REGULATION OF SWARMING MOTILITY BY POLYAMINES IN *ESCHERICHIA COLI* AND ENZYMATIC ASSAY OF D-MANNOSE FROM URINE

by

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This dissertation is dedicated to my family.

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by

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DISSERTATION

Presented to the Faculty of

The University of Texas at Dallas

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY IN

MOLECULAR AND CELL BIOLOGY

THE UNIVERSITY OF TEXAS AT DALLAS

December 2018

ACKNOWLEDGMENTS

This dissertation would not have been complete without the help of so many people in so many ways. I would like to express my deepest gratitude to all those people.

I am greatly indebted to my mentor, Dr. Reitzer for giving me an opportunity to work in his lab and discover the joys of being a scientist. His contribution in my research was immense and I will always be grateful to him for making me the researcher I am today.

I would also like to thank Dr. Barbara Schneider for all her help and guidance throughout. There is so much that I learnt from her about the little details that make a huge difference in the longer course of your research. I want to thank my committee members, Dr. Spiro, Dr. Miller and Dr. Palmer, for their great inputs and continuous support. Also, I was lucky to have very knowledgeable and helping lab members. This journey became very memorable because of their company and motivation. I am very grateful to all of them.

In the end I want to thank my friends, Sindhu, Lalitha and Anvit, for believing in me that I could do this, even when I did not believe in myself. None of this would have been possible without the unconditional love and care from my family. A big thank you to all of them for being my constant pillars of strength.

August 2018

REGULATION OF SWARMING MOTILITY BY

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The University of Texas at Dallas, 2018

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Swarming is a type of surface motility behavior exhibited by a few bacterial families. Polyamines are aliphatic cations known to modulate protein synthesis and impact gene expression and we studied the role of these polyamines in modulating swarming in *Escherichia coli*. There are nine enzymes that synthesize polyamines (putrescine, spermidine and cadaverine) in *E. coli*. Chapter 2 describes the requirement of putrescine in regulating swarming motility in *E.coli*. Analysis of different genetic knockouts in the polyamine anabolic pathways showed that putrescine, but not spermidine, is important for swarming. Putrescine transport and catabolism were also shown to be critical for this type of surface movement behavior. Evidence is presented that suggests that one function of putrescine is to provide resistance to oxidative stress during swarming.

Urinary tract infections are one of the most common bacterial infections in humans. Many studies show that D-mannose ingestion diminishes the frequency of urinary tract infections, perhaps by blocking the binding of pathogenic bacteria to the bladder epithelium. We want to

test whether women susceptible to urinary tract infections have low urinary D-mannose, and whether oral D-mannose increases urinary D-mannose. However, there is no published technique to specifically and sensitively measure D-mannose in urine. I developed a reliable, precise, sensitive, and fast enzymatic method to measure D mannosuria in women, and this assay is described in Chapter 3. This reliable method of measuring D-mannosuria may prove useful to determine the efficacy and optimize the intake (dose, intake frequency, elimination ratio) of D-mannose in women suffering from recurrent urinary tract infections.

Chapter 4 discusses the results from a published article where it is shown that CyuA is a major anaerobic cysteine catabolism enzyme in both *E. coli* and *S. enterica*. My contribution was to examine regulation of *cyuA* in *E. coli*. In this paper, we presented evidence that CyuA was present in the Last Universal Common Ancestor (LUCA).

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ACME	Arginine catabolic mobile element
cAMP	Cyclic adenosine mono phosphate
COG	Clusters of orthologous groups
E. coli	*Escherichia coli*
GAD	Glutamate decarboxylase
GBS	Group B streptococcus
GDAR	Glutamate carboxylase dependent acid resistance
G6PDH	Glucose-6-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HK	Hexokinase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani growth medium
ManA	Mannose-6-phosphate isomerase
NADP	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
PGI	Glucose-6-phosphate isomerase
P. mirabilis	*Proteus mirabilis*
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
rRNA	Ribosomal ribonucleic acid

RSSH	Hydropersulphides
S. enterica	*Salmonella enterica*
S. pneumoniae	*Streptococcus pneumoniae*
TCA	Tricarboxylic acid
tRNA	Transfer ribonucleic acid
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infections

CHAPTER 1

AN INTRODUCTION OVERVIEW OF POLYAMINES AND SWARMING

1.1 Introduction to Polyamines

The polyamines putrescine, spermidine, spermine and cadaverine are aliphatic cations with two or more primary or secondary amines -NH_2 or –NH- (Chattopadhyay *et al.*, 2009) (Figure 1.1). Polyamines are peculiar because of their polybasic character which allows them to interact with acidic components much more efficiently than other positive ions like Na+, Mg++, or monoamines (Tabor & Tabor, 1984). Polyamines are known to interact with nucleic acids and phospholipids playing a role in gene regulation by affecting chromosomal and ribosome structure, mRNA interactions and protein and nucleic acid elongation rates (Sakamoto *et al.*, 2015, Yoshida *et al.*, 1999). The pathways for their synthesis were first studied in prokaryotes but the mechanism of synthesis is quite similar in eukaryotes too (Tabor & Tabor, 1984). Mutants that lack polyamine biosynthetic pathways have been studied a lot in the last twenty years to understand the role and mechanism of how polyamines work.

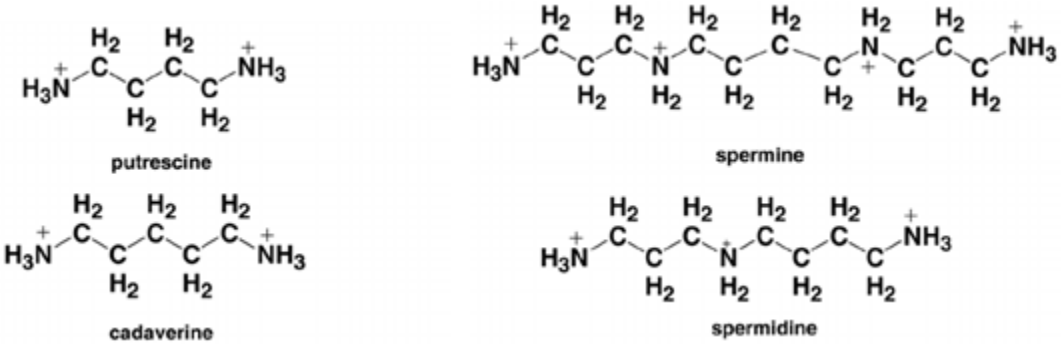

Figure 1.1: Chemical structures of polyamines

1.2 Polyamines in bacteria

Two of the most common polyamines found in bacteria are putrescine and spermidine (Tabor & Tabor, 1985). *E. coli* has high concentrations of putescine and spermidine, and a lower concentration of cadaverine (Herbst *et al.*, 1958). Nine genes synthesize these polyamines (Figure 1.2). Two isozymes of ornithine decarboxylase (SpeC and SpeF) and a two-step pathway of arginine carboxylase (SpeA) and agmatinase (SpeB) synthesize putrescine in micro-organisms (Tabor *et al.*, 1983). There are nine genes for synthesizing polyamines in *E. coli* (Figure 1.2). Bacteria also have multiple active transport systems for polyamine uptake (Holtje, 1978, Igarashi *et al.*, 2001, Igarashi & Kashiwagi, 1999).

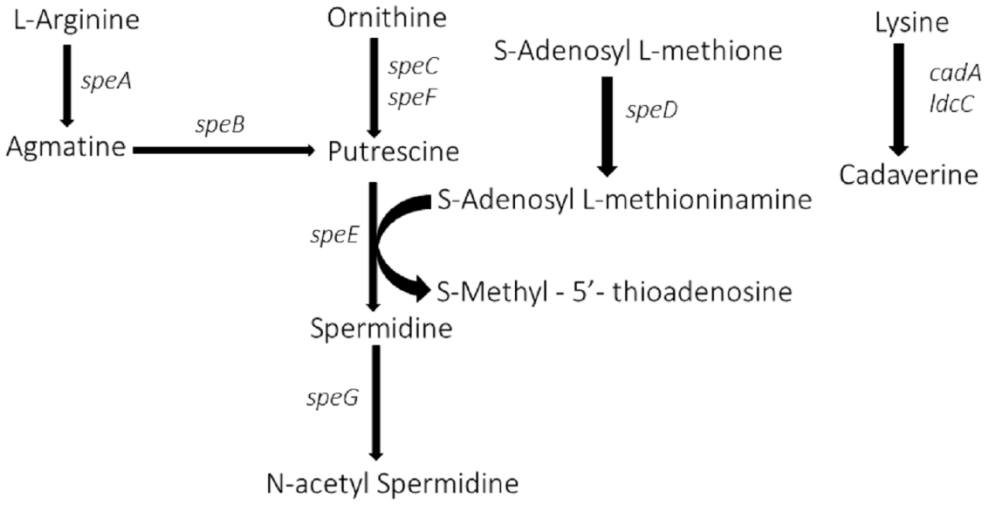

Figure 1.2: Pathways of polyamine synthesis in *E.coli*

1.3 Polyamines as global regulators of gene expression

Polyamines control the expression hundreds of genes in *E. coli* (Yoshida *et al.*, 1999, Igarashi & Kashiwagi, 2000, Capp *et al.*, 1996, Terui *et al.*, 2009). This group of genes has been called the 'polyamine modulon' (Yoshida *et al.*, 2004). These genes are involved in growth control and viability, for example by modulating protein synthesis (Igarashi & Kashiwagi, 2015). Control of these genes often occurs by enhancing levels of transcription factors at the translational level (Yoshida *et al.*, 2004). They have recognized the following genes as directly controlled by polyamines: *oppA, cya, rpoS, fecI, fis, soxR, emrR ,gshA, katE, katG, hns, cra, rpoE* and many more (Sakamoto *et al.*, 2015, Yoshida *et al.*, 2004, Yoshida *et al.*, 1999).

It was also showed that most of the polyamines exist as a Polyamine-RNA complex (Igarashi & Kashiwagi, 2015, Miyamoto *et al.*, 1993). This results in affecting translation at various stages. They are involved in proper assembly of 30S ribosomal subunits (Algranati *et al.*, 1975, Echandi & Algranati, 1975a, Echandi & Algranati, 1975b). Polyamines also induce conformational changes in RNA through the binding of 2 mol of spermidine and 4 mol of putrescine to each 100 nucleotide-long RNA in *E. coli* (Miyamoto *et al.*, 1993). Fis, an important member of polyamine modulon, regulates the expression of many genes involved in translation (rRNA and tRNA genes) (Bradley *et al.*, 2007). Polyamines have a great role to play in cell viability and growth, especially at the translational level. A similar type of modulation has also been observed in eukaryotic cells but a detailed mechanism has not been explained yet (Igarashi & Kashiwagi, 2015). Some of these results also strongly suggest that polyamines play an important role not only in modulating growth, but also help bacteria fight oxidative an acid stress (Sakamoto *et al.*, 2015, Chattopadhyay & Tabor, 2013).

1.4 Specific functions of polyamines in micro-organisms

Because polyamines affect the expression of hundreds of genes, it is not surprising that polyamines are associated with a broad range of biological functions (Di Martino *et al.*, 2013, Shah & Swiatlo, 2008, Wortham *et al.*, 2007). This sections describes some specific functions that have been firmly established.

Acid resistance. *E. coli* maintains its internal pH between 7.4 and 7.8 during aerobic growth with external pH ranging from 5.0–9.0 (Zilberstein *et al.*, 1984). Polyamines have been reported as being key mediators in retorting to acidic stress in bacteria. They are responsible for inducing a glutamate carboxylase dependent acid resistance (GDAR) response in *E.coli* (Chattopadhyay & Tabor, 2013). Important genes involved in acid resistance response (*gadA* and *gadB*) are regulated via polyamines by controlling cAMP levels (Jung & Kim, 2003a). A polyamine deficient mutant was completely devoid of the GAD activity and was very sensitive to low pH if exogenous polyamines were not provided (Jung & Kim, 2003a). This mutant had higher cAMP levels than wild type, and cAMP negatively regulated the expression of *gadA* and *gadB* (Jung & Kim, 2003a). Polyamines also increase the synthesis of the *gadE*, an important regulator of this GDAR system, by increasing the *rpoS* levels (Chattopadhyay *et al.*, 2015). A similar regulation for acid resistance was observed in *Salmonella* where the *cad* operon was shown to be important in response to low pH (Lee *et al.*, 2007).

Synthesis of siderophores. The production of iron scavenging molecules known as siderophores has been known to be of importance for virulence in many pathogens (Litwin & Calderwood, 1993). A class of these siderophores have polyamines as their backbones (Wortham *et al.*, 2007). *Vibrio cholarae* is unable to form vibriobactin in absence of norspermidine (Griffiths *et al.*,

1984). Similarly, a mutant strain of *Bordetella*, unable to produce putrescine, is deficient in alcaligin, an important siderophore (Brickman & Armstrong, 1996).

Biofilm formation. Polyamines have been linked to biofilm levels in *Y. pestis,* a pathogen that causes plague (Patel *et al.*, 2006). Putrescine levels were correlated to that of the biofilm levels and higher concentrations of putrescine were required in comparison to that needed for the bacteria to grow (Patel *et al.*, 2006). A polyamine transport related protein PotD was also shown important for biofilm formation in *Vibrio cholerae* (Karatan *et al.*, 2005).

Resistance to oxidative stress. Many pathogens are known to encounter oxidative stress *in vivo*. Reactive oxygen species resulting from this stress can cause DNA damage and mutations. Polyamine deficient mutants are more sensitive to oxygen as compared to wild type *E. coli* (Chattopadhyay *et al.*, 2003, Jung & Kim, 2003b). This effect is shown to be reversed by adding exogenous polyamines (Jung & Kim, 2003b). Another study from our lab also showed that a polyamine catabolism mutant has increased sensitivity towards oxidative stress, temperature as well as antibiotics (Schneider *et al.*, 2013).

Polyamines regulate a number of genes involved in oxidative stress response including catalase, hydroperoxidase, and polyphosphate kinase (Murata *et al.*, 1988, Sakamoto *et al.*, 2015). They are also being considered for being a very effective therapeutic agent in order to treat diseases where production of reactive oxygen species is very high due to inflammation, for example, arthiritis, asthma and even cancer (Hussain *et al.*, 2017). Polyamines were shown to be significantly anti-inflammatory in acute as well as chronic models of inflammation and this effect was attributed to their anti-oxidant properties (Lagishetty & Naik, 2008). These effects are

being taken so seriously that a recent article in Science discusses the possibility of polyamines as agents that extend life and prevent age-associated diseases (Madeo *et al.*, 2018).

1.5 Polyamines in eukaryotes

Polyamines are known to have fundamental roles in various biological systems including their association in growth and proliferation of eukaryotic cells (Bachrach *et al.*, 1983). Depletion of cellular polyamines, spermidine and spermine, causes a total arrest in translation and growth in mammalian cells (Mandal *et al.*, 2013). The rate limiting enzyme in polyamine synthesis has been shown to be of great importance in cell migration and helps with epidermal wound healing also (Kheng *et al.*, 2018). They have been known to be associated with several critical diseases like leukemia, brain tumors, cystic fibrosis, diabetes, sickle cell anemia as well as neoplasia (marton, 1977, park & igarashi, 2013, mastracci *et al.*, 2015). Polyamine pathways also contribute to the pathogenesis of parkinson's disease (lewandowski *et al.*, 2010). Polyamine analogs that increased the activity of a specific polyamine catabolism enzyme showed decreased parkinson's disease histopathology (lewandowski *et al.*, 2010). Changes in polyamine levels have been linked with aging and diseases and the levels drop constantly with age (kiechl *et al.*, 2018, madeo *et al.*, 2018, schwarz *et al.*, 2018). These studies might be important in terms of dealing with prevention of these age associated diseases. Scientists are also working on novel therapies to treat cancer by targeting polyamine pathways. The use of polyamines synthesis inhibitors has been shown important clinically in cancer chemotherapy (aziz *et al.*, 1996, murray-stewart *et al.*, 2016).

1.6 Polyamines and virulence

In addition to affecting growth and several physiological functions, polyamines are essential for bacterial virulence and pathogenesis (Di Martino *et al.*, 2013). More studies in this field are not only leading to a better understanding of polyamine functions, but also have a great potential towards progress in developing new anti-bacterial therapeutic methods.

Alterations in polyamine metabolism affect a lot of bacterial species' ability to cause infections and fight the host immune response. The capacity to survive in macrophages in *Shigella* is facilitated by high accumulation of spermidine (Barbagallo *et al.*, 2011). It has adapted itself to undergo certain genetic changes that has altered its polyamine profile to better suit its ability to endure the stress and maintain fitness inside the host (Barbagallo *et al.*, 2011). Putrescine is also involved in restoring virulence in some of *Shigella flexineri* mutants by altering gene expression of some of the virulence genes (Durand & Bjork, 2003).

Staphylococcus aureus infections are difficult to treat due to resistance to multiple antibiotics (Chambers & Deleo, 2009, Cosgrove *et al.*, 2009). A specific methicillin-resistant strain, USA-300, is responsible for a lot of related infections all over the world (Diep *et al.*, 2006). Generally *S.aureus* does not have genes for synthesizing polyamines and exogenous polyamines kill the bacteria at concentrations which are normally found within the host (Joshi *et al.*, 2011). However, USA-300 clone is resistant to spermidine (Joshi *et al.*, 2011). The unique thing about this clone is that it has a rare arginine catabolic mobile element (ACME) which is not found in other resistant clones (Diep *et al.*, 2006). This ACME element has two genetic loci which are involved in polyamine metabolism- *arc* system and *speG* gene (Diep *et al.*, 2006, Joshi *et al.*, 2011). The *arc* system encodes a constitutive arginine deaminase which converts extracellular

arginine to ornithine, a key intermediate in the synthesis of spermidine and spermine. Polyamines resistance was shown in non USA-300 clones by introducing a functional *speG* gene in them (Joshi *et al.*, 2011).This study also showed that polyamines were involved in synchronizing the healing response in *S.aureus* infections.

In *S. pneumoniae*, silencing of PotD which is a polyamine transport protein, significantly decreases the progression of disease in mouse models (Ware *et al.*, 2006). Besides *potD*, there are other genes associated with polyamines which are also involved in causing disease. In a recent study, it was shown that strains which do not synthesize polyamines have reduced virulence and expression of many proteins involved in virulence and replication are downregulated (Shah *et al.*, 2011).

Gene expression studies in *Salmonella typhimurium* have also shown upregulation in putrescine and spermidine biosynthesis genes during infection of epithelial cells and macrophages (Eriksson *et al.*, 2003, Hautefort *et al.*, 2008).

In summary, accumulating evidence stresses the role of polyamines in bacterial pathogenicity.

1.7 Introduction to Swarming

There are different kinds of motilities observed in bacteria (Figure 1.3). They use their flagella to move in liquid cultures or over surfaces. Flagella dependent movement in liquid media is referred to as swimming, and flagella dependent movement over surfaces is called swarming. Twitching and gliding are some other known forms of motion in bacteria.

Twitching is pili mediated whereas in gliding, bacteria form surface adhesions and push their bodies forward.

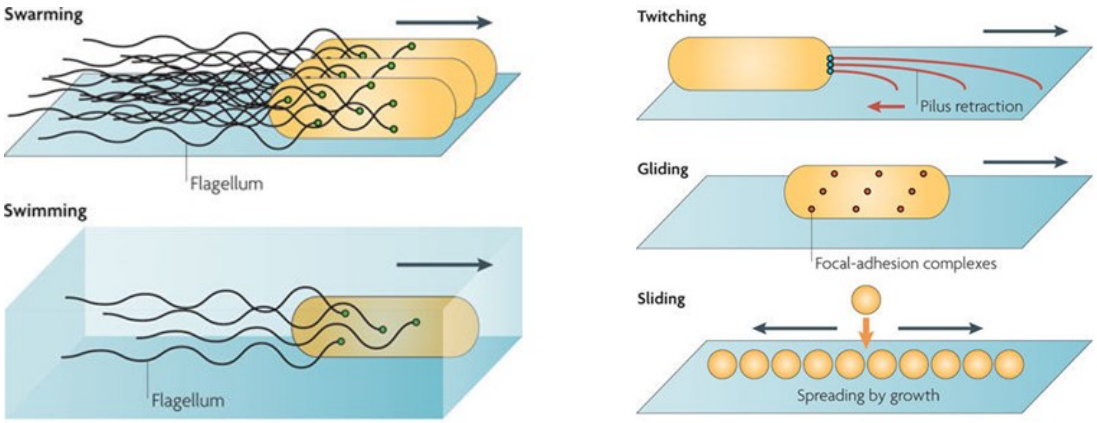

Figure 1.3: Types of motilities found in bacteria. Adapted by permission from Springer Nature, Nature Reviews Microbiology (Kearns, 2010), ©2010 Macmillan Publishers Limited. All rights reserved.

Swarming is a flagella mediated form of migration in which the cells differentiate from planktonic to swarmer cells. These swarmer are cells are hyper-elongated and multi-flagellated. These type of movement is powered by rotating flagella and formation of rafts. The bacteria come together as groups and push forward to move in a particular direction (Kearns & Losick, 2003, Young *et al.*, 1999, Eberl *et al.*, 1999). It is quite unclear as to why these rafts are formed. Only three families in the bacterial domain seem to demonstrate swarming (Figure 1.4).

Although it seems like swarming is conserved very narrowly, the general observation is that swarming is eliminated in laboratory strains due to domestication (Kearns & Losick, 2003, Patrick & Kearns, 2009). It is possible that these strains disappeared because in laboratory setting, stable and small compact colonies were selected over bacteria that would just move all over the surface of the plate.

A study in *Bacillus* showed that the swarming in laboratory strains was stunted and only the natural isolates were able to swarm over a semi-solid surface (Ghelardi *et al.*, 2007). These natural isolates produced toxins and this production was coupled with the flagella synthesis in

those particular swarming strains. A similar study was done in *Salmonella* and it was shown that the most robust swarmer strains were the least domesticated (Kim & Surette, 2005).

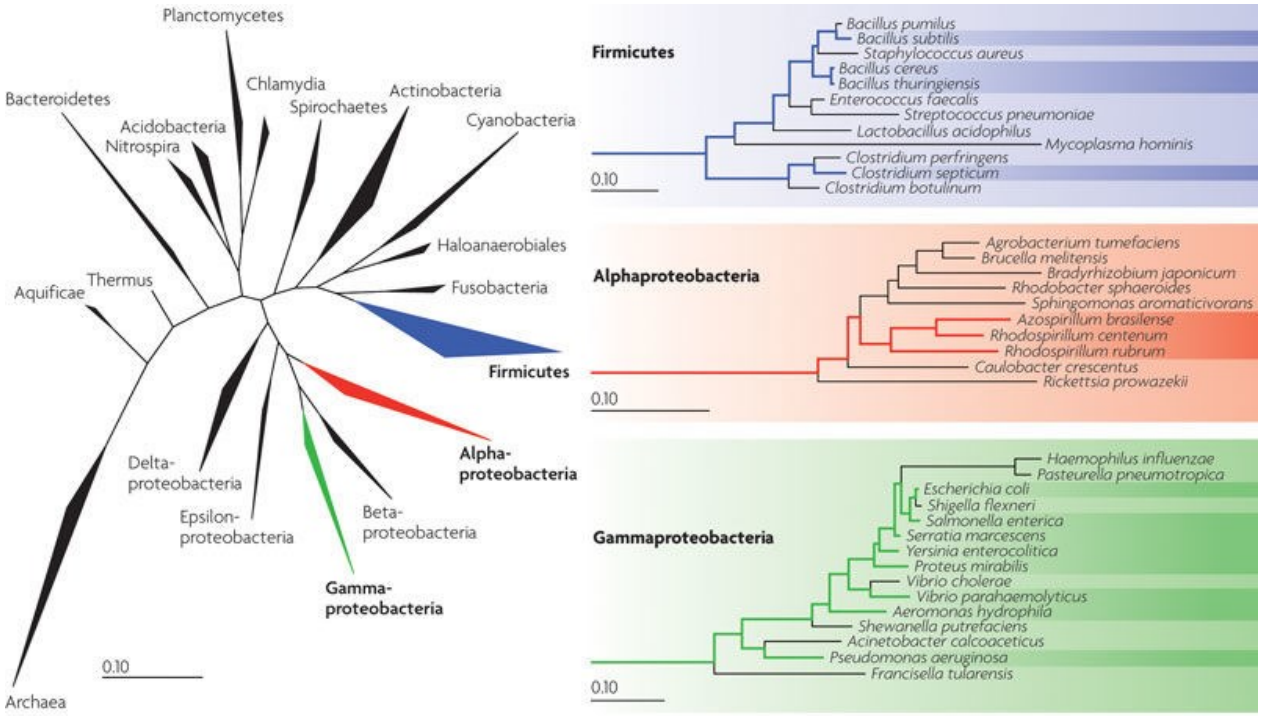

Figure 1.4: Phylogenetic distribution of swarming motility - The highlighted species are the ones able to exhibit swarming motility. These results are based on their phylogeny results with regard to the 16s ribosomal RNA gene. Reprinted by permission from Springer Nature, Nature Reviews Microbiology (Kearns, 2010), ©2010 Macmillan Publishers Limited. All rights reserved.

