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Elevated Temperatures Perturb Lipopolysaccharide Leading to Increased Serum Complement Sensitivity in Most Gram-Negative Bacteria

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ELEVATED TEMPERATURES PERTURB LIPOPOLYSACCHARIDE LEADING TO INCREASED SERUM COMPLEMENT SENSITIVITY IN MOST GRAM-NEGATIVE BACTERIA

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This thesis is submitted in partial fulfillment of the requirements for the degree of

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ABSTRACT

In this study I continued and expanded on the work begun by Heather Wilson in 1993, to include 82 genetically different E.coli strains for the effect of febrile temperatures on their various lipopolysaccharide layers. This was accomplished by growing samples of bacterial strains at temperatures simulating endothermy and fever. Using SDS-PAGE and an LPS-specific silver stain on detergent lysed cells, LPS perturbation was manifested as either diminished amount per cell or significantly different average molecular weights or both.The LPS-specific silver stained SDS-PAGE gels showed perturbations in the LPS bands at elevated temperatures. Seven different forms of perturbation were noted.

Testing was done to examine how the LPS changes over a range of temperatures. These electrophoretic stain patterns fell into four different modes.

Further testing of the strains revealed that subsequent generations grown at elevated temperatures had notably less stained LPS in each passing generation.

Using standard tests, serum sensitivity was shown to increase in stains grown at elevated temperature over those with normal growth temperatures. The increases in sensitivity related to the electrophoretic band patterns of the LPS.

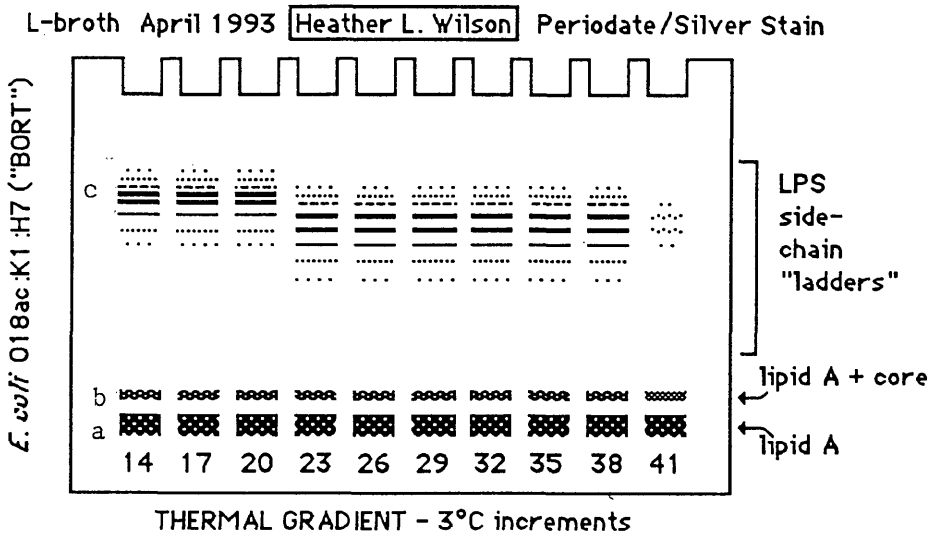
The results observed are apparently due to the reversible thermal inactivation of one or more enzymes in the LPS synthesis pathway. This was tested using streptomycin to inhibit de novo protein synthesis and by elimination of other possibilities.

ELEVATED TEMPERATURES PERTURB LIPOPOLYSACCHARIDE LEADING TO INCREASED SERUM COMPLEMENT SENSITIVITY IN MOST GRAM-NEGATIVE BACTERIA

INTRODUCTION

A serendipitous observation by Heather Wilson in 1993, portended for the first time a general theory of how animals utilize fever to control bacterial infection. In the last week of her undergraduate training she saw that the lipopolysaccharide (LPS) of a clinically derived strain of E.coli O18ac:K1:H7 was diminished in cultures growing at 41°C and above. While conducting an experiment on the effect of temperature variation on LPS, Wilson's water-bath overheated by a few degrees. After subjecting her cultures to LPS analysis using gel electrophoresis, her gel (Figure 1) showed fading bands of LPS O-antigen as the temperature rose to febrile levels. The bands in the lane loaded with bacteria grown at 41°C are significantly lighter than those of the previous, lower temperature. After assuring myself of the potential for this study by reproducing Wilson's results under controlled conditions, I went on to expand the study to consider 82 genetically different E.coli strains for effect of febrile temperatures on their various LPSs. I posed a number of questions concerned primarily with how general among the Gram-negative bacteria the phenomenon was. Only if the function were wide-spread, would I devote time to delving into its mechanism.

