components of the PTS system. According to Stulke et. al., several antiterminators and activators contain a duplicated conserved sequence called the PTS regulation domain (PRD). All regulators of this family contain two copies of the PRD, and these domains can control transcriptional regulators both positively and negatively causing either induction or catabolite repression of operons. The HPr protein exerts this