1.8 Swarming patterns

Swarming bacteria make different types of patterns on the solid media surfaces (Figure 1.5). The patterns vary depending on strains and environmental conditions. The first kind is a featureless swarm in which the cells grow and spread evenly from the center outwards in a uniform fashion. The most studied and famous pattern is the bull's eye or an oscillatory feature. This is formed by alternating cycles of differentiated and de-differentiated phases of movement and growth

respectively. The zone of growth is termed as a 'zone of consolidation' followed by 'zones of migration'. Such a pattern is observed in some strains of *Proteus mirabilis* and our lab strain of *E.coli*. These two zones or phases are very distinct, phenotypically as well as metabolically. In a study in *P. mirabilis*, it was shown that the cells in 'swarmer' phase are elongated and hyper-flagellated and the cells in 'consolidation' phase were short and had very little visible movement when looked under a microscope (Pearson *et al.*, 2010). When gene expression was studied in these two phases, more than 500 genes were shown to be upregulated in the consolidation phase while only nine genes showed increased transcription during swarm phase (Pearson *et al.*, 2010). Although, the swarm phase is the more dynamic one, it is surprising that so many genes were upregulated during the consolidation phase. But on the other hand, it is understandable because cells prepare for the next swarm cycle during this phase collecting all the important proteins in order to proceed further (Pearson *et al.*, 2010).

There is another pattern called as 'dendrites' or tendrils. In this pattern, the cells move out from a common point of origin and branch out (Figure 1.5c). This kind is observed in *P. aeroginosa* and is thought to be based on the presence of surfactants secreted from the bacteria (Caiazza *et al.*, 2005). Some bacteria also form vortices and are sometimes referred as wandering colonies (Henrichsen, 1972). The non-swarming phenotypes can acquire suppressor mutations and be able to swarm and move rapidly all over agar surfaces (Kearns *et al.*, 2004, Kearns & Losick, 2005). These suppressors can then be isolated and studied for swarming and genetic changes to be able to understand swarming better.

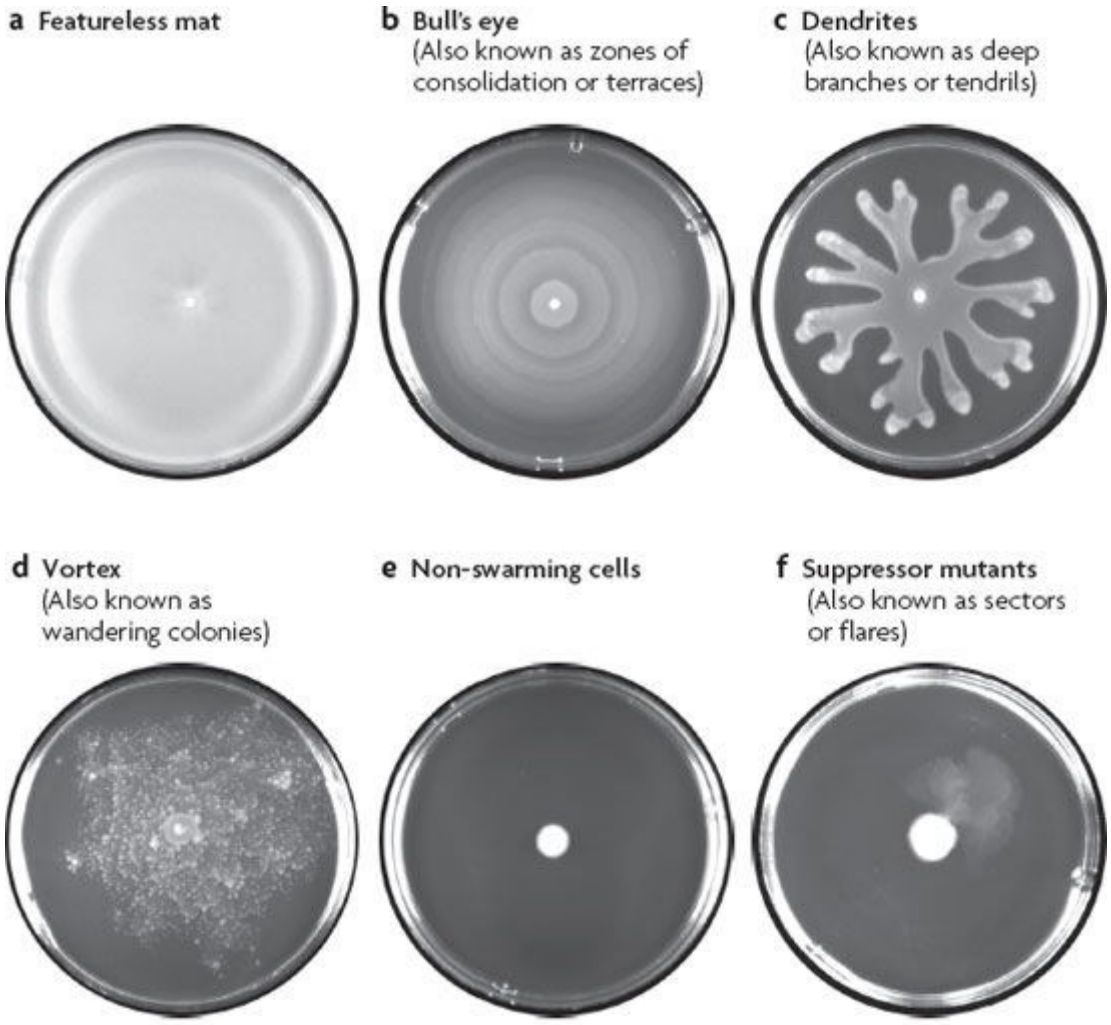

Figure 1.5: Various colony patterns formed by swarming bacteria. Reprinted by permission from Springer Nature, Nature Reviews Microbiology (Kearns, 2010), ©2010 Macmillan Publishers Limited. All rights reserved..

1.9 Requirements for swarming

Flagella

This is one of the most important and the most studied requirements for swarming. Most of the bacteria that swarm have flagella all over their surface (Kearns & Losick, 2003, Hoeniger, 1965, Harshey & Matsuyama, 1994). It is called as a peritrichous arrangement. These flagella come

together when they rotate and generate force more efficiently in viscous environments (Berg & Turner, 1979, Ulitzur & Kessel, 1973, Schneider & Doetsch, 1974). However not all swarming bacteria have this kind of flagellar arrangement. *Pseudomonas aeruginosa*, which has one polar flagellum, make an alternative motor in order to propel the organism to move over a surface (Doyle *et al.*, 2004, Toutain *et al.*, 2005). It has been shown that organisms with mutations in flagellar synthesis genes reduce or even eliminate surface motility (Young *et al.*, 1999, Eberl *et al.*, 1999, Hay *et al.*, 1997, Kearns *et al.*, 2004).

Surfactants

Surfactants are compounds that reduce surface tension between two surfaces. A lot of bacteria synthesize and secrete these compounds in the form of a watery, clear layer, to reduce friction (Kearns & Losick, 2003, Alberti & Harshey, 1990, Calvio *et al.*, 2005). Some bacteria need a special kind of agar because of their inability to produce the surfactant themselves. This special agar helps the bacteria to overcome the frictional force and move over the surface (Zhang *et al.*, 2010, Harshey & Matsuyama, 1994, Matsuyama *et al.*, 1995). The production of these surfactants is generally regulated by quorum sensing systems (Ochsner & Reiser, 1995, Eberl *et al.*, 1996, Magnuson *et al.*, 1994). These surfactants are effective only at a certain, higher concentration so it makes sense for them to be regulated by quorum sensing genes.

Rafts

Swarmer bacteria move side by side in groups termed as rafts (Young *et al.*, 1999, Eberl *et al.*, 1999, Alberti & Harshey, 1990). An image from a swarm front of *Proteus mirabilis* from a scanning electron microscope showed rafting very extensively (Jones *et al.*, 2004). Similar to hyper-flagellation, the reason for rafting is unclear.

1.10 Swarming and virulence

In addition to physiological changes like cell elongation and hyper flagellation, it has also been observed that swarming is an adaptation which leads to changes in gene expression and regulation, showing that swarming is just not another form of motility, but a complex phenomenon (Harshey, 2003, Rather, 2005). To understand this further, a microarray study was done in *Pseudomonas* in which the cells were assayed from the leading edge of the swarm zones. The study showed major shifts in gene expression, along with up-regulation in a large number of virulence related genes (Overhage *et al.*, 2008). This up-regulation in virulence related genes can be an adaptation or a benefit for swarmer cells in order to spread and colonize in new surroundings. They clearly demonstrated that the swarmer cells undergo this adaptation by going into a unique physiological state which is very different from their vegetative or biofilm state. This state helps the bacteria to inhabit and infect the host. In another study, it was shown that the ability of *Proteus* to invade urothelial cells was related to its swarming and related genes (Allison *et al.*, 1992a, Allison *et al.*, 1992b). *Proteus* secretes intracellular urease, extracellular haemolysin and metalloproteases, which are harmful to the host (Allison *et al.*, 1992b). These secretions play a very crucial role in their pathogenicity. Swarming mutants showed reduced haemolytic, ureolytic and invasive phenotypes. Interestingly, there were mutants with various aberrant patterns of swarming migration which were able to form swarm cells. They produced wild-type activities of haemolysin, urease and protease, but were unable to enter the urothelial cells efficiently.

Swarming has also been linked to antibiotic resistance in several bacterial strains (Kim *et al.*, 2003, Kim & Surette, 2003, Lai *et al.*, 2009, Gooderham *et al.*, 2008). These bacteria showed

higher resistance as compared to their vegetative counterparts (Lai *et al.*, 2009). In this study, they performed filter disc assays on *P.aerugenosa, E.coli, Serratia marcescens, Burkholderia thailendensis* and *Bacillus subtilis*. All five bacteria showed resistance against more than ten antibiotics from six different antibiotic families (beta-lactams, aminoglycosides, peptides, macrolides, quinolones and trimethoprim) (Lai *et al.*, 2009). All these studies suggest that motility of these cells might be one of their features for survival in different environmental conditions.

1.11 Common metabolic features between different species that exhibit swarming

Genes are differentially regulated during swarming across different species that exhibit this type of behavior. It can be claimed that there are a lot of differences between these species, however a lot of parallels can also be drawn. We have already discussed the different types of swarm patterns found in various species, but it should also be noted that the requirement of a specific agar concentration is also a peculiar property. *Proteus* swarms on surfaces containing upto 2% agar whereas *Salmonella* and *E. coli* require a much lower concentration of agar (between 0.4 to 0.8%) (Kearns, 2010). Even though they exhibit different patterns and different surface requirements of swarming, when you look carefully into the gene expression and metabolic profiles of these different organisms, a lot of common factors can be observed. TCA cycle genes were upregulated during consolidation phase in *Proteus mirabilis* (Pearson *et al.*, 2010) as well as in actively swarming *Salmonella typhimurium* when compared to its swimming counterparts (Kim & Surette, 2004). A knockout mutant screening study in *E.coli* also reported a similar requirement of TCA cycle genes for swarming (Inoue *et al.*, 2007). Amino acid uptake was also increased during swarming in *Proteus* (Armitage, 1981, Pearson *et al.*, 2010). Biosynthesis and

transport of amino acids was shown to be important in *Salmonella* and *E.coli* too (Kim & Surette, 2004, Inoue *et al.*, 2007). Glucose metabolism was also important for *E.coli* and *Salmonella*. Although there is no requirement of glucose for swarming in *Proteus* (Pearson *et al.*, 2010), the glycolysis mutants exhibited a severe swarming defect (Alteri *et al.*, 2012). Iron uptake and metabolism was shown to be essential for swarming in Salmonella (Wang *et al.*, 2004). Many mutants lacking genes involved in iron acquisition showed repressed swarming in *E.coli* (Inoue *et al.*, 2007). Another common signal that influences swarming is glutamine. *Proteus* is able to swarm on minimal media in the presence of glutamine (Allison *et al.*, 1993). However the requirement is not understood completely, genes for glutamine synthesis are also important for *Salmonella* and *E.coli* (Kim & Surette, 2004, Inoue *et al.*, 2007).

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CHAPTER 2

REQUIREMENT OF PUTRESCINE FOR THE SWARMING MOTILITY OF *ESCHERICHIA COLI*

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Submitted to *Molecular Microbiology* (Manuscript number MMI-2018-17202).

2.1 Summary

Polyamines are biologically ubiquitous compounds that modulate protein synthesis and globally affect gene expression. We examined the relation between polyamines and swarming motility in an *E. coli* strain that exhibited oscillatory swarming. Swarming required two pathways of putrescine synthesis, ornithine decarboxylase-dependent (SpeC/SpeF) and arginine decarboxylase-dependent (SpeA-SpeB), but did not require spermidine. Putrescine supplementation and gene complementation restored swarming in polyamine-deficient mutants. Putrescine transport and catabolism were also shown to affect swarming motility. Genes of oxidative stress, but not genes of acid resistance, partially restored swarming of a polyamine deficient strain, which suggests that one function of putrescine is to provide resistance to oxidative stress during swarming. Despite several differences between the robust swarming of *P. mirabilis* and the much weaker swarming of *E. coli*, both organisms require several of the same pathways and metabolites for swarming, including the polyamine putrescine.

2.2 Introduction

Polyamines are flexible aliphatic cations that are found in virtually all organisms. *E. coli* contains putrescine (1,4-diaminobutane) which is the most abundant polyamine, spermidine (N-(3-aminopropyl)-1,4-diaminobutane), and cadaverine (1,5-diaminopentane) (Tabor & Tabor, 1984). Nine enzymes synthesize polyamines in *E. coli*; the pathways and enzymes are often redundant (Figure 2.1A). Putrescine is synthesized from ornithine by constitutive and inducible ornithine decarboxylases, SpeC and SpeF, respectively, or from arginine by arginine decarboxylase (SpeA) and agmatinase (SpeB) (Tabor & Tabor, 1985). SpeE adds a propylamino group to putrescine to form spermidine. In a minimal medium, a completely polyamine deficient

Escherichia coli strain does not grow anaerobically, and grows about three times slower than wild type aerobically (Chattopadhyay *et al.*, 2009).

Polyamines interact with nucleic acids and phospholipids, and can affect chromosome and ribosome structure, ribosome-mRNA interactions, and protein and nucleic acid elongation rates (Igarashi & Kashiwagi, 2000, Yoshida *et al.*, 1999). Polyamines modulate, but are not absolutely required for, protein synthesis (Igarashi & Kashiwagi, 2015) and expression of hundreds of genes (Yoshida *et al.*, 2004). A major mechanism of polyamine regulation is translational control of 18 genes, including four of the seven σ factors – σ^{18} (FecI), σ^{24} (RpoE), σ^{38} (RpoS), and σ^{54} (RpoN); three histone-like proteins (Fis, H-HS, and StpA); and four major transcription factors (CpxR, Cya, Cra, and UvrY) (Yoshida *et al.*, 2004, Igarashi & Kashiwagi, 2015, Sakamoto *et al.*, 2015, Schneider *et al.*, 2013). Through these proteins, polyamines indirectly affect transcription of hundreds, perhaps thousands, of genes.

Polyamines have been implicated in a few physiological functions, including iron limitation and biofilm formation (Brickman & Armstrong, 1996, Foster, 2004, Price *et al.*, 2004, Richard & Foster, 2003, Balasundaram *et al.*, 1994, Chattopadhyay *et al.*, 2015, Chattopadhyay *et al.*, 2003, Chattopadhyay & Tabor, 2013, Tabor & Tabor, 1985). Certain classes of bacterial siderophores utilize polyamines as their backbone structure. Mutants unable to produce putrescine in *Bordetella pertussis*, a pathogen that causes whooping cough, are unable to produce a siderophore called alcaligin (Brickman & Armstrong, 1996). Furthermore, polyamines have been reported to be involved in the formation of biofilms in Y*ersenia pestis* (Patel *et al.*, 2006). Biofilm levels correlated with putrescine levels in this study. In *Vibrio cholerae*, the polyamine

norspermidine plays a role in biofilm formation in a NspS-dependent manner (Goforth *et al.*, 2013). NspS was proposed to sense polyamines as an environmental signal for biofilm synthesis.

Polyamines are also involved in stress responses, but the effect of polyamines is complex. For *E. coli*, polyamine synthesis is required for responses to oxidative stress and acid stress (Sakamoto *et al.*, 2015, Chattopadhyay & Tabor, 2013). However, polyamine catabolism is also important during stress because high concentrations of polyamines are harmful (Apirakaramwong *et al.*, 1999, Munro *et al.*, 1972, Limsuwun & Jones, 2000, Fukuchi *et al.*, 1995), and polyamine catabolism is a core response to several stresses (Schneider *et al.*, 2013).

Polyamines affect different aspects of bacterial pathogenesis (Di Martino *et al.*, 2013). Alterations in polyamine metabolism affect *Shigella flexneri* (survival in macrophages), *Streptococcus pneumoniae* (resistance to stress), *Vibrio cholerae* (biofilm formation), and *Staphylococcus aureus* (resistance to antibiotics) (Shah & Swiatlo, 2008). Putrescine is also involved in restoring virulence in some *Shigella flexneri* mutants by altering expression of some virulence genes (Durand & Bjork, 2003). In *S. pneumoniae*, silencing of PotD, which is a polyamine transport protein, significantly slows disease progression in mouse models (Ware *et al.*, 2006). Gene expression studies in *Salmonella typhimurium* have also shown upregulation of putrescine and spermidine biosynthesis genes during infection of epithelial cells and macrophages (Eriksson *et al.*, 2003, Hautefort *et al.*, 2008).

Polyamines control swarming and type 1 pili driven motility in *Proteus mirabilis* and *E. coli* (Armbruster *et al.*, 2013, Kurihara *et al.*, 2011, Kurihara *et al.*, 2009). Putrescine has been proposed to be an extracellular signal sensed by *P. mirabilis* in order to initiate swarming (Sturgill & Rather, 2004). Swarming motility is movement of bacteria on a surface facilitated by

flagellar rotation (Jarrell & McBride, 2008, Partridge & Harshey, 2013). It was first reported in a species of *Proteus* in 1885. There are only three bacterial families so far that have been reported to exhibit swarming (Kearns, 2010). Swarming is multicellular and multigenerational, and involves differentiated cells. In this process, the cells move in a coordinated manner, where the differentiation of the vegetative cells into elongated swarmer cells facilitates the movement on solid surfaces (Rather, 2005). The swarming cells not only undergo morphological changes (Hoeniger, 1966) but also transcriptional and metabolic modifications (Armitage, 1981, Wang *et al.*, 2004, Overhage *et al.*, 2008, Kim & Surette, 2004, Inoue *et al.*, 2007).

Swarming has been linked to antibiotic resistance and virulence (Overhage *et al.*, 2008, Butler *et al.*, 2010, Lu *et al.*, 2015). Swarm cells of *E. coli*, *P. aeruginosa*, *Burkholderia thailandensis*, *Bacillus subtilis*, and *Seratia marcescens* showed increased antibiotic resistance compared to their vegetative counterparts (Lai *et al.*, 2009). The ability to invade urothelial cells and the expression of virulence genes was related to the swarming ability of *P. mirabilis* in causing kidney and other linked infections (Allison *et al.*, 1992a, Allison *et al.*, 1992b). *E. coli* is the major cause of urinary tract infections (Stamm, 1991), but a relationship between virulence and swarming motility has not been established.

Swarming of *P. mirabilis* requires putrescine, and swarming of *E. coli* may require spermidine (Sturgill & Rather, 2004, Kurihara *et al.*, 2009). We observed that some putrescine-deficient *E. coli* strains failed to swarm. We show that several aspects of putrescine metabolism affect swarming motility in *E. coli* strain W3110, and that one function of putrescine during swarming may be to overcome oxidative stress. A common putrescine requirement may suggest that *P. mirabilis* and *E. coli* swarming also share other regulatory features.

2.3 Results

Swarming of *E. coli*

We studied swarming motility in *E. coli*, even though swarming has been more extensively studied in *P. mirabilis* and *S. enterica*. Obvious advantages to the use of *E. coli* are the power of the genetic systems, rapid growth, the availability of mutant and plasmid libraries, and several databases on gene expression and mutant phenotypes (Kitagawa *et al.*, 2005, Keseler *et al.*, 2011, Baba *et al.*, 2006).

Different strains from the *E. coli* Genetic Stock Center, mostly derivatives of *E. coli* K-12, were analyzed for swarming. Some strains were non-motile, whereas many swarmed outward, and exhibited great variability in swarm diameter. Our lab strain of W3110 exhibited outward movement that formed concentric rings, with alternating phases of movement and consolidation/growth, and had the least variability in swarm diameter for replicates. Variants of W3110 occasionally appeared that stably moved faster and without ring formation. These variants often had an insertion in the region upstream of the *flhDC* operon (not shown). FlhD and FlhC form a heteromeric transcriptional activator that controls flagella synthesis. Insertions in the *flhDC* promoter region often increase synthesis of flagella (Fahrner & Berg, 2015). We consider these variants and most strains from the Genetic Stock Center, which often have insertions in the *flhDC* promoter region (not shown), to be hypermotile. We chose our lab strain of W3110 for further study because the ring pattern allows observation of subtle changes in movement. This version of W3110 has few genetic markers, does not have an insertion in the *flhDC* promoter region, and has not been highly mutagenized or passaged (Bachmann, 1972).

A major study of *E. coli* swarming examined the entire Keio library of mutants (Inoue *et al.*, 2007). Their parental strain was also W3110, but its swarming pattern differed substantially from that of our strain. We would characterize the movement of this version of W3110 as relatively non-motile. Furthermore, since the movement was not uniformly outward, we suspect the formation of frequent hypermotile variants. They showed that 216 non-essential genes were required for swarming, but not for swimming motility (Inoue *et al.*, 2007). We examined mutants lacking 16 of these 216 genes in our strain background, and could confirm a swarming-deficient phenotype for only 11 of these mutants. It is clear that swarming of the same "strain" from various labs can display different patterns of movement and possibly have different requirements.

E. coli actually has two forms of surface motility, flagella-dependent and pili-dependent, and their relation to each other is apparently complex (Inoue *et al*., 2007, Kurihara *et al.*, 2011). A *fimA* mutant of our strain of W3110, which lacks the major subunit of the type I pili, also had a smaller movement diameter (~ 25% of wild type), and formed two closely-spaced concentric rings. Inoue et al., 2007 had previously reported that a *fimA* mutant fails to swarm, and they suggested that loss of *fimA* reduced flagella synthesis by an unknown mechanism. A *fliC* mutant in our strain background, which lacks the major flagella subunit, had a much smaller movement diameter (~25% of wild type), and failed to form concentric rings. The residual movement is presumably due to pili-mediated movement. To allow comparison with previous published results, we equate surface motility with flagella-dependent swarming, but with the understanding that pili-dependent motility may contribute to the observed movement patterns.

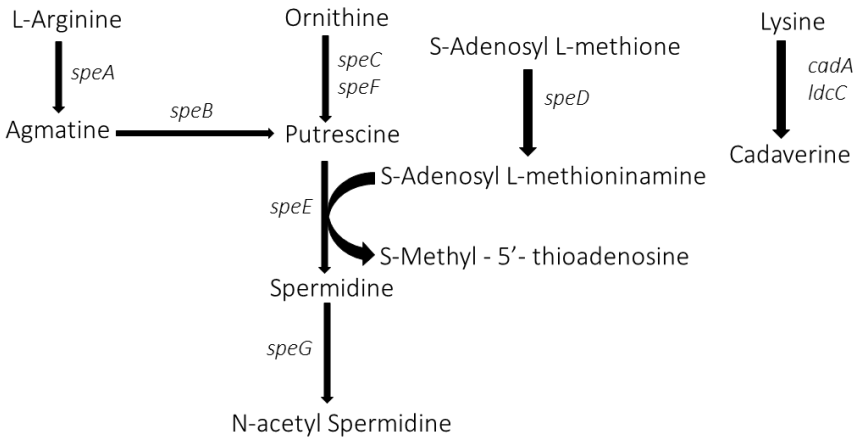
Swarming of mutants defective in polyamine synthesis

The major pathway of putrescine synthesis is a one-step pathway from ornithine to putrescine (Tabor & Tabor, 1985). SpeC is the major ornithine decarboxylase and is considered constitutive, and SpeF is a minor enzyme that is induced under acidic conditions (Tabor & Tabor, 1985, Kashiwagi *et al.*, 1991). *E. coli* Δ*speC* and Δ*speF* mutants swarmed as well as the parental strain, but a Δ*speC* Δ*speF* double mutant failed to swarm (Figure 2.1BC). A secondary pathway of putrescine synthesis is via arginine decarboxylase (SpeA) and agmatinase (SpeB). SpeA is associated with the inner membrane, and the SpeA-SpeB pathway is thought to synthesize putrescine in the presence of extracellular arginine (Tabor & Tabor, 1969, Buch & Boyle, 1985).

Δ*speA* and Δ*speB* mutants could not swarm (Figure 2.1BC). In contrast to these results, Δ*cadA* (partial cadaverine deficiency) and Δ*speE* (complete spermidine deficiency) mutants swarmed as well as W3110. Growth defects could not account for these swarming defects, since the swarming-deficient Δ*speA*, Δ*speB*, and Δ*speC* Δ*speF* strains grew as well as the parental strain (Figure 2.2A). Furthermore, the Δ*speA*, Δ*speB*, and Δ*speC* Δ*speF* mutants had normal swimming motility (Figure 2.2B).

