Order Number 9406073

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The University of Nebraska - Lincoln, 1993

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Detergent Shock Proteins in *Escherichia coli* as Examined by Two-Dimensional Gel Electrophoresis

by

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A Dissertation

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Biological Sciences

Under the Supervision of Professor Kenneth W. Nickerson

Lincoln, Nebraska

August,1993

DISSERTATION TITLE

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GRADUATE COLLEGE
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At least 22 proteins in *Escherichia coli* W3110 were found to increase a minimum of 2-fold after cells were shocked with 0.5% sodium dodecyl sulfate (SDS). Among these 22 proteins, 5 were not observed during normal growth, they were only synthesized during SDS stress thus making them unique. These 22 proteins were also found to follow a time-dependent pattern in their synthesis, with various proteins being synthesized at their peak levels within 10 minutes of SDS shock. Other proteins did not appear in elevated quantities until 1 hour after SDS shock. The SDS shock proteins did not show significant overlap with any of the established shock stimulons, however the DnaK chaperonin protein and the Universal Shock Protein have been tentatively identified as being SDS shock proteins. The SDS resistant phenotype could be at least partially eliminated by transposon mutagenesis to create a mutant strain of *Escherichia coli* which grew poorly after SDS shock. This mutant strain also failed to show a wild-type pattern of protein synthesis after SDS shock. Only 3 of the 22 SDS shock proteins showed significant increases in synthesis after SDS shock, all of the others increased very little or not at all. The synthesis of proteins, including the SDS shock proteins, was not found to be required for survival of *E. coli* after SDS shock. Cultures treated with

chloramphenicol, which halted protein synthesis, showed no decrease in viability after 4 hours of SDS shock. This result may indicate that SDS resistance is a constitutive property of enteric bacteria and that the SDS shock proteins may serve to facilitate growth in an SDS containing environment, but are not essential for short term survival when cells are exposed to detergents.

Acknowledgments

I wish to thank Dr. Ken Nickerson for bringing me into his lab and making me feel not only welcome, but appreciated and respected. I would also like to thank him for giving me the latitude to pursue this project in my own way and the freedom to grow as a scientist and as a person. I would also like to thank the members of my advisory committee; Dr. George Veomett, Dr. Tyrrell Conway, Dr. Leslie Lane, and Dr. Tyler Kokjohn for their time, help, and most of all their interest and personal attention to this project. None of my work here would have been possible without the guidance of these men.

I also wish to thank all of the people in the Nickerson lab; Brad, Meenakshi, Chung, Kristen, Arden and all of the other students who have spent time with me here. This lab has been a great place to work because of you.

I owe much to my parents Michael and Linda, who have continually nurtured and encouraged my curiosity and desire to go further. I also greatly appreciate the support of my new family, Charles and Joan, who made me feel at home when I was far from my family.

Most of all this work has been made possible by my beloved wife Wendy, who kept my spirits up when nothing seemed to be working and has filled my life with more joy and purpose than I can express.

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Introduction

Bacterial cells are continually challenged by environmental factors which cause damage to cellular components and could kill cells if not protected against. Among the many factors that can stress a bacterium are heat, pH, oxidizing agents, extremes of solute concentration and starvation. Each condition elicits a specific cellular response which will allow the cell to survive the duration of the stress relatively unscathed and, in some cases, even grow and divide under stress. A common feature of these responses to stress is the production of new proteins which aid in protecting the cell against stress-factor induced damage or serve to rapidly repair that damage. These proteins are usually described in the context of the stress that elicits them, such as osmotic-stress proteins. While some overlap does occur among groups of stress proteins, the newly synthesized peptides are more often unique to the factor that caused their induction.

To the list of conditions and agents that induce stress proteins must now be added anionic detergents. These molecules have a negatively charged head group and a non-polar lipophilic tail which combine to make a reactive amphiphilic molecule that can disrupt lipid membranes. Detergents such as sodium dodecyl sulfate can disrupt membranes, denature proteins and interfere with protein-protein interactions. Any of these actions can result in cell damage or death, and so it is not surprising that many bacteria have evolved defense mechanisms against the anionic detergents. Part of this

defense involves the outer membrane of the Gram negative bacteria. Another part involves the synthesis of proteins which help adapt the cell to live and reproduce in the presence of high concentrations of anionic detergents. This detergent shock adaptation appears to be fundamentally similar to those of the other shock responses, but upon further examination has proven to be unique.

Literature Review

The ability of Gram negative enteric bacteria to survive in many harsh environments has long been recognized (89). Organisms such as *Escherichia coli* and *Salmonella typhimurium* can variously withstand extremes of temperature (94, 132), pH (148, 48), osmolarity (28) and even the caustic interior of a macrophage (99, 23) to name but a few of these extreme conditions. Anionic and neutral detergents of many types are environmental stress factors that have long been recognized (151, 33), but the mechanism of cellular adaptation to these chemicals has never been closely examined. One natural environment of the enteric bacteria is the highly anaerobic mammalian gut, which contains a rich variety of chemicals including detergent-like bile salts and fatty acids. These molecules can occur locally in high concentrations and can have cytolytic effects on unprotected cells (27, 76). Outside the mammalian body, detergents like sodium dodecyl sulfate have become a significant environmental stress during the last 50 years as industrial wastes have accumulated in the worlds bodies of water. It is clear that to survive in these vastly differing environments, enteric bacteria have had to evolve mechanisms to cope with detergents and avoid their lethal effects.