Together these results suggest that the defect is specific for swarming. To further confirm the requirement for putrescine, the swarming-deficient Δ*speB* strain was supplemented with 0.1 mM and 1 mM of putrescine, which partially restored the swarming phenotype and the ring pattern (Figure 2.3A).

A



B

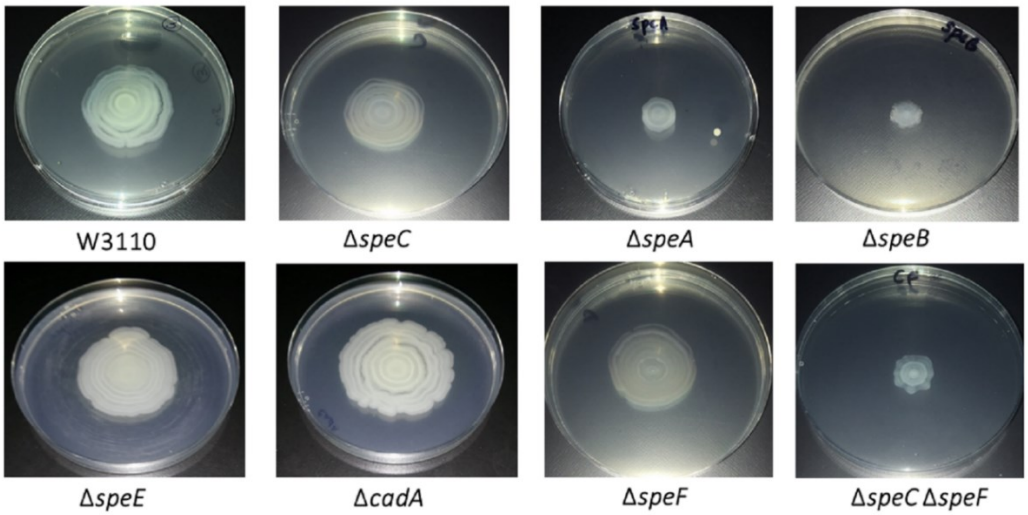


Figure 2.1: Swarming motility and polyamine synthesis. (A) Pathways and genes of polyamine synthesis. (B) Swarming of mutants with defects in polyamine anabolic genes. (C) Diameter of swarm colonies of polyamine mutants after 36 hours. Error bars represent standard deviations for three independent replicates. Statistical analysis was performed using Student's two-tailed t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

C

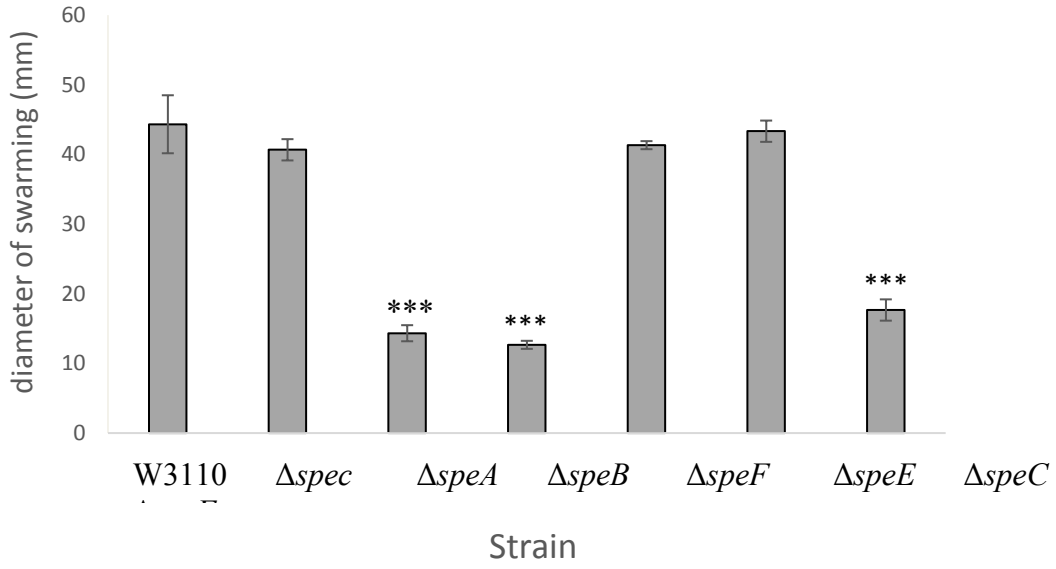
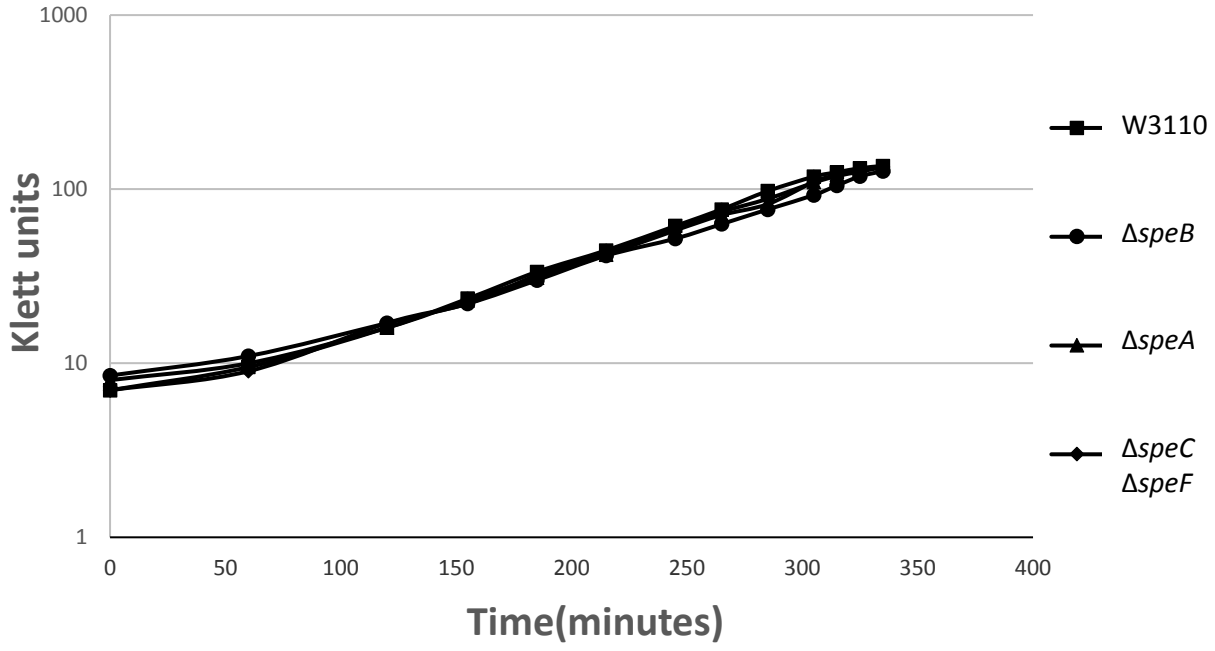


Figure 2.1: (cont.) Swarming motility and polyamine synthesis. (A) Pathways and genes of polyamine synthesis. (B) Swarming of mutants with defects in polyamine anabolic genes. (C) Diameter of swarm colonies of polyamine mutants after 36 hours. Error bars represent standard deviations for three independent replicates. Statistical analysis was performed using Student's two-tailed t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

A similar result was observed for a Δ*speA* mutant (not shown). In contrast to these results, spermidine did not restore wild type swarming. Instead, spermidine caused an unusual pattern of movement to the Δ*speB* and wild type strains (Figure 2.3B).

These exogenous concentrations of putrescine and spermidine were not toxic to the growth of these cells (not shown). Finally, addition of the *speB* gene on a plasmid to the Δ*speB* strain restored the ring pattern and 80% of the diameter (Figure 2.4). In summary, these results show that the swarming motility of W3110 requires two independent pathways of putrescine synthesis, but does not require spermidine.

A



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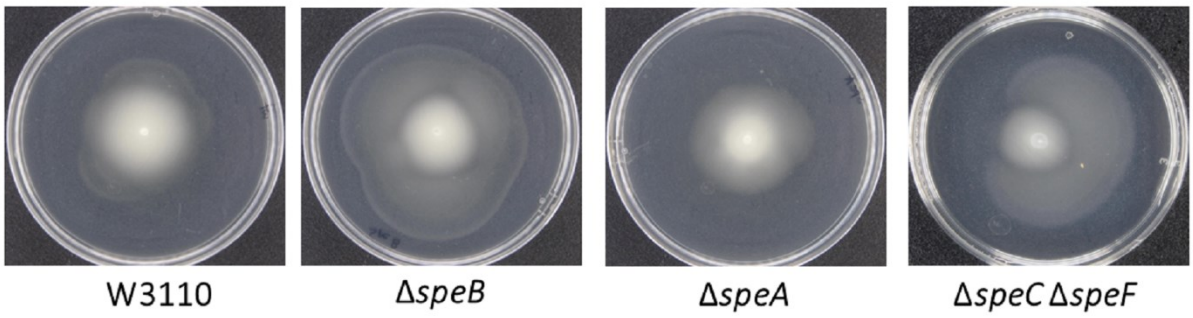
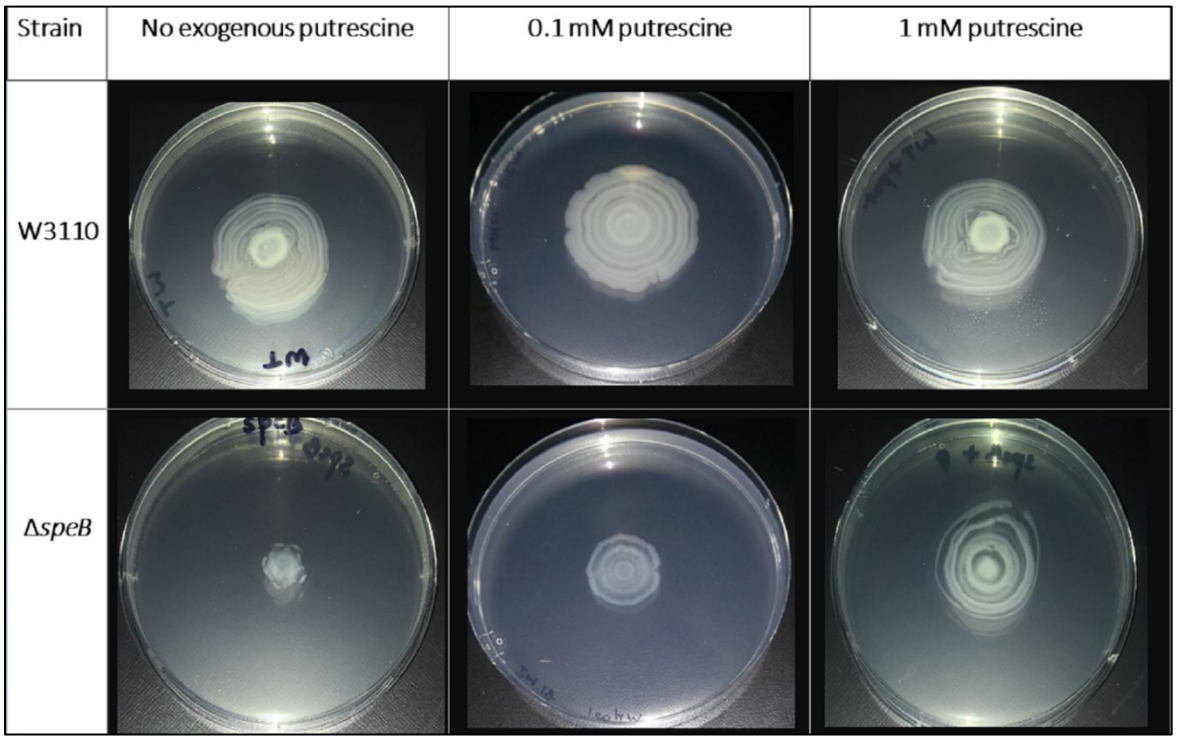


Figure 2.2: Growth and swimming of polyamine anabolic mutants. (A) Growth curves of wild type and putrescine deficient mutants in a shaking aerobic culture. (B) Representative images of swimming.

A



B

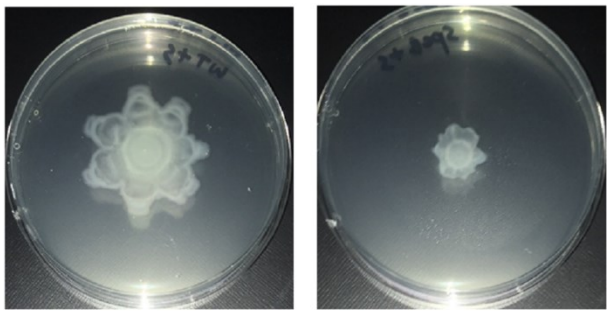


Figure 2.3: Putrescine supplementation of wild type and Δ*speB* strains. Images were taken at 36 hours. All assays were performed in triplicates and representative images are shown.

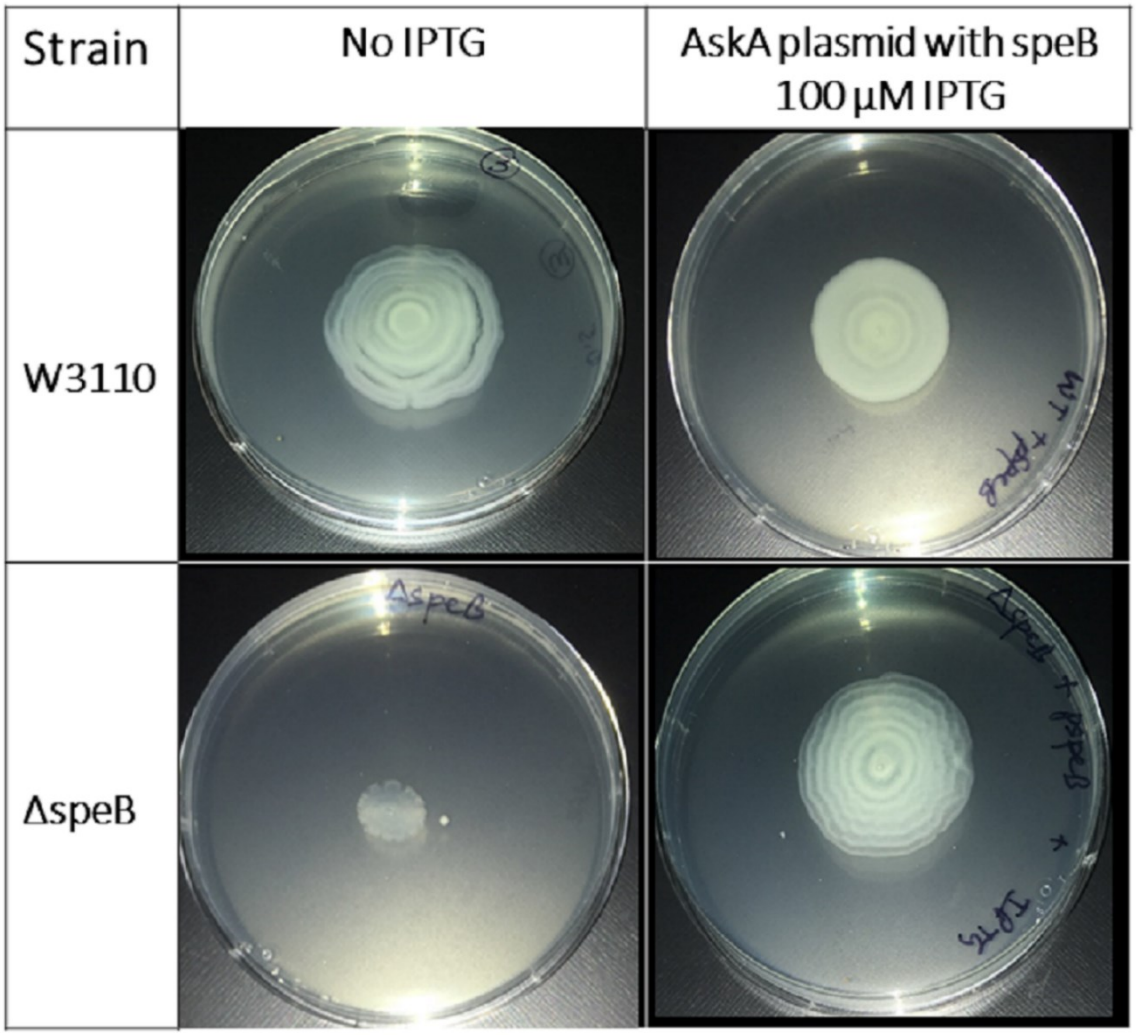


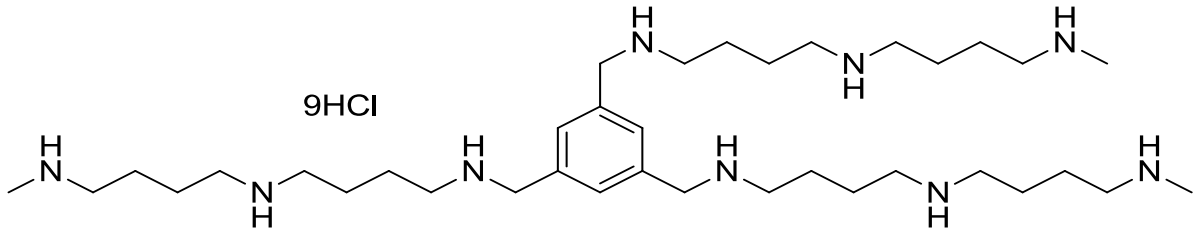
Figure 2.4: The effect of a plasmid containing *speB* on wild type and Δ*speB* strains. At 36 hours after inoculation, plates were imaged. 0.1 mM IPTG was used for induction of *speB*.

Putrescine transport and swarming

Polyamine transport has the potential to modulate intracellular polyamines, if extracellular polyamines are present. *E. coli* has several polyamine transport systems with different preferences for putrescine and spermidine: PotABCD, PotFGHI, PotE, PuuP, and PlaP (Igarashi *et al.*, 2001). The PotABCD and PlaP transport systems have been shown to be important for

swarming in *E. coli* and *P. mirabilis*, respectively (Kurihara *et al.*, 2013, Kurihara *et al.*, 2009). We examined the effect on swarming of trimer44nme, which is a putrescine analog that selectively inhibits putrescine transport (Muth *et al.*, 2014). The tri-substituted 1, 3, 5-benzene design of trimer44nme presents three putrescine motifs to the cell surface receptor and trimer44nme is a competitive inhibitor of polyamine uptake (Figure 2.5A). We examined the effect of trimer44nme on the Δ*speB* mutant, which requires exogenous putrescine for swarming. 100 μM trimer44nme reduced swarming with 1 mM putrescine 40% (Figure 2.5B). Trimer44nme also decreased the swarming diameter of the parental strain by 35% (Figure 2.5B). This concentration of trimer44nme was not toxic for the growth of the bacterial cells (not shown). We also examined swarming in mutants with deletions of four putrescine transport systems. Deletion of *plaP* and *potF* reduced the swarming diameter 50% and 40%, respectively (Figure 2.5C). Although the *plaP* and *potF* mutants moved outward, they did not form concentric rings (Figure 2.5D).

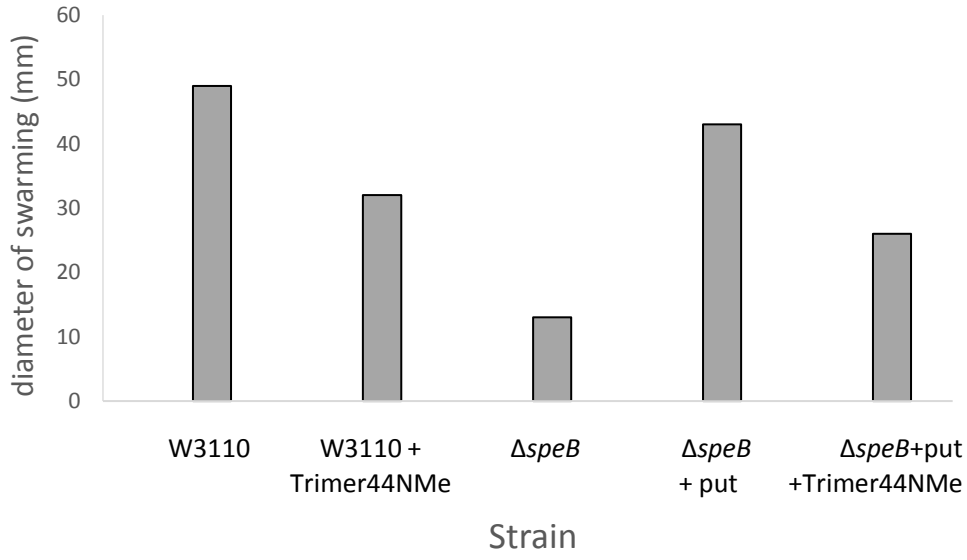
A



trimer44NMe

Figure 2.5: Putrescine transport and swarming (A) Structure of polyamine analog trimer44nme. (B) Effect of trimer44nme on swarming. Swarming diameter at 36 hours is shown with the indicated supplements. Putrescine and T44 were present at 1 mM and 0.1 mM, respectively. (C) Swarm diameters in Δ*plaP*, Δ*potF* Δ*puuP* and Δ*potE* strains. Statistical analysis was performed using Student's two-tailed t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

B



C

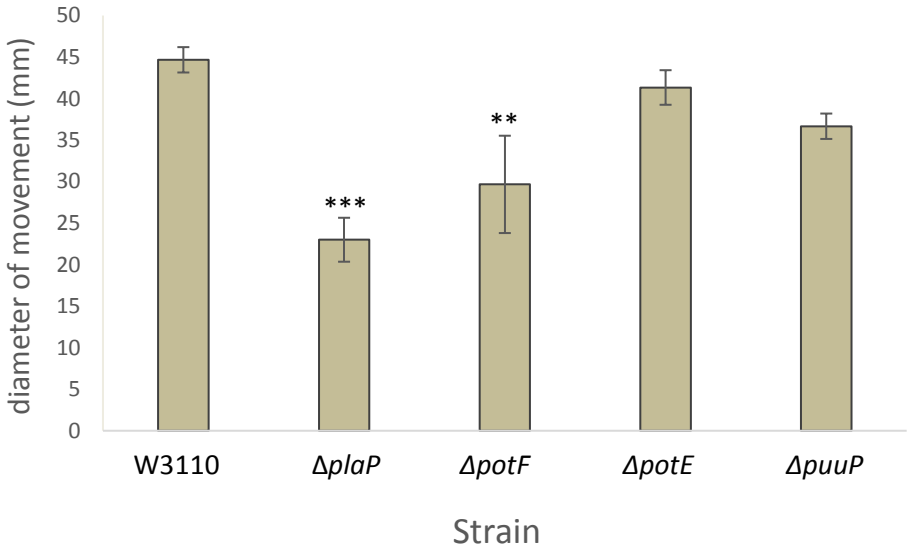


Figure 2.5: (cont.) Putrescine transport and swarming (A) Structure of polyamine analog trimer44nme. (B) Effect of trimer44nme on swarming. Swarming diameter at 36 hours is shown with the indicated supplements. Putrescine and T44 were present at 1 mM and 0.1 mM, respectively. (C) Swarm diameters in Δ*plaP*, Δ*potF* Δ*puuP* and Δ*potE* strains. Statistical analysis was performed using Student's two-tailed t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

D

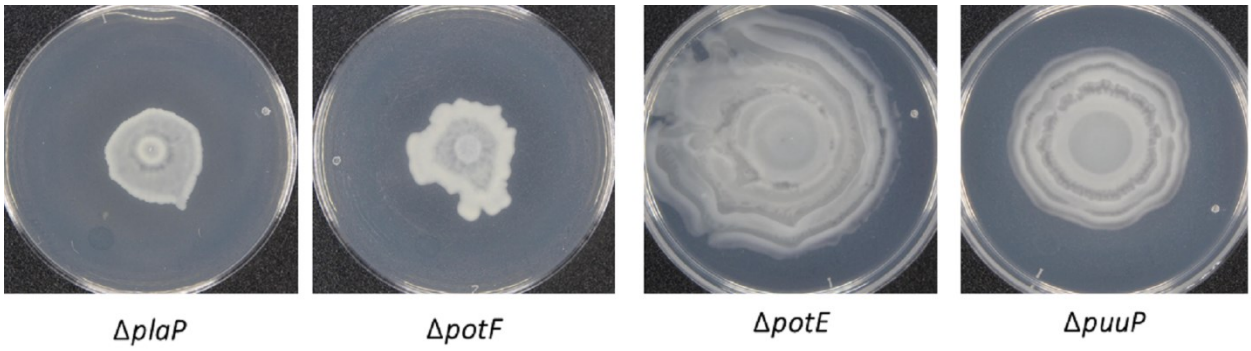


Figure 2.5: (cont.) Putrescine transport and swarming (A) Structure of polyamine analog trimer44nme. (B) Effect of trimer44nme on swarming. Swarming diameter at 36 hours is shown with the indicated supplements. Putrescine and T44 were present at 1 mM and 0.1 mM, respectively. (C) Swarm diameters in Δ*plaP*, Δ*potF* Δ*puuP* and Δ*potE* strains. Statistical analysis was performed using Student's two-tailed t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

In contrast, deletions of *potE* and *puuR* did not affect the swarming diameter (Figure 2.5C), and did not affect concentric ring formation (Figure 2.5D). Nonetheless, these results show that putrescine transport is important for *E. coli* swarming, and suggests that extracellular putrescine is present, even though putrescine was not a medium component.

Putrescine catabolism and swarming

Intracellular putrescine levels are not only controlled by synthesis and transport, but also by catabolism. The evidence that catabolism affects putrescine homeostasis is that mutants defective in putrescine catabolism have altered regulation of putrescine-responsive genes (Schneider *et al.*, 2013). *E. coli* has two pathways of putrescine degradation (Schneider & Reitzer, 2012): the dominant PuuA pathway which glutamylates putrescine, and a less dominant PatA pathway which deaminates putrescine (Figure 2.6A). Putrescine catabolism can be controlled through alteration of the levels of PuuR, which represses genes of the dominant PuuA-dependent pathway. In a strain without PuuR (Δ*puuR*), elevated putrescine catabolism should lower

intracellular putrescine. In such a strain, swarming was diminished (Figure 2.6B). PuuR can be increased in a strain containing a plasmid with *puuR*, and high PuuR should impair putrescine catabolism and elevate intracellular putrescine. In a high PuuR/high putrescine strain, swarming was enhanced (Figure 2.6C). In summary, swarming positively correlated with putrescine content.

A

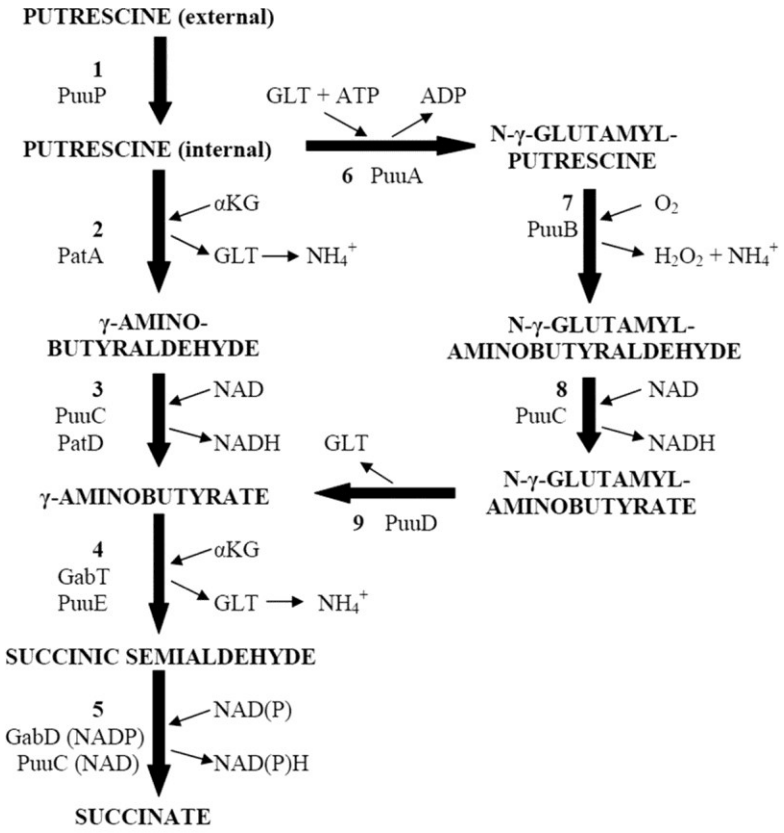
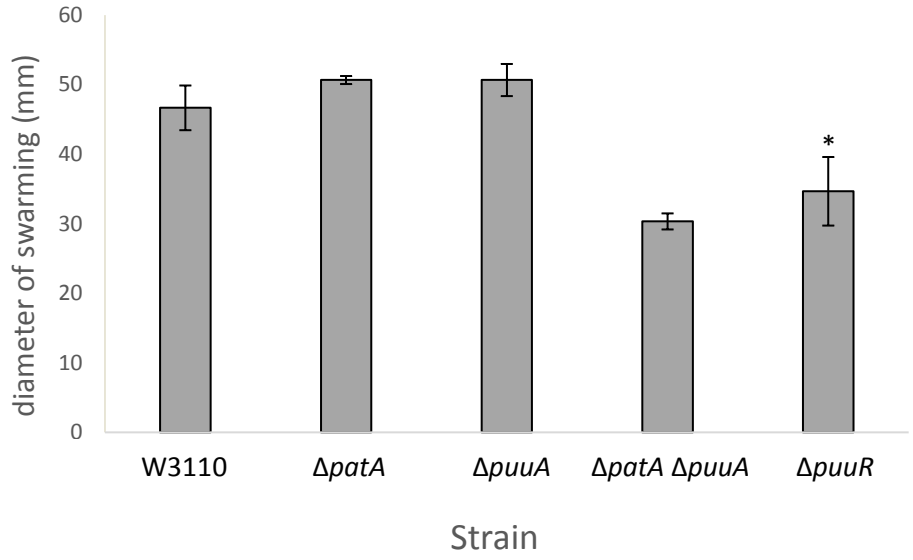


Figure 2.6: Swarming and putrescine catabolism. (A) Pathways and genes of putrescine catabolism. Adapted by permission from ASM, Journal of Bacteriology (Schneider and Reitzer, 2012), ©2012 American Society for Microbiology. (B) Swarm diameter of putrescine catabolic mutants after 36 hours. (C) Swarm diameter in a strain with excess PuuR expressed from a *puuR* plasmid. For B and C, error bars represent standard deviations for three independent replicates. Statistical analysis was performed using Student's two-tailed t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. (D) Representative images of swarming for strains with altered spermidine metabolism.

B



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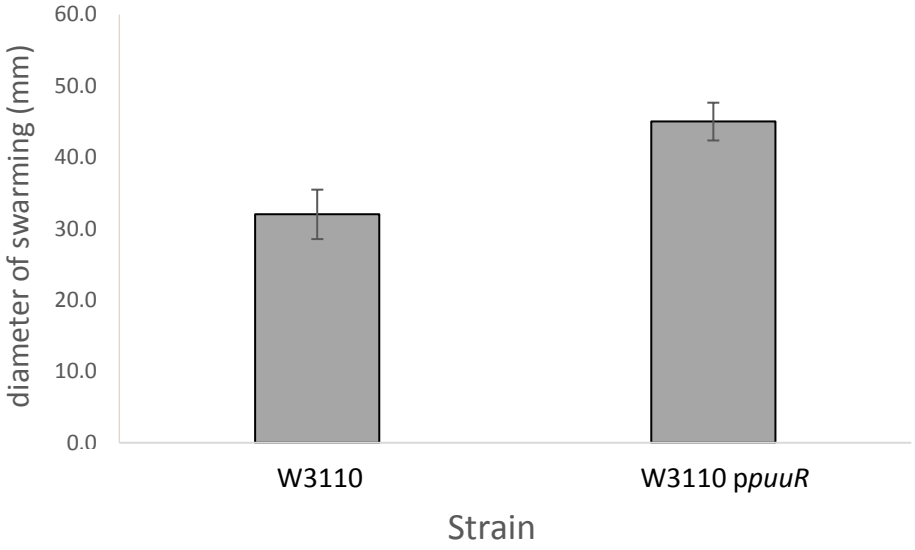
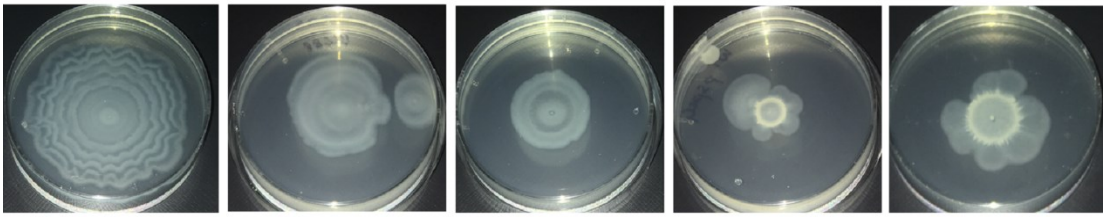


Figure 2.6: (cont.) Swarming and putrescine catabolism. (A) Pathways and genes of putrescine catabolism. Adapted by permission from ASM, Journal of Bacteriology (Schneider and Reitzer, 2012), ©2012 American Society for Microbiology. (B) Swarm diameter of putrescine catabolic mutants after 36 hours. (C) Swarm diameter in a strain with excess PuuR expressed from a *puuR* plasmid. For B and C, error bars represent standard deviations for three independent replicates. Statistical analysis was performed using Student's two-tailed t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. (D) Representative images of swarming for strains with altered spermidine metabolism.

D



patA	+	-	-	+	-
puuA	+	-	-	+	-
speE	+	+	-	+	+
p*speG*	-	-	-	+	+

Figure 2.6: (cont.) Swarming and putrescine catabolism. (A) Pathways and genes of putrescine catabolism. Adapted by permission from ASM, Journal of Bacteriology (Schneider and Reitzer, 2012), ©2012 American Society for Microbiology. (B) Swarm diameter of putrescine catabolic mutants after 36 hours. (C) Swarm diameter in a strain with excess PuuR expressed from a *puuR* plasmid. For B and C, error bars represent standard deviations for three independent replicates. Statistical analysis was performed using Student's two-tailed t-test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. (D) Representative images of swarming for strains with altered spermidine metabolism.

In the high PuuR/high putrescine strain, the PatA-dependent pathway is still intact. Elimination of both catabolic pathways would be expected to increase putrescine and swarming even more. Contrary to expectation, a Δ*patA* Δ*puuA* double mutant swarmed less well, and the swarming pattern was distinctly different from a wild type pattern (Figure 2.6B). Δ*patA* and Δ*puuA* mutants swarmed as well as the parental strain (Figure 2.6B). One possible explanation for defective swarming of the double mutant is the known toxicity of spermidine, which is derived from putrescine (Fukuchi *et al.*, 1995). This possibility predicts restoration of swarming by either loss of spermidine synthesis or more rapid spermidine degradation. A deletion of *speE*, which codes

for spermidine synthase, was introduced into the Δ*patA* Δ*puuA* double mutant, and loss of *speE* did not suppress the defect of the double mutant (Figure 2.6D). A *speG*-containing plasmid was introduced into the Δ*patA* Δ*puuA* double mutant. SpeG acetylates spermidine, which will prevent binding of spermidine to its targets. The *speG* plasmid did not restore swarming to the Δ*patA* Δ*puuA* double mutant (Figure 2.6D). Instead, the *speG* plasmid altered the swarming pattern of both the double mutant and the parental W3110 strain. Examination of the effect of the *speG* plasmid on swarming was not considered further.

In summary, putrescine catabolism contributes to control of swarming. An unusual result was that complete loss of putrescine catabolism, which is predicted to increase putrescine pools, impaired swarming. Loss of putrescine catabolism impairs several stress responses (Schneider *et al.*, 2013), and the possibility that swarming requires responses to oxidative and acid stress is considered next.

The function of polyamines: oxidative stress and acid resistance during swarming

One difficulty in assigning biological functions to polyamines is the difficulty of genetic analysis: polyamine deficient mutants tend to have little or no phenotypes. The strong swarming defect of polyamine deficient mutants, at least for our strain of W3110, allows rapid testing of possible functions for polyamines. Numerous studies suggest that polyamines contribute to resistance to oxidative stress (Tkachenko & Nesterova, 2003, Sakamoto *et al.*, 2015, Rhee *et al.*, 2007, Jung & Kim, 2003, Tkachenko *et al.*, 2012). Polyamines regulate several genes involved in oxidative stress, including those for hydroperoxidase I (KatG), catalase (KatE), and polyphosphate kinase (Ppk) (Murata *et al.*, 1988, Sakamoto *et al.*, 2015). To test whether the swarming defect in putrescine-deficient mutants results from a defective response to oxidative

stress, we complemented the *speB* mutant with two putrescine-modulated oxidative stress genes, *katE* and *katG*, on plasmids. Some movement was observed in strains with *katE* and *katG*. The diameter of the swarm was more than that of the *speB* mutant, and two more rings were observed in the mutant strain with *katG* complementation at 36 hours (Figure 2.7). When the complemented strain was incubated further, it continued to move over the surface, whereas the uncomplemented strain did not move. OxyR controls expression of *katG*, and polyamines modulate expression of *oxyR*. However, complementation of a *speB* strain with *oxyR* did not restore swarming. The partial restoration of the swarming-deficient phenotype of *speB* by oxidative stress genes suggests that one function of polyamines during swarming may be resistance to oxidative stress, but such resistance is not the only function of putrescine.

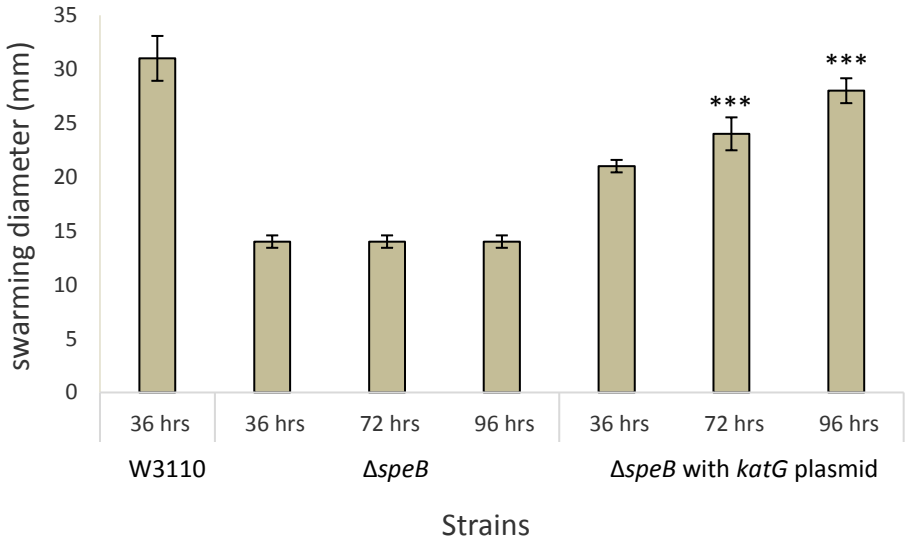


Figure 2.7: Swarming of a *speB* strain complemented with *katG* plasmid. The diameters were measured at 36, 72 and 96 hours. Error bars represent standard deviations for three independent replicates. Statistical analysis was performed using Student's two-tailed t-test: ***, $P<0.001$.

Polyamines are also important for inducing glutamate decarboxylase-dependent acid resistance in *E. coli* (Chattopadhyay & Tabor, 2013). Polyamines have been shown to increase the level of RpoS (σ^S), which in turn, via GadE, stimulates synthesis of two glutamate carboxylases (Chattopadhyay *et al.*, 2015). However, an *rpoS* mutant showed normal swarming (not shown). Furthermore, when the *speB* mutant was complemented with various genes of this acid response system (*gadE*, *gadW*, *gadX*, *gadA* and *gadB*), no restoration of the swarm phenotype was observed (not shown). We conclude that polyamine control of acid resistance genes is not required for swarming.

2.4 Discussion

Swarming requires two independent pathways of putrescine synthesis, but does not require spermidine synthesis. The requirement for two putrescine anabolic pathways may suggest that putrescine content is higher for swarming cells than for growing planktonic cells, which do not require either pathway. In addition to putrescine synthesis, putrescine transport and degradation also affects swarming. The involvement of both putrescine synthesis and degradation suggests fluctuating putrescine concentrations during swarming, and such fluctuations may account for the oscillatory movement. The physiological function of putrescine for swarming is not entirely clear. Our results suggest that putrescine may contribute to the response to oxidative stress during swarming, but putrescine undoubtedly contributes to an additional physiological function, which has yet to be identified.

The importance of the SpeA-SpeB pathway and putrescine transport suggests specific extracellular requirements for swarming. SpeA is mostly periplasmic (Tabor & Tabor, 1969), which implies that extracellular arginine is the substrate. The contribution of putrescine transport

to swarming, though not absolutely required, suggests the presence of extracellular putrescine. Since putrescine is not a medium component, extracellular putrescine implies putrescine export. *E. coli* exports putrescine during osmotic stress (Kashiwagi *et al.*, 1992), which may suggest that swarming cells encounter osmotic stress during swarming, or perhaps another stress that results in putrescine export.

Putrescine affects swarming for both *E. coli* and *P. mirabilis* in similar ways. For both organisms, putrescine synthesis is required, and putrescine transport contributes to swarming. The arginine decarboxylase/agmatinase (SpeA-SpeB) pathway is important for both organisms (Armbruster *et al.*, 2013, Kurihara *et al.*, 2013). One difference is that the ornithine decarboxylase pathway of putrescine biosynthesis is required for *E. coli* swarming, but not for *P. mirabilis* swarming. Another similarity between *E. coli* and *P. mirabilis* swarming is the proposed involvement of extracellular arginine and putrescine (Sturgill & Rather, 2004, Armbruster *et al.*, 2014).

In addition to a polyamine requirement, the swarming of *E. coli, P. mirabilis*, and *S. enterica* have several common metabolic features. First, the citric acid cycle is required for swarming. Citric acid cycle genes were upregulated during consolidation phase during swarming in *P. mirabilis* (Pearson *et al.*, 2010), as well as in actively swarming *S. enterica* (Kim & Surette, 2004). A similar requirement for citric acid cycle genes for *E. coli* swarming has also been reported (Inoue *et al.*, 2007). Second, glucose metabolism is necessary for *E. coli* and *S. enterica* swarming (Inoue *et al.*, 2007, Kim & Surette, 2004). Although there is no glucose requirement for *P. mirabilis* swarming (Pearson *et al.*, 2010), glycolytic mutants exhibited a severe swarming defect (Alteri *et al.*, 2012). Third, iron uptake and metabolism are essential for swarming in *S.*

enterica and *E. coli* (Wang *et al.*, 2004, Inoue *et al.*, 2007). Finally, glutamine or glutamine synthetase are required for swarming of *P. mirabilis*, *S. enterica*, and *E. coli* (Kim & Surette, 2004, Inoue *et al.*, 2007, Allison *et al.*, 1993, Rather, 2005). There are important differences in swarming between these organisms (Kearns, 2010, Harshey, 2003, Harshey & Matsuyama, 1994, Partridge & Harshey, 2013, Pearson *et al.*, 2010). Nevertheless, a common core set of metabolic requirements also exists.

In summary, this report demonstrated the requirement of putrescine for the swarming of *E. coli* W3110 strain, the involvement of putrescine catabolism and transport, and suggested modulation of oxidative stress as one role that polyamines play in swarming *E. coli*.

2.5 Experimental Procedures

Strains and plasmids

All strains used for growth rate determinations and swarm assays are derivatives of *E. coli* K-12 strain W3110, and are listed in Table 2.1. There is great variation in strains of W3110, a common wild type lab strain. Our strain originated from the lab of Boris Magasanik in the 1960s, and had not been highly mutagenized. To construct the mutant strains, an antibiotic resistance gene replaced most of a gene of interest. The altered allele was transferred into W3110 by P1 transduction (Miller, 1972), and the antibiotic gene was removed as described (Baba *et al.*, 2006). All deletions are in-frame deletions, if the antibiotic resistance gene is removed. The plasmids for complementation (*speB*, *puuR*, *katG* and *katE*) genes were from the ASKA(−) clone library of the National Bio Resource Project (National Institute of Genetics, Japan) (Kitagawa *et al.*, 2005).

Media and Growth Conditions

For P1 transductions and plasmid transformation experiments, cells were grown in standard LB liquid medium (1% tryptone, 1% NaCl and 0.5% yeast extract, pH 7.0) overnight or on LB plates (1.5% agar) at 37° C. Antibiotics were used for selection at concentrations of 25 μg/mL (both chloramphenicol and kanamycin).

For swarm assays, all cultures were grown in liquid swarm media (0.25% NaCl, 1% tryptone and 0.5% glucose) and swarm media plates with 0.45% agar (Eiken, Tokyo, Japan) were used.

For growth studies, liquid swarm media was used. Starter cultures (typically 6–12 h incubation) were typically grown in the same medium as the experiment.

Table 2.1: Strains

Strain Name	Genotype	Reference
IM26	W3110 Δ*speB::kan fliC-lacZ*	This study
IM27	W3110 Δ*speE::kan fliC-lacZ*	This study
IM28	W3110 Δ*speF::cat fliC-lacZ*	This study
IM29	W3110 Δ*speC::kan fliC-lacZ*	This study
IM34	W3110 Δ*speC* Δ*speF::cat fliC-lacZ*	This study
IM60	W3110 Δ*cadA::kan fliC-lacZ*	This study
IM61	W3110 Δ*speA::kan*	This study
BLS77	W3110 Δ*puuR*::*cat*	Schneider et al (2012)
BLS80	W3110 Δ*puuA*::*cat*	Schneider et al (2012)
BLS88	W3110 Δ*patA* Δ*puuA*	Schneider et al (2012)
CP2	W3110 Δ*patA*	Schneider et al (2012)

IM62	W3110 Δ*patA* Δ*puuA* Δ*speE::kan*	This study
IM63	W3110 Δ*potE::kan*	This study
IM64	W3110 Δ*potF::kan*	This study
IM65	W3110 Δ*plaP::kan*	This study
IM66	W3110 Δ*puuP::kan*	This study

Cells were washed twice with phosphate buffered saline and re-suspended in swarm media before inoculation. The cells are then grown at 37° C in aerobic conditions (shaking at 240 rpm) and the absorbance was measured every thirty minutes. Cell growth was measured in Klett units using a Scienceware Klett colorimeter with a KS-54 filter. 100 Klett units represent an OD_{600} value of about 0.7.

Swarm Assay

For a standard swarm assay, single colonies from fresh plates (streaked out from frozen stocks a day before) were inoculated in the liquid swarm media and grown for six hours at 37° C in aerobic conditions (shaking at 240 rpm). 30 ml of autoclaved swarm media was poured into each sterile polystyrene petri-dish (100 mm X 15 mm) and allowed to solidify at room temperature for approximately six hours. Then, 1 μL of the pre-swarm growth medium was inoculated at the center of this swarm agar plate and incubated at 33° C. Each experiment was performed in triplicate and pictures were taken after 36 hours. For plates supplemented with exogenous putrescine and trimer44nme, filter-sterilized stock solutions were added directly to autoclaved media before the plates were poured. For plasmid genes under the control of the *lac* promoter, 0.1 mM IPTG was added to the pre-swarm growth medium and to swarm plates before pouring.

Swim assay

Pre-swim culture preparation was identical to pre-swarm culture preparation. 15 mL of autoclaved swarm media was poured into polystyrene petri-dishes (60mm x 15mm) and allowed to solidify at room temperature for about one hour. Then, 1 μL of the culture was stabbed in the center of this swim agar plate and incubated at 33° C. Each experiment was performed in triplicate and pictures were taken after 20 hours.

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CHAPTER 3

ENZYMATIC ASSAY OF D-MANNOSE FROM URINE

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Submitted to *Bioanalysis* (Manuscript ID BIO-2018-0121)

3.1 Introduction to Urinary tract infections (UTI) and D-mannose

Urinary tract infections are a public health issue and caused by an array of pathogenic bacteria. The most common bacteria involved in causing such infections are *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Staphlococcus saprophyticus* and *Enterococcus faecalis*. These infections generally have a very high recurrence rate and are one of the most common bacterial infections. These affect almost 150 million people every year all over the world (Stamm & Norrby, 2001). These infections are responsible for morbidity in females of all ages, especially post-menopausal women. Some of the serious after effects of UTIs are frequent reappearances, renal damage, sepsis and the most dangerous of all, complications caused because of development of high levels of antibiotic resistance and colitis (Flores-Mireles *et al.*, 2015). In United States alone, every year approximately 3.5 billion dollars are spent in order to treat these infections and related problems.

Types of UTIs

These infections are categorized into two kinds: uncomplicated and complicated. In uncomplicated UTIs, the affected patients are those who are healthy otherwise, and have no related abnormality (Hooton, 2012, Nielubowicz & Mobley, 2010, Gupta *et al.*, 2001). The complicated ones are the more difficult ones to deal with. These infections have other associated factors which weaken the body's defense and lead to a condition which is more challenging to treat and control. Those factors include renal failure, pregnancy, immunosuppression and most importantly, catheters and other similar devices (Lichtenberger & Hooton, 2008). These catheter based infections account for about 80% of these complicated UTI cases in United States only (Lo *et al.*, 2014). Both Gram positive as well as Gram negative bacteria are responsible in causing

such infections (Figure 3.1). However, uro-pathogenic *E.coli* (UPEC) is the most common agent that causes these UTIs.