Detergent Resistance. In 1980 Kramer *et al* (85) began to examine detergent resistance in Gram negative bacteria. They discovered that *Enterobacter cloacae* could survive and grow in liquid media that contained up to 25%

sodium dodecyl sulfate (SDS) at 32°C (85). They also found that *E. cloacae* could grow in 25% Triton X-100 and 20% Tween 20, 40, 60, or 80. Triton X-100 and the Tweens are neutral detergents. It was shown that cells under SDS stress lyse soon after entering stationary phase and that the addition of metabolic inhibitors (such as sodium azide) to a culture in exponential phase under SDS stress induced rapid lysis to occur. Kramer *et al* (85) also reported several lines of evidence indicating that the cells were not consuming or modifying the SDS; including the fact that no cultures could use SDS as the sole carbon and energy source. The final cell yields were found to be reduced when cultures were grown in the presence of SDS. Further work by Kramer (87) *et al* showed that the ability to grow in high concentrations of SDS was not a rare quality amongst enteric bacteria. Over 170 strains of *Escherichia*, *Salmonella*, *Enterobacter*, *Serratia*, *Citrobacter*, and *Klebsiella* were detergent resistant; detergent resistance is not a rare characteristic.

Additionally, Kramer (87) *et al* performed plasmid screens which revealed that while most of the tested organisms contained plasmids, there were no plasmids which were common to all the bacteria and 8 of the strains contained no detectable plasmids at all. This evidence indicated that detergent resistance was likely a common cellular property of enteric bacteria and plasmid independent. They also found that none of the bacteria that were resistant to anionic or neutral detergents could tolerate cationic detergents such as benzalkonium chloride or hexadecyl-pyridinium chloride. In contrast, the Gram positive bacteria could tolerate neutral detergents but were totally

inhibited by a concentration of even 0.1% of the anionic detergents. Significant changes in the metabolism of cells growing in SDS were also found (86). When grown in liquid medium containing 10% SDS, *Enterobacter cloacae* exhibited an energy burden in the form of a 30% faster rate of glucose utilization, a 70% increase in oxygen consumption and a 20% decrease in the cell yield. This energy burden was examined in greater detail by Aspedon and Nickerson (8). They found the energy burden was caused by two properties of SDS; first the detergent aspect of the molecule and second the role it plays as a solute in liquid media. The detergent property of SDS appeared to be responsible for the rapid lysis of cells that had entered stationary phase due to carbon limitation. However, entering stationary phase due to limitation of either nitrogen or phosphorus did not lead to enhanced lysis of cultures grown with SDS. These results indicated that the cells apparently needed an energy source in order to survive and avoid lysis in SDS. However, the osmotic solute property of SDS was responsible for the other physiological changes observed in growth in 5 and 10% SDS, i.e. longer lag periods, higher oxygen consumption, potassium accumulation inside cells and decreased cell size (evident as lower photometric yields). All of these results in SDS were mimicked by cells growing in equivalent concentrations of polyethylene glycol or sucrose.

Two-Dimensional Gel Electrophoresis. An extremely powerful tool in the examination of bacterial responses to stress conditions has been the

technique of 2-dimensional gel electrophoresis. Extensive mapping of the protein profiles of unstressed *E. coli* cells has been done by Neidhardt's group (153) and many of the spots have been identified as specific proteins to create a gene-protein database. Two-dimensional gels have also been used to examine cellular adaptations to pH (48), pollutants (16), osmotic pressure (30), ingestion by macrophages (23), starvation (60), anaerobiosis (145), and H_2O_2 (29).

The most common form of 2-dimensional gel electrophoresis used today was developed in 1974 by O'Farrell (127). Prior to O'Farrell's method, several other researchers had attempted to separate proteins in two-dimensions (13, 105, 106, 113, 129, and 134) but their work yielded no better results than the current one-dimensional techniques already in use. O'Farrell combined the two existing separation methods; isoelectric focusing and Laemmli's type of discontinuous SDS polyacrylamide gel electrophoresis (90), to attain a new level of protein separation. The system is based on the fact that IEF separates molecules by their isoelectric point whereas SDS PAGE separates them by their molecular weight (size). These two parameters are completely independent of one another and thus one separation does not interfere with or alter the other. The combination of these two techniques allowed the proteins to be spread across the entire surface of the second dimension (the SDS PAGE gel) and not just arranged in a diagonal pattern as they would be if the separation techniques influenced one another. The result of uniting these two separation strategies has yielded an extremely sensitive technique that can

resolve hundreds, even thousands of individual protein spots on a single gel. When combined with autoradiography, it is sensitive enough to resolve proteins that constitute as little as .00001% of total cell protein. That could mean a total of as few as 10 molecules. Two-dimensional gels can also clearly show the effects of missense mutations that alter a proteins charge, or post-translational modifications such as phosphorylation.