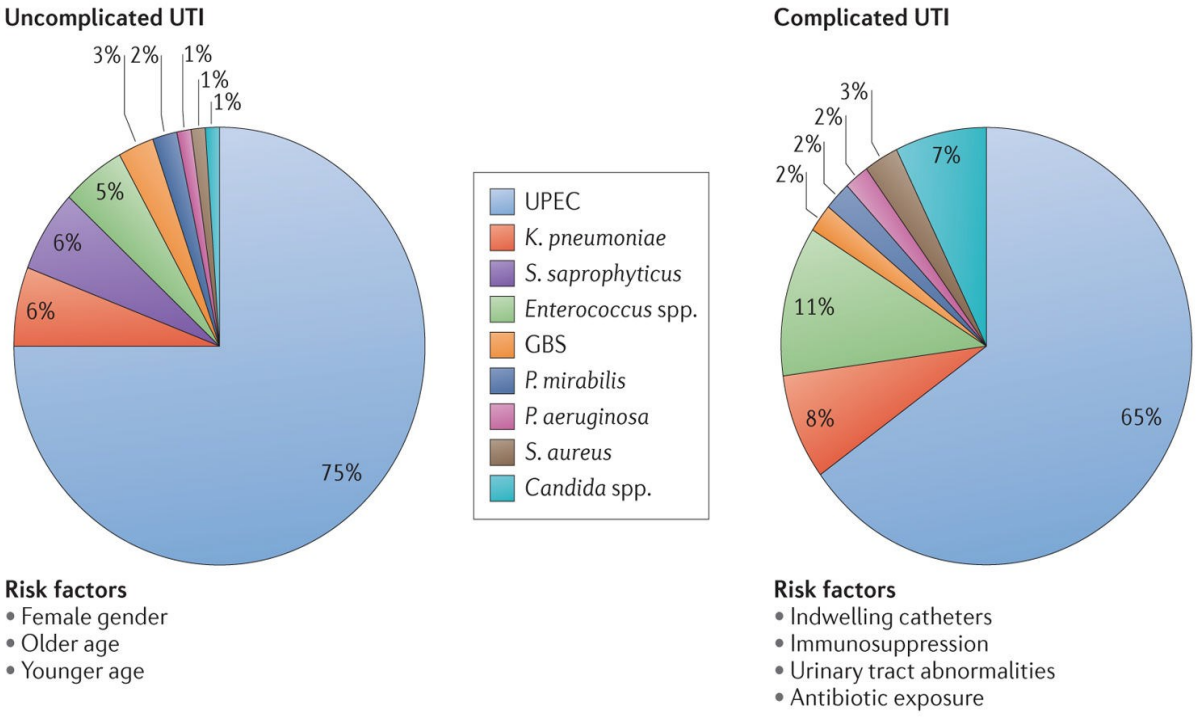

Figure 3.1 Epidemiology of urinary tract infections. Urinary tract infections (UTIs) are caused by a wide range of pathogens, including Gram-negative and Gram-positive bacteria, as well as fungi. Uncomplicated UTIs typically affect women, children and elderly patients who are otherwise healthy. Complicated UTIs are usually associated with indwelling catheters, urinary tract abnormalities, immunosuppression or exposure to antibiotics. The most common causative agent for both uncomplicated and complicated UTIs is uropathogenic *Escherichia coli* (UPEC). For uncomplicated UTIs, other causative agents are (in order of prevalence) *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, group B *Streptococcus* (GBS), *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida* spp. For complicated UTIs, the other causative agents are (in order of prevalence) *Enterococcus* spp., *K. pneumoniae*, *Candida* spp., *S. aureus, P. mirabilis*, *P. aeruginosa* and GBS. Reprinted by permission from Springer Nature, Nature Reviews Microbiology (Flores-Mireles et al., 2015),

Factors affecting UTIs

Adherence: An infection generally starts around the urethral area by a pathogen, where it recruits itself and colonizes the urethra and somehow reaches the bladder. These steps present the requirement of pili or flagella to facilitate the attachment and migration. Bacteria identify the receptors present on the epithelium wall of the bladder through some adhesion molecules and intercede colonization. These uropathogens also produce toxin molecules and synthesize siderophores to make the environment more conducive for them to stick and spread (Flores-Mireles *et al.*, 2015, Nagamatsu *et al.*, 2015, Wright & Hultgren, 2006).

3.1.1 Treatment of UTIs

Antibiotics such as trimethoprim, ciprofloxacin, ampicillin and sulphamethoxazole are the most commonly used therapeutics for UTIs presently. However, there are increasing cases of recurrence and antibiotic resistance reported every day. There is a need for new and alternative therapies that will help the society recuperate from this health burden.

Multidrug resistance: Bacteria like *E.coli* and *K.pneumoniae* have acquired beta-lactamases related plasmids which help in spreading resistance to many antibiotics like cephalosporins (Chen *et al.*, 2013). This type of resistance is also commonly seen in enterococci (Chen *et al.*, 2013). They are naturally resistant to a number of antibiotics like penicillins and trimethoprim but now they have also started developing resistance to vancomycin which is considered as the last line of defense treatment (Pendleton *et al.*, 2013). Therefore there is a need to come up with alternative or combination therapies to deal with this problem. One of them is molecules targeting bacterial adhesion and D-mannose is one of them. Several small clinical trials have

shown that D-mannose is helpful in controlling severity and frequency of recurrent urinary tract infections.

3.1.2 D-mannose and UTIs

D-mannose is a sugar that plays a great role in glycosylation of proteins in humans. The rationale for mannose therapy is that uropathogenic bacteria bind to mannose-containing glycolipids and glycoproteins on the bladder epithelium(Mobley *et al.*, 2009). In one of the clinical studies, mannose was shown to be as effective as nitrofurantoin, an antibiotic used to prevent the recurrence of UTIs (Kranjcec *et al.*, 2014). However they do not report any results pertaining to the amount of D-mannose found in urine samples or any mechanism by which mannose is helping this prevention. Another study showed mannose treatment comparable to another antibiotic called as prulifloxacine (Palleschi *et al.*, 2017). It was also shown in patients with multiple sclerosis that regular D-mannose intake reduced UTI recurrence in patients using catheters (Phe *et al.*, 2017). A similar effectiveness resulting from D-mannose was also reported in acute cystitis patients (Vicariotto, 2014). There are more clinical trial studies that emphasize on the role of D-mannose in prevention of recurrence of these urinary tract infections but do not throw light on the mechanism or role of mannose in detail (Porru *et al.*, 2014, Panchev *et al.*, 2012).

3.1.3 Measurement of D-mannose

There are not much studies that report the concentration of mannose in urine. In a report to study anti-bacterial defense mechanisms in urinary bladder, high performance liquid chromatography (HPLC) was used to measure mannose in urine in 186 samples (Toyota *et al.*, 1989). Due to

technical difficulties because of unidentified peaks, they were only able to detect urine in 80 samples and measure it in 24 of those samples. They also reported that mannose is involved in preventing the binding of bacteria to the mucosa. Another report which measured mannose levels using gas chromatography-mass spectroscopy method had complicated sample preparation steps and variability was very high (Shoemaker & Elliott, 1991). There is no published paper that studies the effect of oral ingestion of mannose on urinary mannose concentration. Although there are reports on measurement of mannose in serum (Etchison & Freeze, 1997). There is a need to establish an assay which enables us to measure urinary mannose concentrations efficiently and reliably.

3.2 Enzymatic assay of D-mannose from urine

3.2.1 Abstract

Background: Several studies indicate that D-mannose ingestion reduces the frequency of urinary tract infections (UTIs). A reliable assay for urinary D-mannose is needed to assess the effects of supplemental D-mannose on urinary D-mannose and UTIs. **Results:** We developed an enzymatic assay for D-mannose in urine. Hexoses in urine were phosphorylated, sequentially isomerized, and oxidized, and the increases in NADPH were measured in a spectrophotometer. Urinary mannose from five volunteers was well above the detection limit and ranged from 8 to 700 μM. **Conclusions:** A rapid, reliable, and sensitive assay readily detected urinary D-mannose. If a correlation exists between urinary D-mannose and UTIs, then the assay could clinically assess susceptibility to UTIs and direct D-mannose therapy.

3.2.2 Introduction

Urinary tract infections (UTIs) are the most common bacterial infections in humans (Foxman, 2014). After an initial infection, the risk of a second infection is around 25-30% in the ensuing 6 months (Ejrnaes, 2011). Uropathogenic *E. coli* (UPEC) causes 80-90% of community-acquired UTIs, 30-50% of nosocomially-acquired UTIs, and most recurring UTIs (Ejrnaes, 2011, Foxman, 2014). Several small clinical trials consistently indicate that D-mannose (mannose) supplementation reduces the severity and frequency of UTIs (Domenici *et al.*, 2016, Porru *et al.*, 2014, Kranjcec *et al.*, 2014, Palleschi *et al.*, 2017, Phe *et al.*, 2017, Panchev *et al.*, 2012, Vicariotto, 2014). The rationale for mannose therapy is that uropathogenic bacteria bind to mannose-containing glycolipids and glycoproteins on the bladder epithelium (Mobley *et al.*, 2009). UPEC binding to the urothelium requires the FimH adhesin, which binds mannose with a dissociation constant of 2.3 μM (Bouckaert *et al.*, 2005). It is possible that women who are susceptible to UTIs have low urinary mannose, and mannose ingestion may help these women. Mannose supplementation is a non-antibiotic therapy, and such therapies are becoming increasingly important as UTIs become antibiotic resistant (Flores-Mireles *et al.*, 2015).

Only three sources report mannose concentrations in urine. The human metabolomic database reports a range from 12.5 to 166 μM (Wishart *et al.*, 2018). A high-performance liquid chromatography assay, which could measure mannose in only 13% of individuals, showed a range from 14 to 600 μM (Toyota *et al.*, 1989). A gas chromatography-mass spectroscopy assay had large variability and concentrations of 116 ± 154 μM (Shoemaker & Elliott, 1991). When measurements were reported as a ratio to creatinine, which is common for measurement of urine components, a creatinine concentration of 10 mM was used for the calculation. We could find no

published reports on the effects of mannose ingestion on urinary mannose. Mannose concentrations have been more often measured in serum, and in contrast to the results above, the range of values from serum is much smaller. Serum mannose concentrations ranged from 20 to 80 μM (Miwa & Taguchi, 2013, Sharma *et al.*, 2014, Wishart *et al.*, 2018). Oral mannose transiently increased serum mannose 3-fold, with a clearance $t_{½}$ of about 4 hours (Sharma *et al.*, 2014).

A variety of methods have been used to measure mannose in complex mixtures (Hu *et al.*, 2016, Miwa & Taguchi, 2013). Chromatographic methods often involve deproteinization, derivatization, and detection with expensive equipment. Enzymatic assays do not require these processing steps, and detection can use a spectrophotometer, which is relatively inexpensive. An enzymatic assay for mannose in serum has been described, and it required prior removal of glucose (Etchison & Freeze, 1997). The suitability of an enzymatic assay for urinary mannose has not been assessed. We describe a rapid and reliable enzymatic assay for urinary mannose that does not require sample processing and is not impaired by urinary components.

3.2.3 Experimental

Enzymes and reagents

Mannose for biochemical assays, glucose-6-phosphate dehydrogenase (G6PDH) from yeast (1750 U per ml), and hexokinase (HK) from yeast (1500 U per ml) were purchased from Sigma-Aldrich. Both enzymes were ammonium sulfate suspensions, and were dialyzed at 4ºC against 1000 volumes of 25 mM HEPES buffer (pH 8.0), 50 mM KCl, and 20% glycerol to remove the ammonium sulfate. The dialyzed enzymes were aliquoted in 200 μl batches and frozen at -80°C. Mannose for ingestion was from Now Foods.

Glucose-6-phosphate isomerase (PGI) was purified from *Escherichia coli* MG1655, a standard laboratory strain, with plasmid ECK4017 from the ASKA plasmid library (Kitagawa *et al.*, 2005). The plasmid codes for N-terminally His-tagged PGI that is expressed from an inducible *lac* promoter. The strain was grown overnight at 37°C in 4 ml of Luria-Bertani (LB) medium: a pH 7.0 mixture of 10 gm/l tryptone (Fisher Scientific), 10 gm/l NaCl, 5 gm/l yeast extract (Fisher Scientific), and 25 μg/ml chloramphenicol to maintain the plasmid. 1 ml from this overnight culture was inoculated in 250 ml LB medium in a one-liter flask, and shaken at 240 rpm for 12 hours on a platform shaker. The medium contained 1 mM isopropyl-β-D-thiogalactopyranoside to induce PGI expression. Cells were harvested by centrifugation at late exponential phase (A_{600} ~ 0.6), resuspended in 20 ml of 150 mM NaCl, centrifuged at 3,220 *g*, and the pellet was stored at −80°C. All subsequent steps were performed at 4°C. The pellet was resuspended in 10 ml PBS, and then sonicated in tubes partially submerged in an ice water bath using a Vibra-cell sonicator for seven 10-second bursts, with 1 min between intervals. The extract was centrifuged at 16,000 *g* for 10 minutes at 4°C to remove whole cells and insoluble material, and the supernatant was added to a column containing 100 μl HisPur™ cobalt resin (Thermo Scientific). Protein was bound and eluted by the gravity-flow method according to the manufacturer's instructions. The fractions were assayed for activity. Protein purity was visually estimated to be close to 100% after gel electrophoresis. The enzyme concentrations for G6PDH, HK, and PGI were 5 mg/ml, 3.3 mg/ml and 2.38 mg/ml respectively.

Mannose-6-phosphate isomerase (ManA) was expressed from *E.coli* strain JW2011, which has a deletion in the *gnd* gene (lacks 6-phosphogluconate dehydrogenase) (Baba *et al.*, 2006). JW2011 contained the *manA*-containing ASKA plasmid ECK1608 (Kitagawa *et al.*, 2005). The ManA-

containing strain (IM52) was grown as described above for the PGI-containing strain. Activity was readily detected from a crude extract, but ManA activity did not survive affinity chromatography. Therefore, the sonicated crude extract was used as the source of ManA. The deletion of the *gnd* gene was necessary to prevent a slow reaction that resulted in more than one NADPH for each mannose. HK, G6PDH, and PGI were free of 6-phosphogluconate dehydrogenase activity.

Urine

Collection of urine had institutional review board approval. Urine from female volunteers was collected mid-stream and immediately placed on ice. The samples were stored in aliquots at -80°C until analysis. Prior to assay, one aliquot was thawed, filter sterilized to remove particulates, and centrifuged at 16,000 *g* at 4°C for ten minutes.

Creatinine

Creatinine in urine was assayed by a standard laboratory procedure.

Mannose assay

Figure 3.2 describes the reactions involved in the assay. Glucose, fructose, and mannose were converted to the corresponding sugar-phosphates by HK. Glucose-6-phosphate, but not the other sugar-phosphates, was oxidized by G6PDH with the stoichiometric generation of NADPH. NADPH, but not $NADP^+$, absorbs at 340 nm (Lowry & Passonneau, 1972). The other sugar-phosphates were sequentially converted to glucose-6-phosphate by PGI and ManA. The A_{340} was measured in a Hitachi U-2000 spectrophotometer. The molar extinction coefficient of NADPH is 6270 (Lowry & Passonneau, 1972). The mM concentration of the sugar in the urine sample is the ΔA_{340} times the dilution factor divided by 6.27 (the mM extinction coefficient). For a dilution

factor of ten, this reduces to the ΔA_{340} x 1.593. For example, an ΔA_{340} of 0.100 indicates 0.159 mM sugar in urine. A ΔA_{340} of 0.005, or 8 µM mannose was considered the detection limit.

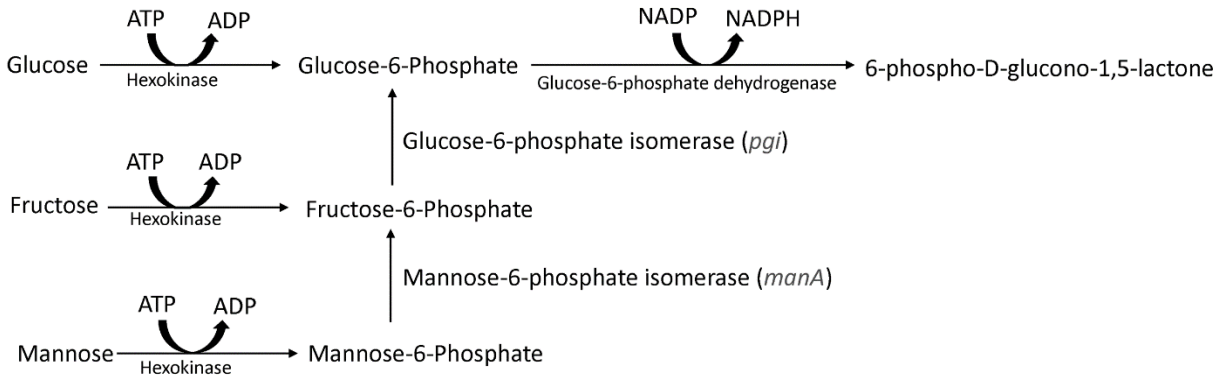

Figure 3.2: Schematic diagram of the assay for mannose.

The reactions for assay of one sample are summarized in Table 3.1. All reactions had a total volume of 1.0 ml and contained the following basic components: 50 mM HEPES buffer (pH 7.5), 2 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM $NADP^+$, 1 mM dithiothreitol, and 10 mM KCl. The reactions were incubated at 37°C for 45 minutes, after which A_{340} was measured. Tube A contains the basic components and 0.1 ml urine. Tube B contains tube A components, HK (1.5 units), and G6PDH (1.75 units). All the hexoses are phosphorylated, but only glucose-6-phosphate is converted to 6-phosphogluconate with NADPH formation. The ΔA_{340} between tubes A and B measures glucose. Tube C contains the tube B components, and PGI (7.1 µg), which converts fructose-6-phosphate to glucose-6-phosphate. The ΔA_{340} between tubes B and C measures fructose. Tube D contains the tube C components, but also contains ManA (5 µl extract), which converts mannose-6-phosphate to fructose-6-phosphate. The ΔA_{340} between tubes

C and D measures mannose. Tube E contains all the components of tube D, but also contains an addi tional 20 μmoles of D-mannose.

Table 3.1: The mannose assay components. The enzyme abbreviations are defined in the Experimental section.

Tube	G6PDH	HK	PGI	ManA	mannose	comment
A	-	-	-	-	-	background
B	+	+	-	-	-	B minus A measures glucose
C	+	+	+	-	-	C minus B measures fructose
D	+	+	+	+	-	D minus C measures mannose
E	+	+	+	+	+	E minus D is the linearity control

3.2.4 Results & Discussion

Linearity of mannose concentration and ΔA_{340}.

Figure 3.3 shows the linear relationship between mannose concentration and A_{340}. The correlation coefficient (R^2) was 0.996. Interfering factors in urine, such as light scattering, enzyme inactivation, or high background absorbance, could vary between urine samples and cause non-linearity in the assay. To control for this possibility in each urine sample, one reaction contained an additional 20 nmoles of mannose. This powerful control ensures that the ΔA_{340} was in the linear range (if the A_{340} increased by 0.125), and the urine in the reaction did not inhibit the enzymes. A non-linear response was observed when the ManA extract lost activity due to repeated freeze-thawing cycles. A new batch of enzyme solved the problem.

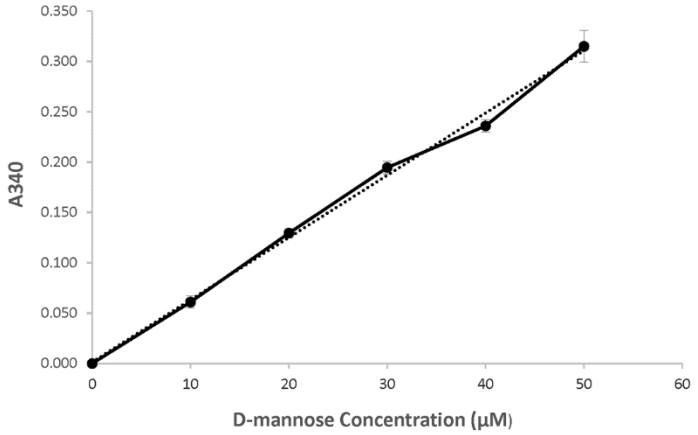

Figure 3.3: Linearity of A_{340} with mannose concentration. These reactions were performed in triplicate in separate tubes with different enzyme combinations as described in Table 3.1. The correlation coefficient (R^2) was 0.996.

The reactions can be in the non-linear range if urinary glucose is high, because the glucose concentration will result in a high A_{340} after addition of HK and G6PDH. In this case, not only is the high ΔA_{340} a problem, but the subsequent ΔA_{340} with ManA addition may be within experimental variation of the glucose measurement. The mannose control described above would detect such non-linearity. Nonetheless, this assay is not considered suitable for diabetics.

The effect of urea.

The normal range of urea, a protein denaturant, in human urine has been reported to be 342 ± 67 mM (Liu *et al.*, 2012). While performing the assay, urine is diluted tenfold, which will result in urea concentration of 34 ± 7 mM. ≤ 50 mM urea had little effect on the assay (Figure 3.4), which implies that normal urea concentrations should have no effect on the assay.

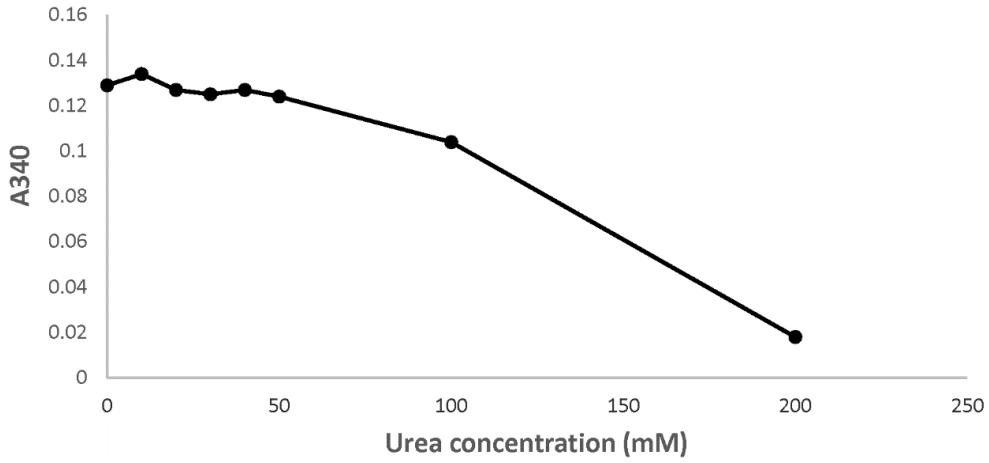

Figure 3.4: Effect of urea. 20 μM mannose was assayed as a function of urea concentration. The ΔA_{340} for 20 μM mannose should be 0.125.

Mannose levels in human urine.

Urine samples were taken from two female volunteers on two different days. The mannose levels for volunteer 1 were 470 and 710 μM, and for volunteer 2 were 230 and 450 μM. Several months later, volunteer 2 provided urine on 13 different days. The glucose, fructose, and mannose levels were 150 ± 77 μM, 83 ± 23 μM, and 62 ± 33 μM, respectively. From these volunteers, the mannose concentrations ranged from 8 to 710 μM. The 710 μM concentration is the highest reported, and may result from a procedure that does not require sample processing. Mannose concentrations from volunteer 2 ranged from 8 to 450 μM, which shows that appreciable variations can occur from one individual. These large ranges are consistent with previously published results, which show a range from 12 to 600 μM (Wishart *et al.*, 2018, Shoemaker & Elliott, 1991, Toyota *et al.*, 1989). Mannose levels were also assayed from three postmenopausal women with a UTI. Their urinary mannose levels were 13, 35, and 62 μM.

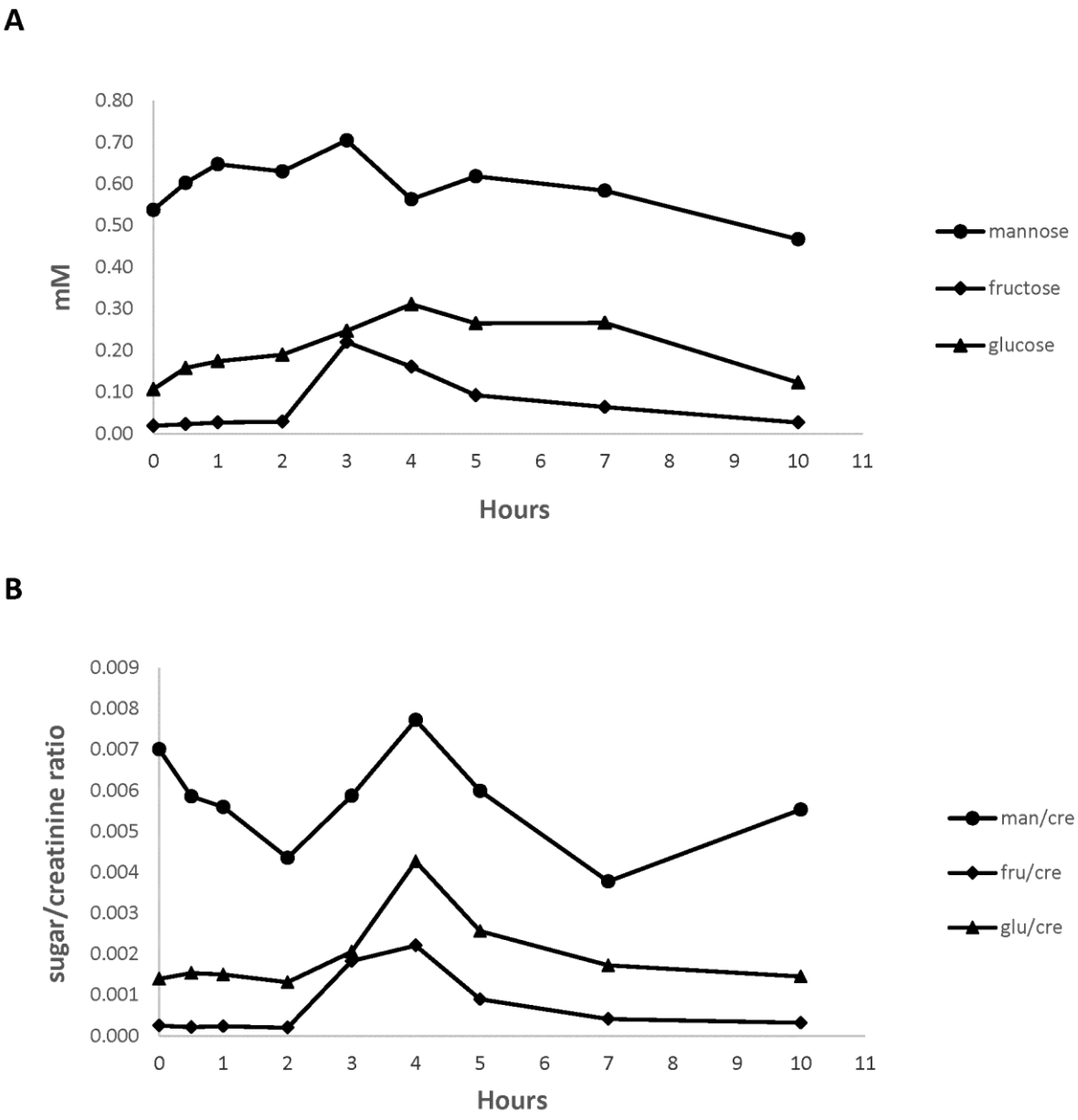

Figure 3.5 Urinary mannose after mannose ingestion. Urine samples were obtained from volunteer 1 several times up to ten hours after ingesting 2 grams of mannose. (A) Glucose, fructose, and mannose concentrations. (B) Sugar concentrations normalized to urinary creatinine concentrations

We conclude that urinary mannose levels were above the detection limit and readily measured. Furthermore, the urinary mannose concentrations were within a physiologically relevant range, since the dissociation constant between the FimH receptor and mannose is 2.3 μM.

Mannose levels after oral mannose ingestion.

Urine samples from the same two uninfected volunteers described above were taken from 0-10 hours after ingesting 2 grams of mannose. The mannose concentrations from volunteer 1 are shown in Figure 3.5A. These results are normalized to urinary creatinine in Figure 3.5B. Similar results were observed for volunteer 2. Oral intake of 2 grams of mannose had no obvious effect on urinary mannose. The basal urinary mannose levels at time zero were at the high end of the observed range. The lack of an obvious effect does not imply a similar effect for women with low basal mannose.

Suitability for high throughput analysis.

The assay is easily adapted for high throughput analysis, which requires smaller volumes and a plate reader instead of a standard spectrophotometer. The number of reactions can also be reduced, since tubes A and B can be omitted if glucose and fructose are not measured.

3.2.5 Conclusions

We developed an enzymatic assay for urinary mannose. The advantages of this procedure over previously published procedures are (a) reliability, because urine components do not inhibit the assay reactions; (b) speed, because of minimal sample processing and simple instrumentation; (c) sensitivity down to 8 μM; and (d) adaptability to high throughput analysis.

3.2.6 Future perspective

A rapid and reliable assay for urinary mannose is necessary to examine the relationship between mannose and UTIs. We will test whether women with UTIs, especially women with recurring UTIs, have low urinary mannose, and whether supplemental mannose increases urinary mannose. Positive results would imply that the assay can identify women who are susceptible to UTIs, especially elderly or hospitalized women, and that such women could benefit from mannose therapy. More nuanced results may suggest that low urinary mannose is necessary, but not sufficient, for UTIs. Oral mannose may not only affect urinary mannose, but also protein mannosylation (Alton *et al.*, 1998). Elevated mannosylation of urinary glycoproteins, such as the Tamm-Horsfall protein, may prevent binding of uropathogens to the bladder epithelium. Our assay could be used to assess the extent of glycoprotein mannosylation after appropriate sample processing. In summation, the mannose assay could become clinically important for assessing and determining mannose ingestion as a therapy option, especially when a recurring UTI becomes antibiotic resistant.

3.2.7 Executive summary

- Several clinical studies suggest that mannose ingestion reduces the frequency of UTIs.
- A possible mechanism by which mannose reduces UTIs is to prevent binding of bacteria to the bladder epithelium.
- The effect of mannose ingestion on urinary mannose is unknown.
- We developed an enzymatic assay for urinary mannose that is rapid and does not require sample processing.

- The assay involves hexose phosphorylation, isomerization to glucose-6-phosphate, oxidation to 6-phosphogluconate, and measurement of NADPH generation.
- The assay was linear with respect to mannose concentration, and linearity was tested in each urine sample by adding a known amount of mannose to the sample.
- Urea in the sample did not affect the reactions.
- Urinary mannose was readily detected from five female volunteers and ranged from 8 to 700 μM.
- The assay is adaptable to high throughput analysis, and will allow testing of the relationship between urinary mannose and UTIs.

Financial disclosure/Acknowledgements

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter.

Ethical conduct of research

The authors obtained institutional review board approval for the collection of urine samples. For the investigations involving human subjects, informed consent was obtained from the participants.

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CHAPTER 4

ANAEROBIC CYSTEINE CATABOLISM BY CUTR-REGULATED CUTA (YHAM/N) IN *ESCHERICHIA COLI* AND *SALMONELLA ENTERICA**

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Partial contribution to the publication

 [Journal of Bacteriology, Volume number 199, 2017, 10.1128/JB.00117-17]

Preface: This chapter is based on the joint work done by a team of researchers listed in the author list above. Only a partial contribution to the published manuscript was made by the author of this thesis. The strains IM35, IM36, IM37, IM38, IM39 and IM40 were constructed and all the experiments resulting in Figures 4.5A, 4.6A, 4.7A and 4.7B were performed by the author. The regulator *cutR* and operon *cutPA* were later renamed as *cyuR* and *cyuPA* respectively.

4.1 Abstract

Salmonella enterica has two CutR-activated enzymes that degrade cysteine: the aerobic CdsH and an unidentified anaerobic enzyme; *E. coli* only has the latter. To identify the anaerobic enzyme, transcript profiling was performed from *E. coli* without *cutR* and with overexpressed *cutR*. 37 genes showed at least a five-fold change in expression, and the *cutPA* (formerly *yhaOM*) operon had the greatest differential. Homology suggested that CutP and CutA are a cysteine transporter and an iron-sulfur-containing cysteine desulfidase, respectively. *E. coli* and *S. enterica cutA* mutants grown with cysteine generated substantially less sulfide and had a lower growth yield. Oxygen affected the CutR-dependent genes reciprocally: *cutP-lacZ* expression was higher anaerobically, whereas *cdsH-lacZ* expression was higher aerobically. In *E. coli* and *S. enterica*, anaerobic *cutP* expression required *cutR* and cysteine, and was induced by L-cysteine, D-cysteine, and a few sulfur-containing compounds. Loss of either CutA or RidA, both of which contribute to cysteine degradation to pyruvate, increased *cutP-lacZ* expression, which suggests that CutA modulates intracellular cysteine concentration. Phylogenetic analysis showed that CutA homologs are present in obligate and facultative anaerobes, confirming an anaerobic function, and in archaeal methanogens and bacterial acetogens, suggesting an ancient origin. Our

results show that CutA is the major anaerobic cysteine catabolic enzyme in both *E. coli* and *S. enterica*, and it is proposed that anaerobic cysteine catabolism can contribute to coordination of sulfur assimilation and amino acid synthesis.

4.2 Importance

Sulfur-containing compounds such as cysteine or sulfide are essential and reactive metabolites. Exogenous sulfur-containing compounds can alter the thiol landscape and intracellular redox reactions, and are known to affect several cellular processes, including swarming motility, antibiotic sensitivity, and biofilm formation. Cysteine inhibits several enzymes of amino acid synthesis, and so increasing cysteine could increase the levels of the inhibited enzymes. This inhibition implies that control of intracellular cysteine, which is the immediate product of sulfide assimilation, can affect several pathways and coordinate metabolism. For these and other reasons, cysteine and sulfide concentrations must be controlled, and this work shows that cysteine catabolism contributes to this control.

4.3 Introduction

Cysteine and sulfide are metabolically related and reactive, and either can have cytotoxic or beneficial effects. Cysteine can impair bacterial growth (Harris, 1981, Sorensen & Pedersen, 1991), inhibit several enzymes (e.g., threonine deaminase and homoserine dehydrogenase I) (Reitzer, 2005), enhance oxidative stress (Berglin *et al.*, 1982, Park & Imlay, 2003), stimulate swarming motility, and increase antibiotic resistance (Turnbull & Surette, 2008, Turnbull & Surette, 2010). Sulfide inhibits several enzymes (e.g., cytochrome oxidases and other metalloenzymes) (Lloyd, 2006, Korshunov *et al.*, 2016), ameliorates antibiotic-induced oxidative stress (Shatalin *et al.*, 2011), may serve as a regulatory signal for intestinal tract bacteria (Lloyd,

2006), and reacts with sulfhydryls to form persulfides (RSSH), which may modify regulatory proteins and affect gene expression (Lu *et al.*, 2013, Ida *et al.*, 2014).

Intracellular levels of cysteine are controlled by synthesis (Kredich, 1996), import and export (Dassler *et al.*, 2000), and possibly degradation (Oguri *et al.*, 2012). Cysteine catabolism is the least studied, yet several enzymes have been proposed to degrade cysteine in *E. coli* and *S. enterica*. CdsH is a pyridoxal-5'-phosphate-dependent cysteine desulfhydrase that degrades cysteine aerobically to pyruvate, ammonia, and sulfide in *S. enterica*, but not in *E. coli* (Oguri *et al.*, 2012, Guarneros & Ortega, 1970, Kredich *et al.*, 1972, Collins & Monty, 1973). *E. coli* has at least five enzymes with cysteine desulfhydrase activity as a secondary activity: TnaA, MetC, CysK, CysM, and MalY (Awano *et al.*, 2005), but their total activity (27 nmoles min^{-1} mg^{-1} protein at 37ºC) is much less active than CdsH in *S. enterica* (260 nmoles min^{-1} mg^{-1} protein at 23ºC) (Awano *et al.*, 2005, Kredich *et al.*, 1972).

The products of cysteine catabolism are often depicted as pyruvate, ammonia, and sulfide; however, cysteine catabolism is actually a two-step process. Cysteine desulfhydrase degrades cysteine to sulfide and the toxic intermediate 2-aminoacrylate (an enamine), and while 2-aminoacrylate may spontaneously decompose, RidA degrades 2-aminoacrylate to pyruvate and ammonia (Ernst *et al.*, 2014). A different two-step pathway of cysteine catabolism has also been proposed in which aspartate transaminase deaminates cysteine to 3-mercaptopyruvate which SseA (a mercaptopyruvate sulfurtransferase) further cleaves to sulfide and pyruvate (Shatalin *et al.*, 2011).

Loss of CdsH in *S. enterica* eliminated aerobic cysteine degradation, but had no effect on anaerobic cysteine degradation (Oguri *et al.*, 2012). The activator CutR, which is homologous to

the leucine-responsive regulatory or Lrp, is required for synthesis of both CdsH and the anaerobic enzyme (Oguri *et al.*, 2012). The goals of this work were to identify and characterize the regulation and function of the CutR-regulated gene for the anaerobic cysteine catabolic enzyme in *S. enterica* and *E. coli*.

4.4 Materials and Methods

Bacterial strains. *E. coli* and *S. enterica* strains used in this study are listed in Table 4.1. The deletion alleles for *E. coli* strains were obtained from Keio collection strains (Baba *et al.*, 2006), and then transduced into various strains with phage P1. All results were reported from a strain W3110 background. *S. enterica* gene deletions were constructed by the one-step inactivation method in strain TT22971 as described (Datsenko & Wanner, 2000), and alleles with the antibiotic resistance cassettes were transduced with P22 into TR10000. For both *E. coli* and *S. enterica* strains, the antibiotic resistance cassettes were removed as described (Datsenko & Wanner, 2000). The primers for the *cutP/yhaO* deletion were GAATTTTATG GAATCGGCAA GTAATACCAG CGTAATCCTC ATTCCGGGGA TCCGTCGACC and AATGCCAGGA ACGGAGAAAC GCATAGCAGC AGACCGGTAA GTGTAGGCTG GAGCTGCTTC. The primers for the *cutA/yhaN* deletion were TCCATTGTGG CAAAGTTTTA TTCTGGCCGT ACAGGAAGAA ATTCCGGGGA TCCGTCGACC and ATAATCTCGA TGATCTGCTT GTCGGTCTGC TGCATTGAGC GTGTAGGCTG GAGCTGCTTC. The subsequent *S. enterica* strains were shown to be phage-free by cross-streaking against P22-H5 on green-agar plates (Chan & Botstein, 1976).

Bacterial growth

(i) Media. Cultures for enzyme assays were grown in Luria-Bertani (LB) broth with a variety of supplements, whereas cells for analysis of growth characteristics were grown in a modified LB, which was called 25% LB. 25% LB contained 0.25X LB with 5.25 g/liter K_2HPO_4, 2.25 g/liter K_2HPO_4, and 0.025 g/liter $MgSO_4$. When added, supplements were from concentrated stocks, except for the more insoluble supplements which were added directly to the culture. Supplemental amino acids were L-amino acids, unless otherwise indicated.

(ii) Overnight cultures. Prior to experiments, cells were streaked for singles from frozen stocks to check for uniformity. All experiments for growth analysis or enzyme assays involved cells from three separate single colonies. Shaking overnight cultures were grown in 20 ml culture tubes with 4 ml of LB without supplements. The cultures were placed in a test tube rack on a rotating shaker (240 rpm) at 37ºC. The overnight cultures were probably microaerobic or anaerobic.

(iii) Culture conditions for growth rates and enzyme assays. Overnight cultures were diluted so that the initial turbidity was five klett units or less ($\leq$0.03 OD_{600}), and cultures for enzyme assays were grown to at least 50 klett units in order to establish steady state aerobic or anaerobic growth. Anaerobic cultures were grown in 9 ml screwcap tubes containing 8.5 ml of medium, and incubated in a 37ºC water bath in a test tube rack without agitation. The tubes were inserted directly into a colorimeter to measure turbidity. Aerobic cultures contained 10 ml of medium in 125 ml sidearm flasks, which allows turbidity measurements, and shaken at 240 rpm at 37ºC. Turbidity was measured using a Scienceware Klett colorimeter with a 54 filter. 100 klett units are about 0.7 OD_{600}.

Table 4.1: Strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Source or reference
S. enterica		
TR10000	*S. enterica* serovar Typhimurium LT2	48
TO1	TR10000 Δ*cdsH*	15
TO2	TR10000 Δ*cdsH* Δ*metC*::*cat*	15
TO3	TR10000 Δ*cutR*	15
TO11	TR10000 *att*λ::[Φ(*cdsH'-lacZ*) Kan^r]; *cdsH* region from −138 to +57***a***	15
TO20	TR10000 Δ*cutP* (Δ16-432/443)***b***	This study
TO21	TR10000 Δ*cutA* (Δ21-416/436)***b***	This study
BLS143	TR10000 *att*λ::[Φ(*cutP'-lacZ*) Kan^r]; *cutP* region from −381 to +44***a***	This study
BLS145	BLS143 Δ*cdsH*	This study
BLS147	BLS143 Δ*cutR*	This study
BLS149	BLS143 Δ*cutA*::*cat*	This study
E. coli		
MG1655	K-12 reference strain	Laboratory stock
ML1	MG1655 Δ*cutR*	This study

W3110	K-12 *lacL8 lacI*^{q}	Laboratory stock
ML2	W3110 Δ*cutR*	This study
ML3	W3110 Δ*cutP*	This study
ML4	W3110 Δ*cutA*	This study
BW25113	K-12 reference strain	38
JW2505	BW25113 Δ*sseA*::*kan*	37
JW3467	BW25113 Δ*gor*::*kan*	37
ML5	W3110 Δ*aspC*	This study
PL41	W3110 Δ*aspC* Δ*tyrB*	This study
JOEY281	MG1655 *attλ*::[Φ(*cutP′-lacZ*) Kan^{r}]; *cutPA* region from −306 to +100***a***	Stephen Spiro
ML6	W3110 *attλ*::[Φ(*cutP′-lacZ*) Kan^{r}]	This study
ML7	ML6 Δ*cutR*	This study
IM35	ML6 Δ*arcA*::*kan*	This study
IM36	ML6 Δ*arcB*::*kan*	This study
IM37	ML6 Δ*ridA*::*kan*	This study
IM38	ML6 Δ*soxR*::*kan*	This study
IM39	ML6 Δ*ggt*::*kan*	This study
IM40	ML6 Δ*cutA*::*kan*	This study

Plasmids		
pCP20	FLP (FRT-specific) recombinase; Amp^r, Cam^r ***c***	38
p*cutR*	Same as JW0437; pCA24N carrying *cutR*	49
pBLS18	*lacZ* transcriptional fusion vector; Kan^r	50
pBLS45	[Φ(*cutP′-lacZ*)] Kan^r in pBLS18; *cutP* region from −381 to +44 ***a***	This study

Non-standard chemical. *S*-Nitrosocysteine was synthesized and quantified as described (Lonart & Johnson, 1995, Grossi & Montevecchi, 2002).

Enzyme assays and sulfide detection. Aerobic cysteine degradation was qualitatively assessed with an activity stain that detected sulfide production (Awano *et al.*, 2003, Oguri *et al.*, 2012). Sonicated cell extracts were run into a non-denaturing gel, and incubated with cysteine, pyridoxal-5'- phosphate and $BiCl_3$. Bismuth sulfide forms a black precipitate at the site of the enzyme.

Three different qualitative methods (assays 1-3) and one quantitative method (assay 4) detected anaerobic cysteine-dependent sulfide production. The qualitative methods produced a black metal sulfide precipitate. Assay 1 was a stab culture assay in which cells were stabbed into tubes filled with LB containing 0.6% agar, 0.1% $FeSO_4 \cdot 7\ H_2O$ and 5 mM cysteine (Oguri *et al.*, 2012). Assay 2 was an overlay plate assay with the following layers from top to bottom: LB in 0.6% agar, a filter disc with 5 µl 1 M cysteine and 5 µl 10% $FeSO_4 \cdot 7\ H_2O$, 0.5 ml cells from an overnight culture mixed with LB in 0.6% agar, and LB in 1.5% agar (Oguri *et al.*, 2012). Assay 3 detected sulfide from lead acetate strips (Fisher Scientific), which was sealed on top of an 1.7 ml

Eppendorf centrifuge tube. Cells were grown in the tube containing 1.3 ml LB and 0.5 mM cysteine without shaking (Shatalin *et al.*, 2011, Oguri *et al.*, 2012). Assay 4 measures the sulfide-dependent reaction with *N*'-*N*'-dimethyl-*p*-phenylenediamine to form methylene blue, which was measured at 670 nm (Soutourina *et al.*, 2001).

β-Galactosidase assays were performed as previously described (Kim *et al.*, 2010). Specific activity is nmoles min^{-1} mg^{-1} total protein. The numbers are the mean ± the standard deviation from three separate determinations.

Growth and RNA isolation for microarray analysis. For RNA isolation, 4 ml overnight cultures (see section on overnight cultures) were grown in LB then diluted 100-fold into 25 ml of LB. The cultures were grown aerobically, and at OD_{600} 0.3 cells were challenged with 5 mM cysteine for 30 minutes. 500 µl of cells were then incubated with 2.5 ml of RNA protect (Qiagen), the mixture centrifuged (14,000 x g for 5 min at 4°C), and total RNA purified using the RNEasy mini kit and On-Column DNase kit (Qiagen).

RNA was extracted from ML2 (Δ*cutR*) and W3110 (wild type) with the p*cutR* plasmid. Ten µg RNA was used for the microarray analysis. The cDNA synthesis, labeling, hybridization to the Affymetrix *E. coli* gene chip were performed by the Core Microarray Facility at UT Southwestern Medical Center (Dallas, TX), and the data was processed by RMA and LIMMA from the Bioconductor package (Irizarry *et al.*, 2003, Ritchie *et al.*, 2015, Huber *et al.*, 2015). The complete results have been submitted to NCBI.

Phylogenetic analysis of CutA in archaea and bacteria. The protein sequences were collected from the database of Clusters of Orthologous Groups (COGs) (ftp://ftp.ncbi.nih.gov/pub/COG/COG2014/static/byCOG/COG3681.html). Only organisms

without multiple paralogs were selected for tree construction. The rooted UPGMA tree was constructed with PHYLIP software (version 3.69; J. Felsenstein, Department of Genome Sciences, University of Washington, Seattle [http://evolution.genetics.washington.edu/phylip.html]). 100 bootstrapping samples were used to estimate the robustness of the tree topology.

4.5 Results

CutR activates the major anaerobic pathway of cysteine catabolism in both *E. coli* and *S. enterica*. Anaerobic cysteine degradation was readily detected by cysteine-dependent sulfide production from *S. enterica* with a stab culture assay (Figure 4.1A) and an overlay assay (Figure 4.1B). Neither assay detected cysteine-dependent sulfide production from wild type *E. coli* (Figure 4.1A and (Oguri *et al.*, 2012)), which was instead detected with lead acetate strips above the medium inside closed microcentrifuge tubes, when the culture contained at least 0.5 mM cysteine (Figure 4.1C).

Loss of CutR eliminated anaerobic cysteine-dependent sulfide production from both *E. coli* and *S. enterica* (Figure 4.1ABC), and *E. coli cutR* on a plasmid largely restored sulfide production from *S. enterica* (Figure 4.2B). The reason for only partial restoration is unknown, but could result from a non-wild type level of CutR. Previous results showed that aerobic CdsH expression in *S. enterica* requires CutR (Oguri *et al.*, 2012), and *E. coli cutR* restored CdsH activity in an *S. enterica* Δ*cutR* mutant (Figure 4.2A). These results show that the *E. coli* and *S. enterica* CutRs are interchangeable, and that CutR is likely to regulate the same anaerobic cysteine catabolic enzyme in both organisms.

Transcript profiling of potential *cutR*-regulated genes in *E. coli*. The gene for the CutR-regulated anaerobic enzyme was analyzed initially from *E. coli* because of the available microarray chip. To identify CutR-regulated genes, transcripts were analyzed from an *E. coli* Δ*cutR* strain and a parental wild type *E. coli* strain with *cutR* on a plasmid. qRT-PCR analysis indicated that the level of CutR in the plasmid-containing strain was ~55 times higher than that found in a wild type strain (M. Loddeke, unpublished observation). Cells in exponential phase were grown aerobically at 37ºC in LB, and challenged with 5 mM cysteine for 30 minutes (see Methods for more details). Although the goal was to identify the gene for an anaerobic enzyme, we assumed that overexpression of *cutR* in an aerobic envirorment would permit detection of an anaerobically expressed gene. In addition, a secondary goal was to identify other CutR-regulated genes involved in metabolism of *S*-containing compounds.

Plasmid *cutR* increased expression of 20 genes at least five-fold, and diminished expression of 17 genes at least five-fold (supplemetal data-not shown). The two most up-regulated genes were the adjacent *yhaO* (94-fold) and *yhaM* (56-fold) (supplemental data-not shown); qRT-PCR analysis confirmed this regulation and showed a 40-fold difference for these transcripts with and without overproduced CutR (M. Loddeke, unpublished data).