Isoelectric focusing takes advantage of the fact that all proteins carry a net electrical charge. This charge results from adding all of the positive and negative charges carried on the component amino acids (and other side groups such as cabohydrates and sulfates) together. When a protein is suspended in a pH gradient and an electrical field is applied to that gradient, the protein will migrate to the area of the gradient where the pH is such that the protein has no net charge (i.e. the protein has an equal number of negative and positive charges). This point is termed the isoelectric focusing point or more simply the isoelectric point, and when reached the protein will no longer migrate appreciably. The exact isoelectric point of a protein is specific, conserved, and usually unique and because of these properties IEF can be used to separate proteins. The pH gradient is established through the use of small molecules called carrier ampholytes, which are composed of isomers and homologues of polyaminopolycarboxylic acids with isoelectric points that range from pH 2.5 to 11. Each ampholyte molecule carries a net charge and essentially mimics the behavior of a protein when placed in an electrical field. When quantities of differing ampholytes are blended together

and an electrical field is applied to them, the ampholytes will position themselves to form a pH gradient into which proteins can then be added. Important characteristics of carrier ampholytes are that they 1) have even conductivity, including the production of an even field strength with no areas of local high resistance wherein overheating may occur, 2) have a high buffering capacity, 3) are soluble at their isoelectric point and 4) do not interact chemically with focused proteins (42a). IEF separation is carried out in low percentage (less than 3%) polyacrylamide gels which are usually cast in small diameter glass tubes, although strip gels can also be used. In IEF gels the polyacrylamide does not function as a molecular sieve, as it does in SDS PAGE gels, but acts more as a stabilizing matrix in which the proteins and ampholytes can migrate and arrange themselves according to the established charge gradient. Proteins that are run on IEF gels are denatured using a mixture of 8 M urea and a non-ionic detergent such as Triton X-100. Not only do these agents serve to denature the protein and expose all charged groups, but the neutral detergent strips off any bound SDS molecules that had been used to lyse the bacteria when the protein preparations were made. This mixed micelle of SDS and neutral detergent ends up in the very acidic end of the gel where no proteins focus and thus they are are rendered harmless. IEF gels are usually run for 5000 to 10,000 Volt-hours to allow complete focusing to occur. Further electrophoresis would not alter the separation because once a protein reaches its isoelectric point it will have a net charge of 0 and will cease migration. In fact ampholyte gradients drift with time and small amounts

of protein migration will continue unless the gradient is immobilized via binding the ampholytes to the polyacrylamide (immobilines). This drift is ususally not significant.

The second dimension separates proteins on the basis of their size or molecular weight. This is done by first immersing the tube gel from the IEF dimension in a Tris-HCL SDS equilibration buffer which is identical to that used to lyse the bacteria when making the protein preparation. The equilibrated tube gel is then placed directly onto the top of a polyacrylamide slab gel. In the O'Farrell method the slab gel is that of Laemmli (90) combined with the stacking gel system of Ornstein (128) and Davis (38). This stacking gel system uses a lower pH (pH 6.8) than the running buffer (pH 8.3) or the separating gel (pH 8.8) to create a region of high-voltage gradient between the leading chloride ions and the lagging glycine zwitterions which are nearly immobile at acidic pH values (31). The stacking gel uses a lower percentage of acrylamide than the separating gel and the resulting larger pore size allows the proteins in this high-voltage gradient to easily and rapidly migrate until they come to the leading edge of the chloride ions. This rapid migration in a small area creates a "stacking" effect in which all of the proteins are piled up in a tight disc, with the smallest at the bottom and the largest at the top. Upon entry into the basic separating gel the glycine zwitterions become fully mobile and overtake the protein. When this happens the charge gradient disappears as the chloride and glycine ions migrate together, creating a constant field strength throughout the gel. At this point the proteins are separated only by

their size as they migrate through the pores of the gel which work like a molecular sieve. Smaller molecules can pass through the pores more easily and so travel more quickly than larger proteins. In this way the tightly stacked disc is spread throughout the gel. The pore size of the separating gel can be controlled by varying the amount of acrylamide used per unit volume and the degree of crosslinking. Larger quantities of acrylamide per unit volume will decrease the pore size. Increasing the ratio of bis-acrylamide (a crosslinker) to the amount of acrylamide will increase the pore size in the gel. The more crosslinker added the larger the pore size will be and the less restrictive the gel. A detailed review of 2-dimensional electrophoresis and many related techniques was done by Dunbar (42).

Two-dimensional gel electrophoresis is an extremely powerful technique for analyzing cellular responses to any factor which will elicit a protein response. However there are problems with the technique. Most importantly, because two separation strategies are employed, there are far more opportunities for experiment to experiment variation. The vagaries of equipment, temperature, current flow, and time that the gels were run can all alter the results to a significant degree. Much difficulty has been found in trying to relate gels done in one lab to those done in another. Even when using the same organism grown under the same conditions, results are often significantly different. These discrepancies should be expected. Different labs often use different sources of equipment and reagents and, in a technique which is so sensitive to experimental detail, homology of results is the exception rather than the