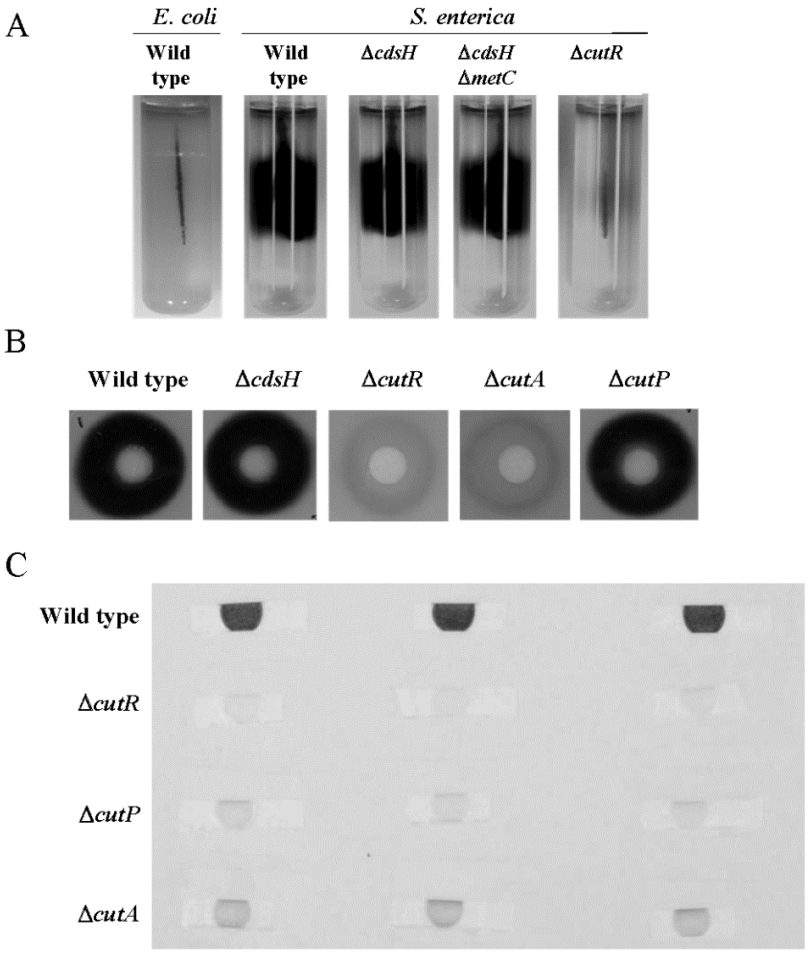


Figure 4.1: Anaerobic cysteine-dependent sulfide generation. (A) Sulfide detection with the stab method. (B) Sulfide detection from *S. enterica* strains with the overlay method. (C) Sulfide detection from *E. coli* strains with the lead acetate strip method in triplicate. The *S. enterica* strains are TR10000 (wild type), TO1 (Δ*cdsH*), TO2 (Δ*cdsH* Δ*metC*), TO3 (Δ*cutR*), TO20 (ΔcutP), and TO21 (Δ*cutA*). The *E. coli* strains are W3110 (wild type), ML2 (Δ*cutR*), ML3 (Δ*cutP*), and ML4 (Δ*cutA*).

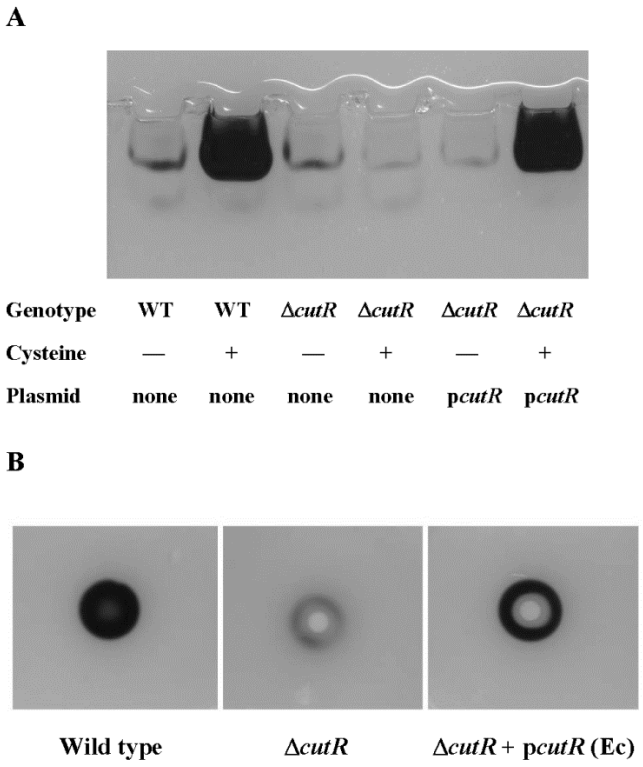


Figure 4.2: Complementation of *S. enterica* Δ*cutR* strain with a plasmid containing *E. coli cutR*. Strains TR10000 (wild type) and TO3 (Δ*cutR*) were used. (A) Aerobic cysteine-dependent sulfide production. Cysteine desulfhydrase (CdsH) activity in a non-denaturing gel from aerobic *S. enterica* extracts. The presence of the inducer cysteine and a plasmid containing *cutR* from *E. coli* are indicated. (B) Anaerobic sulfide generation from *S. enterica* by the agar overlay method.

The other genes activated at least five-fold were four genes of aromatic amino acid metabolism (both genes of the *tyrA-aroF* operon, *aroP*, and *aroL*), and several genes of carbohydrate metabolism (*lacI*, two genes of the *srlA* operon, and all the genes of the *uidABC* and *dhaKLM* operons). Six of the repressed genes degrade glucarate and galacturate – *gudP* and the adjacent *garPLRK* and *garD* operons. Nine of the down-regulated genes are in the maltose regulon: *malEFG*, *malK-lamB-malM*, *malPQ*, and *malS*. Except for *yhaO* and *yhaM* which are described below, none of the *cutR*-regulated genes with a five-fold change in expression has an obvious

connection to cysteine or sulfur metabolism, or has C-S lyase activity (i.e., degrades cysteine) as a secondary activity. A possible explanation for most of the apparent CutR control is that overexpressed CutR artifactually interacts with other regulators: an interfering interaction with two activators, CdaR and MalT, could explain repression of 15 of the 17 most repressed genes.

The highly regulated *yhaO* and *yhaM* will be shown to participate in cysteine catabolism, and we propose the designations *cutP* (**c**ysteine **ut**ilization **p**ermease) for *yhaO* homologs, and *cutA* (**c**ysteine **ut**ilization **A**) for *yhaM* homologs for both *E. coli* and *S. enterica*. Alternate names exist for *cutP* (*cdsA* and *dlsT*), *cutA* (*cdsB*), and *cutR* (*decR*) (Mendez *et al.*, 2011, Shimada *et al.*, 2016, Connolly *et al.*, 2016). The *Y. ruckeri* designations of *cdsA* and *cdsB* (for *cutP* and *cutA*) were first (Mendez *et al.*, 2011), but the *E. coli cdsA* designation is already taken for the gene for CDP-diglyceride synthetase of phospholipid metabolism. The next chronological precedent is the designation of *cutR* for the cysteine utilization regulator (Oguri *et al.*, 2012), and we propose the designations *cutR*, *cutP*, and *cutA* to indicate their involvement in cysteine utilization.

Homology suggests *E. coli* CutP and CutA specify a cysteine transporter and a cysteine desulfidase. *E. coli* CutP is a putative 422-residue protein with 87% amino acid identity with *Y. ruckeri* CdsA which transports cysteine (Mendez *et al.*, 2011). CutP is different from the two previously described cysteine/cystine transporters in *E. coli*. The *E. coli* TcyJ-TcyL-TcyN system transports both cystine and diaminopimelate, is sensitive to osmotic shock (Deutch *et al.*, 2014, Berger & Heppel, 1972, Quadroni *et al.*, 1996, Kertesz, 2001), and probably corresponds to the osmotic shock-sensitive CST-1 in *S. enterica* (Baptist & Kredich, 1977). The *E. coli* TcyP transporter is specific for cystine and resistant to osmotic shock (Berger & Heppel, 1972, Deutch

et al., 2014). Regulation is a major distinguishing feature of these systems: cysteine limitation and CysB control TcyJLN and TcyP (Quadroni *et al.*, 1996, Deutch *et al.*, 2014, Baptist & Kredich, 1977, Chonoles Imlay *et al.*, 2015), whereas exogenous cysteine and CutR regulates CutP.

E. coli CutA is a putative 436-residue protein with homology to YhaN from *S. enterica* (84% amino acid identity), CdsB from *Yersinia ruckeri* (67% identity), and MJ1025 from the archaea *Methanocaldococcus jannaschii* (25% identity) (Mendez *et al.*, 2011, Tchong *et al.*, 2005). The *M. jannaschii* CutA homolog catalyzes the same reaction as cysteine desulfhydrase, but the former was called cysteine desulfidase because it contains an iron-sulfur cluster instead of pyridoxal-5'-phosphate (Tchong *et al.*, 2005). The *M. jannaschii* cysteine desulfidase contains four conserved cysteines that bind the iron-sulfur cluster, and these cysteines are present in the *E. coli* and *S. enterica* homologs (Tchong *et al.*, 2005).

Anaerobic cysteine catabolism: role of CutA and CutP

(i) Cysteine-dependent sulfide production. *E. coli* and *S. enterica* Δ*cutA* mutants produced little sulfide when grown anaerobically for one day in LB with cysteine (Figures 4.1BC), although the *E. coli* mutants did produce sulfide after two days (not shown). A quantitative assay showed that the *S. enterica* Δ*cutA* mutant produced only 43% of the sulfide as a wild type strain in LB with cysteine. An *E. coli* Δ*cutP* mutant failed to produce sulfide from cysteine, in contrast to an *S. enterica* Δ*cutP* mutant which did produce sulfide (Figure 4.1BC).

An alternate cysteine catabolic pathway proposes that aspartate transaminase deaminates cysteine and generates 3-mercaptopyruvate, which is further degraded to pyruvate and sulfide by SseA, a 3-mercaptopyruvate sulfurtransferase (Shatalin *et al.*, 2011). However, an *E. coli* Δ*sseA*

mutant and a Δ*aspC* Δ*tyrB* double mutant, which lacks all aspartate transaminase activity, generated wild type levels of sulfide both anaerobically and aerobically after one day (not shown).

(ii) Anaerobic cysteine sensitivity. Anaerobic growth with and without cysteine was examined in broth instead of glucose-containing minimal medium because glucose represses anaerobic sulfide production from cysteine (Oguri *et al.*, 2012). Growth inhibition was observed in 25% LB (see Methods for medium composition), but not in 100% LB (not shown). 10 mM cysteine inhibited growth of *E. coli cutR* (triangles) or *cutA* (diamonds) mutants, but had no effect on Δ*cutP* (squares) or wild type (circles) strains (Figure 4.3). Cysteine (≥ 5 mM) inhibited the growth yield of *S. enterica* Δ*cutR* or *cutA* mutants (Figure 4.4DE), but 2 mM cysteine did not inhibit (not shown). Cysteine actually stimulated growth of *S. enterica* wild type, Δ*cutP*, and Δ*cdsH* strains (Figure 4.4ABC). The growth stimulation did not result from cysteine catabolism, since neither pyruvate, ammonia nor sulfide stimulated growth; sulfide was actually inhibitory (Figure 4.4F).

Transcriptional regulation of *cutPA* expression. To examine *cutA* expression, β-galactosidase was assayed from strains with a *cutP-lacZ* fusion. The *cutP* promoter probably controls *cutA* expression because (a) these genes are separated by only 25 bases in *E. coli*, which suggests that *cutP* and *cutA* probably form an operon, and (b) a global analysis of *E. coli* transcription start sites indicates a *cutP* transcription start site that is 159 bases from the start codon, but no start site for *cutA* (Thomason *et al.*, 2015).

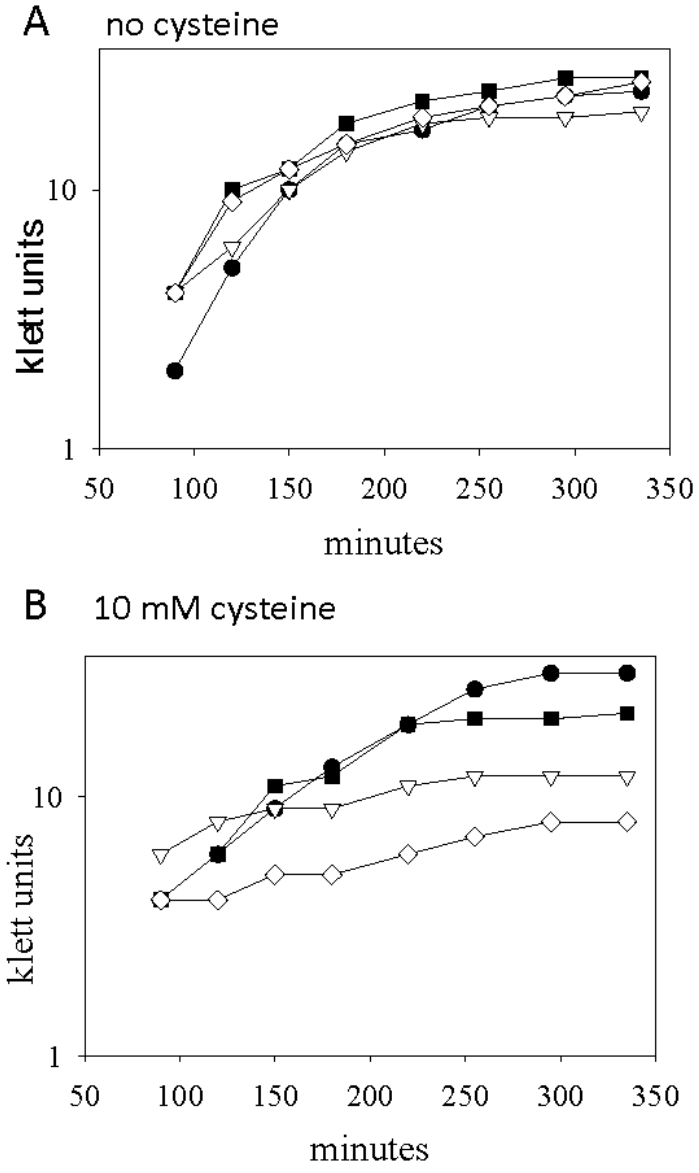


Figure 4.3: Anaerobic growth of *E. coli* strains with and without cysteine. Anaerobic growth cultures containing 25% LB medium without exogenous cysteine (panel A) or with 10 mM cysteine (panel B). The results are from one experiment. This experiment was done three times with similar results. The strains used were W3110 (wild type) (filled circles), ML2 (Δ*cutR*) (open inverted triangles), ML3 (Δ*cutP*) (filled squares), and ML4 (Δ*cutA*) (open diamonds).

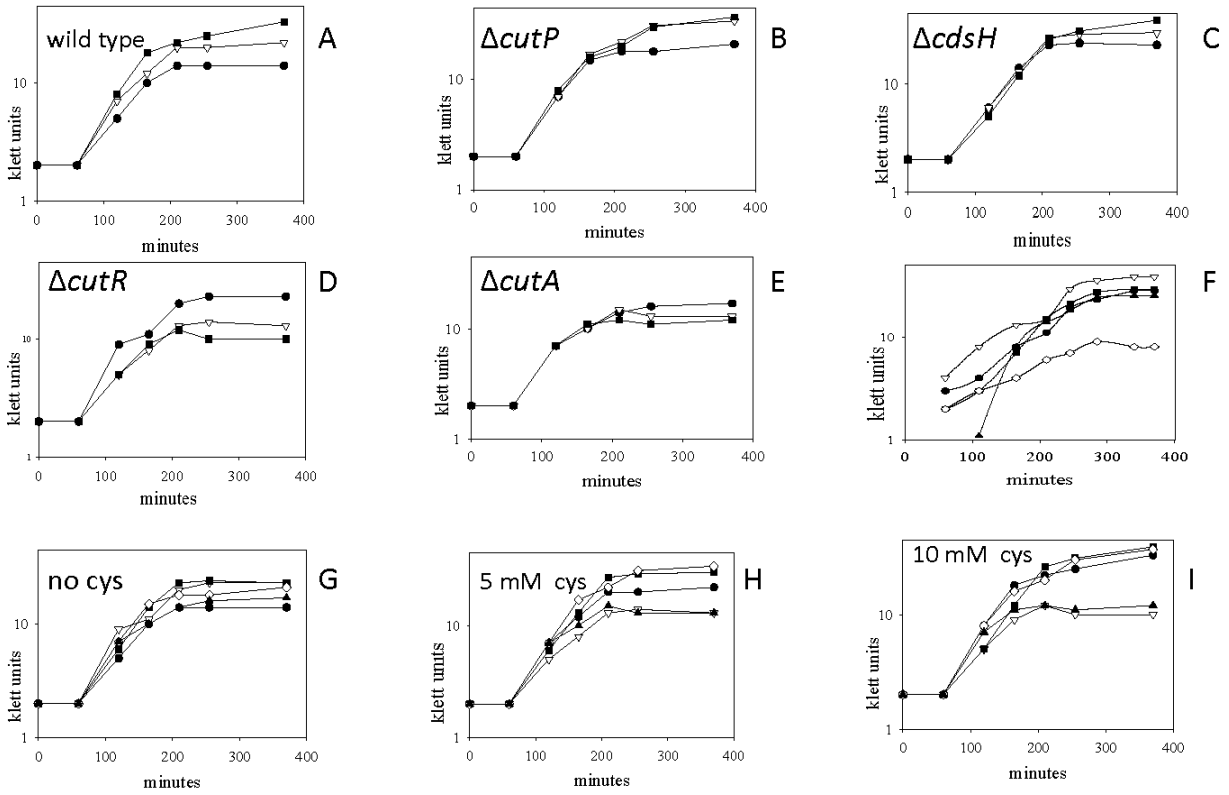


Figure 4.4: Anaerobic growth of *S. enterica* strains with and without cysteine. Panels A-E: Mutants grown with no exogenous cysteine (filled circles), 5 mM cysteine (inverted triangles), and 10 mM cysteine (filled squares). Panel F: Wild type *S. enterica* grown with no cysteine (filled circles), 5 mM cysteine (open inverted triangles), 5 mM pyruvate (filled squares), 5 mM Na2S (open diamonds), 5 mM (NH4)2SO4 (filled triangles). Panels G-I: Comparison of mutants with 0, 5, and 10 mM cysteine. Wild type (filled circles), Δ*cutP* (open diamonds), Δ*cutA* (filled triangles), Δ*cutR* (open inverted triangles), and Δ*cdsH* (filled squares). Anaerobic growth cultures contained 25% LB medium with the indicated supplement. The results are from one experiment. This experiment was done three times with similar results. The strains used were TR10000 (wild type), TO1 (Δ*cdsH*), TO3 (Δ*cutR*), TO20 (Δ*cutP*), and TO21 (Δ*cutA*).

Transcriptional and translational *cutP-lacZ* fusions were integrated into *E. coli* and *S. enterica* chromosomes, but no difference was observed between the two types of fusions, and results from only the transcriptional fusions are shown.

(i) Dependence on CutR and cysteine. Anaerobic *cutP-lacZ* expression required cysteine and CutR in both *E. coli* (Figure 4.5A) and *S. enterica* (Figure 4.5B): the numbers above the bars give the ratio with and without cyteine. Expression of *E. coli cutP-lacZ* was five times higher from stationary phase cells (7,000 ± 500 units; Figure 4.5A) than from exponential phase cells (1,200 ± 120 units; Figure 4.6A), and cysteine induced to essentially the same extent: 24-fold and 20-fold, respectively. It should be noted that induction occurred in a medium in which cysteine is not toxic (100% LB).

A **B**

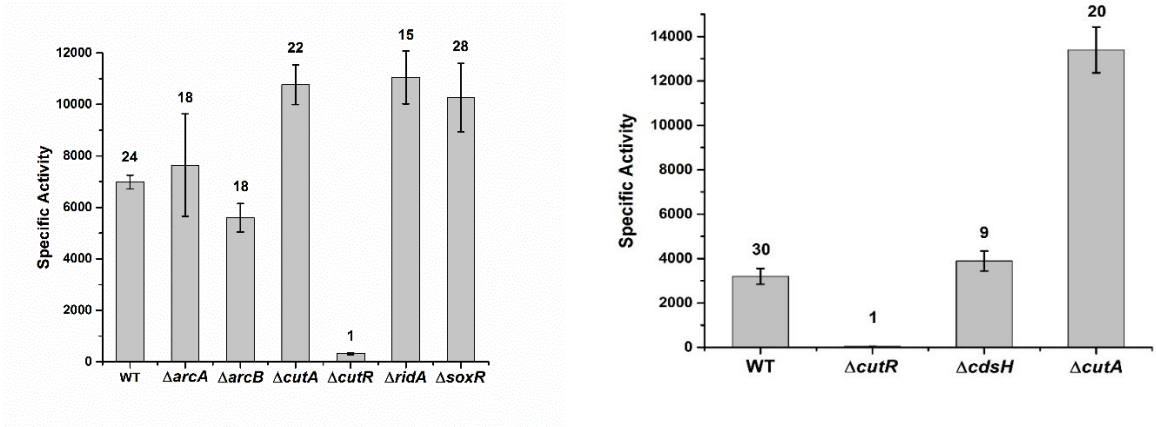


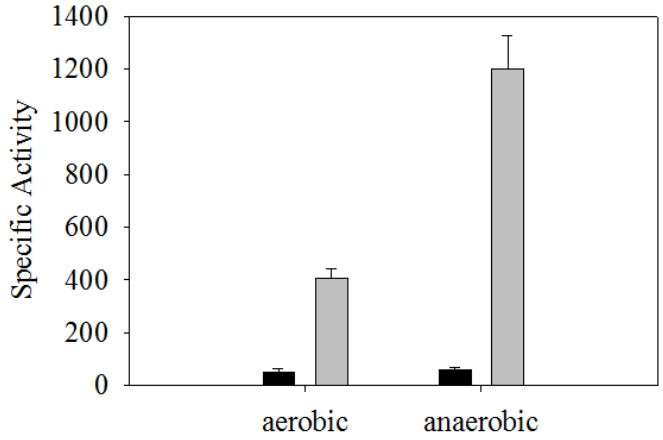
Figure 4.5: Anaerobic *cutP-lacZ* expression in *E. coli* and *S. enterica* mutants. The number over the bar is the ratio of activity with and without 2 mM cysteine. (A) *E. coli* mutants. Cells for β-galactosidase activity were harvested after overnight incubation (in stationary phase). The strains used were ML6, ML7, IM35, IM36, IM37, and IM38. (B) *S. enterica* mutants. Cells were harvested in late exponential phase (~ 40 Klett units). Strains used were BLS143, BLS145, BLS147, and BLS149.

Further confirmation of control by cysteine comes from observations of elevated *cutP-lacZ* expression in *cutA* and *ridA* mutants, which have impaired cysteine catabolism and presumably higher intracellular cysteine. For a *cutA* mutant grown in 2 mM cysteine, *cutP-lacZ* expression was 50% higher in *E. coli* (Figure 4.5A) and four-fold higher in *S. enterica* (Figure 4.5B). Even

without exogenous cysteine, *S. enterica cutP-lacZ* expression was 6.3-fold higher in a Δ*cutA* mutant (670 ± 75 units) than from a wild type strain (106 ± 4 units), which suggests that intracellular cysteine is higher in the *cutA* mutant. Similar results were observed from a mutant without RidA, which degrades the enamine/imine intermediates in the two-step conversion of cysteine to pyruvate (Ernst *et al.*, 2014). Loss of *ridA* in *E. coli* increased *cutP-lacZ* expression 1.6 fold ($p = 0.02$) in medium with 2 mM cysteine (Figure 4.5A), and 2.5 fold without exogenous cysteine (720 ± 20 units from the *ridA* mutant versus 290 ± 25 units from the wild type strain). The probable explanation is that loss of RidA results in enamine/imine accumulation, which in turn inhibits CutA, and increases intracellular cysteine. The results with the *cutA* and *ridA* mutants not only confirm control by cysteine, but also indicate that CutA and RidA modulate the intracellular cysteine concentration. In contrast to these results, loss of the aerobic CdsH did not affect *cutP-lacZ* expression (Figure 4.5B).

(ii) Aerobic versus anaerobic regulation of *cutPA* and *cdsH*. Anaerobic *cutP-lacZ* expression was 3 and 28 times higher than aerobic expression for *E. coli* and *S. enterica*, respectively (Figures 4.6A and 4.6B). For comparison, expression of the *cutR*-regulated *cdsH* was examined from *S. enterica*, and in contrast to *cutP-lacZ* expression, *cdsH-lacZ* expression was six times higher aerobically than anaerobically (Figure 4.6C). One possible explanation for the greater anaerobic versus aerobic expression of *cutP-lacZ* is the participation of a co-regulator that differentially controls anaerobic and aerobic genes. Loss of ArcA, ArcB, and SoxR did not impair anaerobic expression (Figure 4.5C), whereas loss of SoxR unexpectedly elevated *cutP-lacZ* expression 50% ($p = 0.05$). This limited analysis did not identify a potential co-regulator, if it exists.

A



B

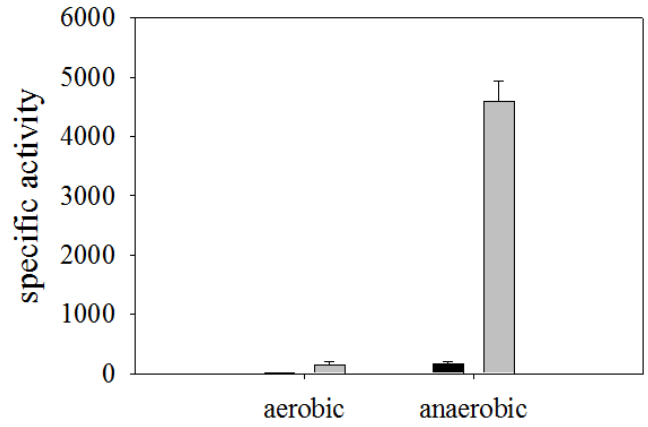


Figure 4.6: Aerobic versus anaerobic expression of cysteine catabolic genes. (A) *E. coli cutP-lacZ* (ML6). (B) *S. enterica cutP-lacZ* (BLS143). (C) *S. enterica cdsH-lacZ* (TO11). Cells were grown in LB without cysteine (black bars) or with 1 mM cysteine (gray bars) as indicated. 0.5 to 1.5 ml samples were removed at late exponential phase (100 Klett units for aerobic cultures and 40 Klett units for anaerobic cultures) for assay of β-galactosidase.

C

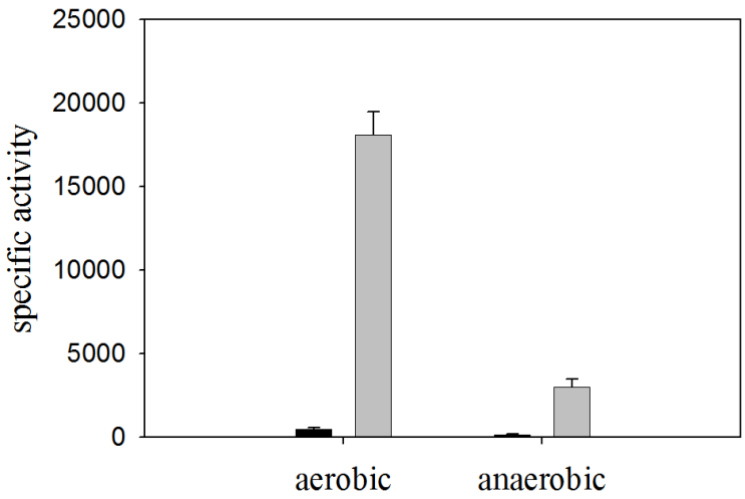


Figure 4.6: (cont.) Aerobic versus anaerobic expression of cysteine catabolic genes. (A) *E. coli cutP-lacZ* (ML6). (B) *S. enterica cutP-lacZ* (BLS143). (C) *S. enterica cdsH-lacZ* (TO11). Cells were grown in LB without cysteine (black bars) or with 1 mM cysteine (gray bars) as indicated. 0.5 to 1.5 ml samples were removed at late exponential phase (100 Klett units for aerobic cultures and 40 Klett units for anaerobic cultures) for assay of β-galactosidase.

(iii) Induction of *cutP-lacZ* by amino acids and *S*-containing compounds. Of the L-amino acids tested, only L-cysteine induced in both *E. coli* and *S. enterica* (Figures 4.7A and 4.7C, respectively). This confirms a recent report that L-cysteine is the only L-amino acid that induces *cutP* expression (Shimada *et al.*, 2016). A few D-amino acids were tested for induction since CutP-dependent D-serine transport was shown to be important for virulence of enterohemorrhagic *E. coli* (Connolly *et al.*, 2016). D-Cysteine induced, about three times less well than L-cysteine in both *E. coli* and *S. enterica*, but D-alanine and D-serine did not induce (Figure 4.7A and C).

In *E. coli*, the following *S*-containing compounds induced with the indicated expression relative to L-cysteine induction: *N*-acetyl-cysteine (40%), homocysteine (21%), cystine (17%), *S*-

nitrosocysteine (14%), and glutathione (11%) (Figure 4.7B). In *S. enterica*, cystine (34%), *N*-acetylcysteine (11%), and homocysteine (9%) induced (not shown). Sulfide and djenkolate did not induce *cutP* in either organism (not shown). The induction by these compounds does not imply direct binding to CutR, because the inducing compound could indirectly affect intracellular cysteine. Glutathione induces *E. coli cutP-lacZ* about four-fold over background (Figure 4.7A). Assuming that glutathione degradation to cysteine is the mechanism of induction, expression was examined in a mutant lacking the periplasmic Ggt (γ-glutamyltranspeptidase) which is the major, but not the only, enzyme of glutathione utilization (Suzuki *et al.*, 1987).

A

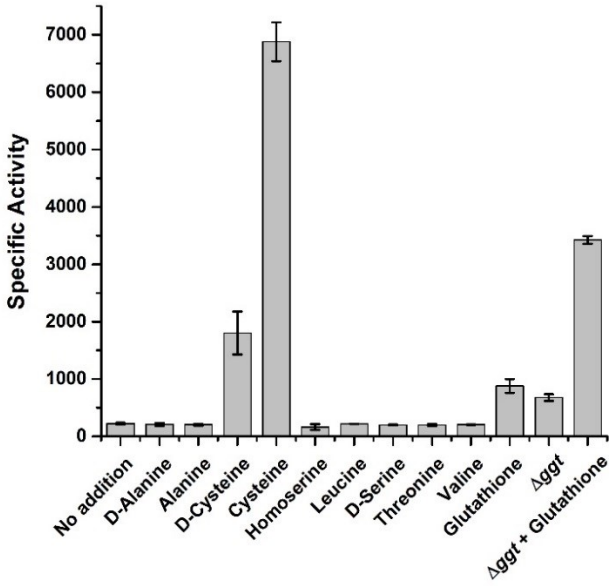
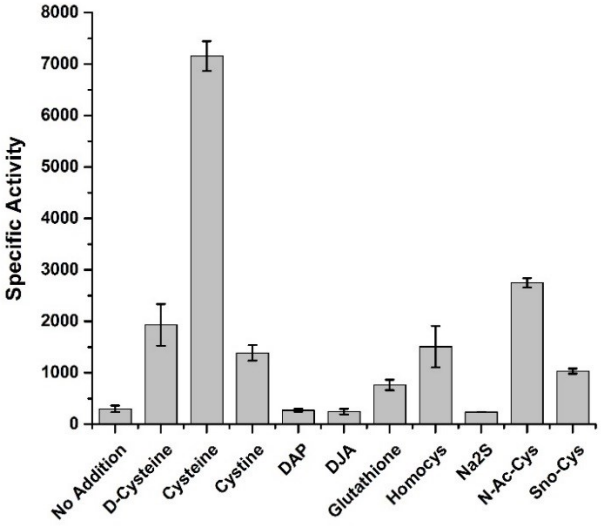


Figure 4.7: Induction of *cutP-lacZ* by amino acids and *S*-containing compounds in *E. coli* and *S. enterica*. (A) Amino acid induction in *E. coli* ML6 (wild type) and IM39 (Δ*ggt*). (B) Induction by *S*-containing compounds in *E. coli* ML6. (C) Induction in *S. enterica* BLS143. All compounds were added at 2 mM, with the following exceptions: Na2S (0.5 mM), N-acetylcysteine (N-Ac-cys, 5 mM), diaminopimelate (DAP, 5 mM), djenkolate (DJA, 5 mM), homocysteine (Homocys, 5 mM), and cystine (10 mM). Other abbreviation: Sno-Cys, *S*-nitroso-cysteine.

B



C

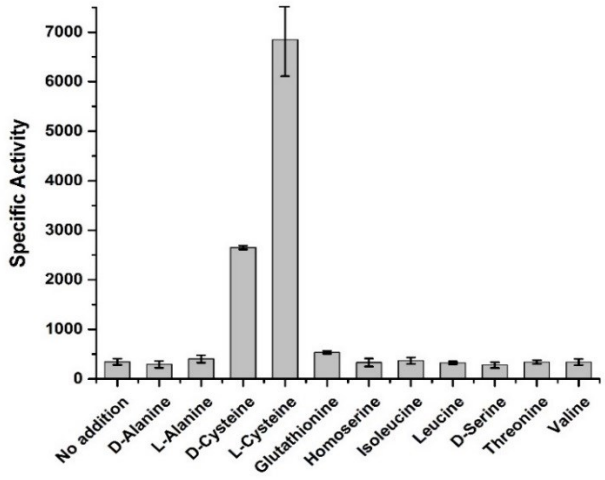


Figure 4.7: (cont.) Induction of *cutP-lacZ* by amino acids and *S*-containing compounds in *E. coli* and *S. enterica*. (A) Amino acid induction in *E. coli* ML6 (wild type) and IM39 (Δ*ggt*). (B) Induction by *S*-containing compounds in *E. coli* ML6. (C) Induction in *S. enterica* BLS143. All compounds were added at 2 mM, with the following exceptions: Na2S (0.5 mM), N-acetylcysteine (N-Ac-cys, 5 mM), diaminopimelate (DAP, 5 mM), djenkolate (DJA, 5 mM),

homocysteine (Homocys, 5 mM), and cystine (10 mM). Other abbreviation: Sno-Cys, *S*-nitroso-cysteine.

Unexpectedly, loss of Ggt increased glutathione-independent and glutathione-dependent *cutP-lacZ* expression (Figure 4.7A). Both results suggest that Ggt-independent glutathione catabolism generates more cysteine than Ggt-dependent catabolism.

CutA phylogeny: presence in anaerobes and possible ancient origin. 84 organisms have CutA homologs. These organisms are in the following phyla with the number of representatives in parentheses: *Euryarchaeota* (5), *Acintobacteria* (3), *Bacteroidetes* (7), *Firmicutes* (16 in class *Clostridia* and 5 in other classes), *Fusobacteria* (2), *Spirochaetes* (3), *Synergistetes* (4), *Thermotogae* (5), *Proteobacteria* (31), and other bacteria (3) (supplemental data-not shown). Excluding the *cutA*-containing β- and γ-proteobacteria, 61 out of 62 organisms are obligate anaerobes, and the single exception is a microaerotolerant anaerobe. Among the *cutA*-containing β- and γ-proteobacteria, 20 of 22 organisms are facultative anaerobes, and the remaining two are obligate anaerobes. No strict aerobe has a CutA homolog. This organismal distribution strongly suggests an anaerobic function for CutA homologs.

A rooted phylogenetic tree of CutA homologs is shown in Figure 4.8. A CutA homolog appeared in five archaea. The CutA homologs from three archaea form an outgroup (group C), and the CutA homologs from two archaea are grouped with bacterial homologs. These observations are consistent with some gene transfer between kingdoms. The bacterial CutAs are separated into two groups, with group A containing the δ-proteobacterial homologs, and group B containing the β-, and γ-proteobacterial homologs (Figure 4.8). Some firmicutes have multiple *cutA* paralogs, such as *Pelosinus fermentas* JBW45 which has four paralogs. CutA homologs are found in archaeal methanogens and bacterial acetogens, which are considered some of the most ancient

organisms (Weiss *et al.*, 2016). This distribution may suggest an origin for CutA that predated the archaeal-bacterial split.

A rooted phylogenetic tree of CutA homologs is shown in Figure 4.8. A CutA homolog appeared in five archaea. The CutA homologs from three archaea form an outgroup (group C), and the CutA homologs from two archaea are grouped with bacterial homologs. These observations are consistent with some gene transfer between kingdoms. The bacterial CutAs are separated into two groups (A and B in Figure 4.8), with group A containing the δ-proteobacterial homologs, and group B containing the β-, and γ-proteobacterial homologs. Some firmicutes have multiple *cutA* paralogs, such as *Pelosinus fermentas* JBW45 that has four paralogs (Figure 4.8). CutA homologs are found in archaeal methanogens and bacterial acetogens, which are considered some of the most ancient organisms (Weiss *et al.*, 2016). This distribution suggests an ancient origin for CutA.

4.6 Discussion

Anaerobic cysteine catabolic pathways and differences between *E. coli* and *S. enterica* sulfur metabolism. CutR-regulated CutA is the major anaerobic cysteine catabolic enzyme in both *E. coli* and *S. enterica*, at least under the conditions tested.Neither CdsH in *S. enterica* nor a transaminase/mercaptopyruvate sulfurtransferase pathway in *E. coli* obviously contributed to anaerobic cysteine catabolism. The failure of CdsH to contribute to anaerobic cysteine catabolism is surprising, because CdsH is expressed anaerobically, although not optimally (Figure 4.6C). For unknown reasons, CdsH is not active during anaerobic growth, even when overexpressed (see Figure 4.4C of (Oguri *et al.*, 2012)).

A comparison of anaerobic cysteine catabolism in *E. coli* and *S. enterica* suggests several subtle differences: CutA is more active in *S. enterica* (Figure 4.1); CutP is the only anaerobic cysteine transporter in *E. coli*, but not in *S. enterica* which must have an additional transporter (Figures 4.3 and 4.4); and the *E. coli cutA* mutant was more sensitive to cysteine (compare Figures 4.3 and 4.4).

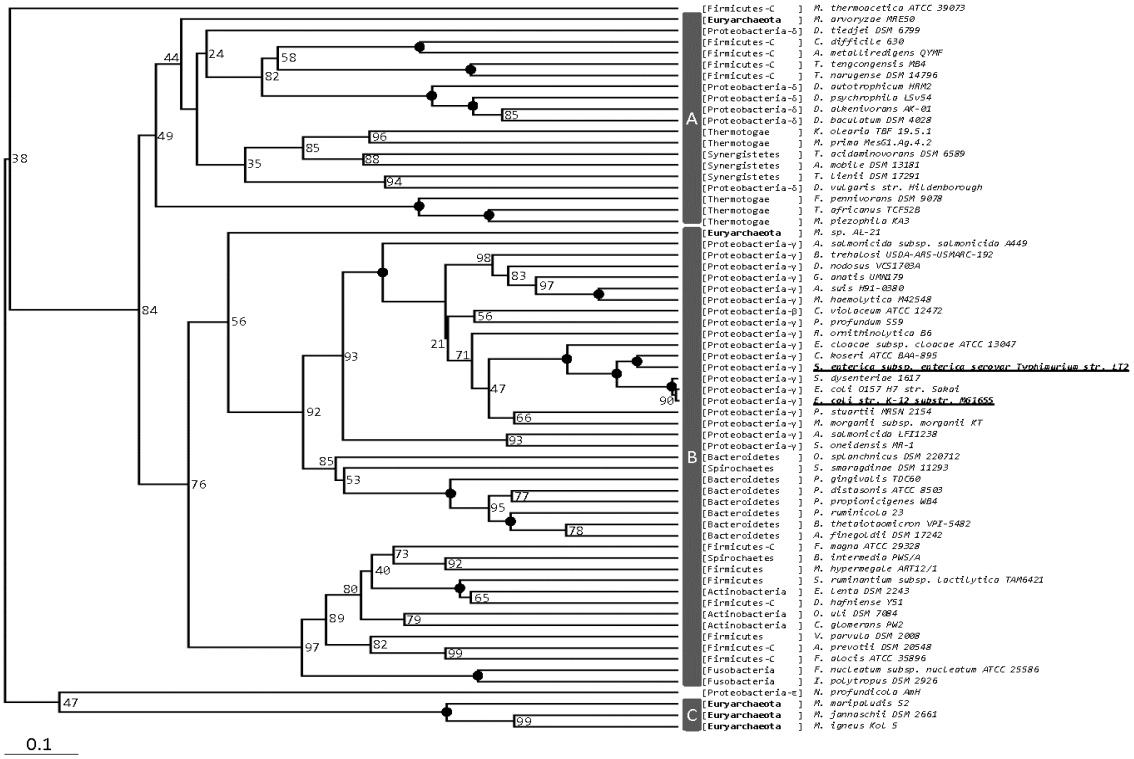


Figure 4.8: Rooted phylogenetic tree of CutA homologs in 60 bacteria and 5 archaea. Three archaea form an outgroup (group C) and the bacteria are separated into two major groups (A and B) with one archaea in each group (bold font). The *E. coli* and *S. enterica* homologs are shown in bold Italic font with underlining. The bootstrapping value from 100-samplings are shown at each fork. Those clades with bootstrapping values of 100 were marked with filled circles.

More generally, *S. enterica* has a greater capacity than *E. coli* to metabolize *S*-containing compounds anaerobically: *S. enterica*, but not *E. coli*, contains reductases for sulfite, thiosulfate, and tetrathionate which allows their utilization as alternate anaerobic electron acceptors (Kredich, 1996). Its greater capacity to utilize a variety of *S*-containing compounds suggests that *S. enterica* is a specialist in anaerobic sulfur metabolism that endows *S. enterica* with unusual capabilities, such as pathogenic outgrowth in the intestinal tract (Winter *et al.*, 2010).

The function of anaerobic cysteine catabolism: potential to coordinate metabolism. At least three possible functions can be proposed: (a) cysteine as a carbon or energy source, (b) detoxification of cysteine at concentrations that inhibit growth, and (c) modulation of intracellular cysteine at non-toxic concentrations. The evidence to support utilization of cysteine as a carbon/energy source is CutA repression by compounds associated with energy generation: oxygen (electron acceptor), glucose (carbon and energy sources), and nitrate (alternate electron acceptor) (Figure 4.6 and (Oguri *et al.*, 2012)). Evidence against this possibility is that pyruvate, which is the energy-generating product of cysteine catabolism, did not stimulate anaerobic growth.

The second proposed function of anaerobic cysteine catabolism is detoxification at cysteine concentrations that inhibit growth. The evidence for this function is enhanced cysteine sensitivity in a Δ*cutA* mutant (this work and (Shimada *et al.*, 2016)). Cysteine sensitivity requires at least 5 mM exogenous cysteine (this work), which is probably not often encountered in nature.

A third possible function for cysteine catabolism is modulation of intracellular cysteine. The elevated *cutP-lacZ* expression in *cutA* and *ridA* mutants of both *E. coli* and *S. enterica* supports this possibility (Figure 4.5). The mutants presumably have elevated intracellular cysteine, which

binds to CutR and increases *cutP-lacZ* expression. If cysteine catabolism modulates intracellular cysteine, then what is the function of this modulation? A possible function of this modulation is control of cysteine-inhibitable enzymes. Cysteine inhibits several enzymes of amino acid synthesis (Reitzer, 2005), and this inhibition is relevant *in vivo*: cysteine impairs growth of *cutA* mutants in 25% LB, but not in 100% LB, which has higher amino acid concentrations. Even sub-toxic cysteine concentrations are likley to inhibit several enzymes, at least partially, and elevated expression of these enzymes can compensate for the inhibition. Cysteine catabolism would minimize the need to increase expression of these enzymes. By this logic, the catabolism of cysteine, the product of sulfur assimilation, affects the synthesis of other amino acids and integrates sulfur assimilation with several pathways of amino acid synthesis. The modulation of intracellular cysteine to integrate metabolism explains the reduced growth yield of *cutA* mutants: with high cysteine, energy is diverted for synthesis of the inhibited enzymes (Figures 4.3 and 4.4). Proteins that coordinate metabolism, as is proposed for CutA, may be similar to products of quasi-essential genes: non-essential, but required for optimal and robust growth (Hutchison *et al.*, 2016).

CutA appears to be an ancient enzyme. CutA homologs are present in archaeal methanogens and eubacterial acetogens which are considered two of the most ancient organisms (Weiss *et al.*, 2016), and their presence might suggest that CutA homologs existed before the archaeal-bacterial divergence and in the last universal common ancestor. The methanogens and acetogens do not share pathways of cysteine synthesis, which implies that the catabolic pathway was present before the anabolic pathways (Grochowski & White, 2008); perhaps a primitive (non-enzymatic?) pathway of cysteine synthesis existed before the archaeal-bacterial divergence.

Regardless of the early mechanism of cysteine synthesis, the fluctuating concentrations of environmental sulfide, which was readily available in a proposed primordial environment (Weiss *et al.*, 2016), probably resulted in fluctuating concentrations of cysteine, and the potential to inhibit other pathways, such as those of threonine or methionine synthesis. It is proposed that ancient CutA homologs not only minimized cysteine toxicity, but also coordinated sulfide assimilation with synthesis of some amino acids.

Final comments: what does it all mean? We have presented evidence that CutA is the major enzyme that degrades cysteine during anaerobic growth in both *E. coli* and *S. enterica*; analysis of gene expression and phylogenetic relationships confirm an anaerobic function. Because of cysteine's ability to inhibit several enzymes of amino acid synthesis, cysteine catabolism has the potential to modulate synthesis of some amino acids. The presence of CutA homologs in the most ancient organisms suggests that cysteine catabolism was an ancient mechanism of metabolic coordination.

4.7 Acknowledgments

This work was supported in part by grant GM085536 from the National Institute of Health. We thank Stephen Spiro (The University of Texas at Dallas) for providing strain JOEY281, and Juan Gonzalez (The University of Texas at Dallas) for the use of equipment.

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BIOGRAPHICAL SKETCH

Iti Mehta was born in Kota, Rajasthan (India), on June 27th, 1988, to Dinesh Dutt Mehta and Hansa Mehta. After completing her schooling from DAV Centenary Public School (2005), she joined the Center for Converging technologies, University of Rajasthan for further studies in 2006. She received her dual degree (B.Tech - M.Tech) in Biotechnology from University of Rajasthan in 2012 and moved to Dallas, Texas to pursue her PhD in Cell and Molecular Biology at The University of Texas at Dallas.

CURRICULUM VITAE

Iti Mehta iti.mehta@utdallas.edu

Education

Ph.D (Molecular and Cell Biology) - UT, Dallas Aug 2012 - Aug 2018
MS (Molecular and Cell biology) - UT Dallas Aug 2012 - May 2015
B.Tech - M.Tech dual degree (Biotechnology)-University of Rajasthan Sept 2006 - June 2012

Teaching Experience

May 2018 –Aug 2018 University of Texas – Dallas
Teaching Assistant for the Microbiology Laboratory course. Contribute in teaching along with helping the students by solving their problems. Also assisted in grading lab reports.

Jan 2018 – May 2018 University of Texas - Dallas
Co-taught Introductory Biology Laboratory course as an Instructor. Demonstrated lab techniques and monitor student participation along with supervising undergraduate and graduate teaching assistants.

Sept 2013 – May 2018 University of Texas – Dallas
Teaching Assistant for the Introductory Biology Laboratory course. Contributed in teaching along with helping the students by solving their problems. Graded exams and weekly lab reports. Also manage the weekly experiment setup and lab design.

May 2013- Aug 2013 University of Texas – Dallas
Teaching Assistant for the Biochemistry Laboratory course. Helped teach laboratory techniques along with grading exams, quizzes, math home-works and weekly lab reports.

Sept 2012 – May 2013 University of Texas – Dallas
Teaching Assistant for the Introduction to Modern Biology course. Taught weekly workshops along with grading quizzes and exams.

Jan 2104 –Aug 2018 University of Texas – Dallas
Mentored several masters and undergraduate students in Dr. Reitzer's lab at the Biological Sciences Department to perform their research projects. Also helped with the project design and plan experimental details.

Research Experience

Jan 2014 – Aug 2018 University of Texas – Dallas
Currently working on my Ph.D. dissertation work in a microbiology lab under the supervision of Dr. Larry Reitzer. My work involves studying the regulation of swarming motility in *E.coli* and the role of polyamines in it.

June 2017 – Present UT Southwestern Medical Center - Dallas
Also working on another project in Dr. Reitzer's lab in collaboration with well-known urologist Dr. Philippe Zimmern at UT Southwestern medical center. This project involves the study of urine composition and the effect of dose dependent D-mannose oral intake on recurrent urinary tract infections. Experience with IRB compliance and sample collection and storage.

July 2013- November 2013 University of Texas – Dallas
Worked in a cancer biology lab to study the effect of hypoxia on breast cancer. Learned sectioning of tumors along with H&E staining along with some immunohistochemistry experiments.

Oct 2011-April 2012 Indian Institute of Science, Bangalore
Worked as a Research Intern at Bioinformatics Lab, Department of Physics. Used protein visualization and modeling tools such as PyMol and Discovery studio to better understand helix turn helix DNA transcription factors.

Conferences and Workshops

- Presented a poster titled 'A reliable, sensitive and fast enzymatic method to measure D-mannosuria' at the Society of urodynamics, female pelvic medicine and urogenital reconstruction (SUFU), 2018 held in Austin (Feb, 2018).
- Invited speaker for the talk titled 'The putrescine requirement for Escherichia coli swarming motility' at the Gordon Research Seminars in Waterville Valley, New Hampshire (June 2017).
- Presented a poster titled 'The putrescine requirement for Escherichia coli swarming motility' at the Gordon Research Conference in Waterville valley, New Hampshire (June 2017).
- Attended ASM Conference 2016, Texas chapter held at University of Texas at Dallas (Oct 2016).
- Presented a poster titled 'Common elements exist in regulation of swarming in different bacterial species' at 2016 Molecular Genetics of Bacteria and Phages Meeting at UW, Madison.
- Presented a poster titled 'Role of polyamines in regulation of swarming motility in *E.coli* at ASM conference 2015 held at Sam Houston State University, Huntsville.
- Attended "3rd Bangalore Nano-Frontiers of Nanotechnology", International Conference (2010).

Technical skills

- Bacterial transformation, transduction, plasmid isolation and other microbiological techniques.
- Enzyme assays and protein estimation.
- Protein purification

- Microscopy
- IHC and basic sectioning of tissue samples.
- UV/Vis Spectrophotometry
- High Performance Liquid Chromatography (HPLC) for alkaloids separation from plants extracts.
- Gene Gun and Agrobacterium mediated gene transfer for plant tissue culture experiments.
- Gel Electrophoresis and Western Blotting for proteins detection and separation.

Computer Skills

- Operating Systems: Windows, Linux
- Languages: C, C++, HTML, PHP, Basic Python
- Software & Applications: MATLAB, Discovery Studio, PyMol, Ape, Microsoft Office'07/13

Awards and Achievements

- PhD small grant in summer 2017 and spring 2018.
- UTD graduate tuition scholarship which entails full tuition waiver.
- Graduate level scholarship (MS) for standing in the top five students of the batch (2006) in CCT, Univ. of Rajasthan, India.
- Honor certificate from Central board of Secondary Education in India for being amongst top 1% students in senior secondary English exam.
- Won various debates and paper presentation competitions during Intra and inter school events.