FIGURE 1
DIAGRAM OF HEATHER WILSON'S GEL



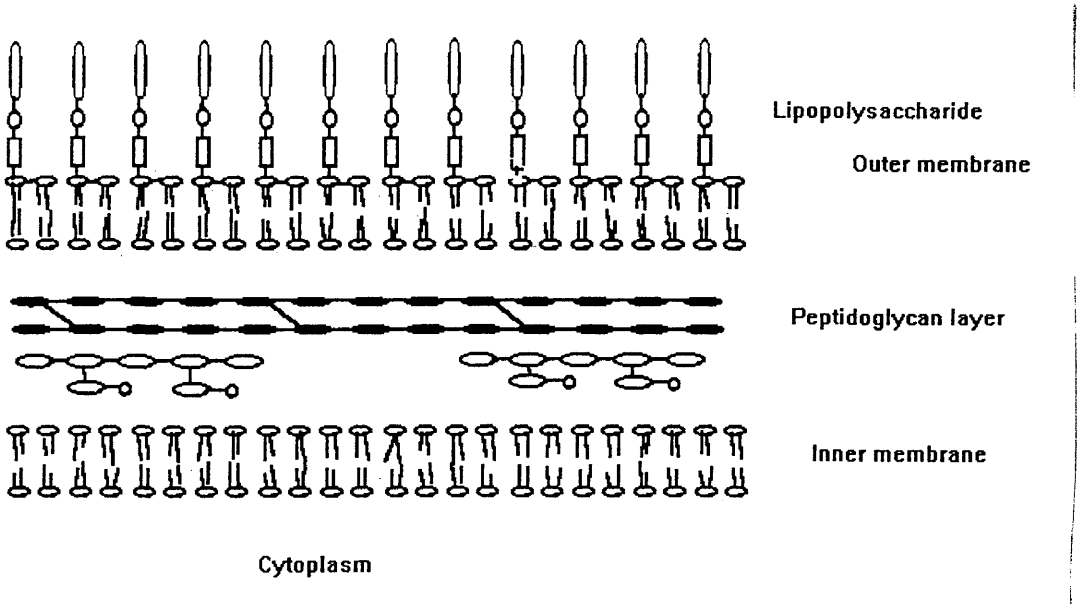
This LPS-specific silver stained SDS-PAGE gel was produced by Heather Wilson in May 1993. The stain shows lipid-A and the molecules bound to it. The bottom two bands are lipid-A (a) and core (b), while the ladder-like bands up the gel are O-antigen (c). Note the changes in the O-antigen bands as the temperature increases. The bands fall lower on the gel between 20°C and 23°C. This indicates that the average molecular weight of the O-antigen at 23°C is lower than that of 20°C. The O-antigen bands begin to fade at 41°C, due to a reduced amount of O-antigen per cell at this temperature. These two changes are the kind of perturbations observed in this study. The original gel was accidently destroyed.

Nearly half of all bacteria - most of the Gram-negative bacteria, including the Enterobacteriaceae - have a unique membrane lipid composition (Lüderitz, et al, 1966). The outer leaflet of the outer membrane consists almost exclusively of LPS (Figure 2), also refered to as endotoxin (Egan, et al, 1982; Lüderitz, et al, 1966; Kastowsky, et al, 1992). The LPS is unique in nature, being limited only to Gram-negative bacteria. As shown in Figure 3, the structure of LPS is that of a complex macromolecule made up of three domains: lipid-A, a phosphorylated oligosaccharide core and the O-antigen polysaccharide side-chains (Lüderitz, et al, 1966; Orskov, et al, 1977). Lipid-A is the portion of the molecule that is deeply anchored in the outer membrane (Figure 2) (Orskov, et al, 1977).

Each different O-serotypic strain of E.coli in this study has a population of unique LPS molecules. The majority of the differences among these many LPS molecules are due to variations in the number and composition of the polysaccharide repeating units, which make up the O-antigen side-chains, that protrude out into the cell's local environment (Peterson, et al, 1985; Munford, et al, 1980; Lightfoot, et al, 1991).

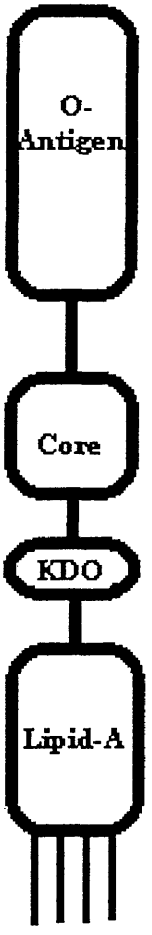
Due to these genetic variations in LPS, I felt a general survey of not only Gram-negative bacteria, but also E.coli strains in specific was necessary to ascertain the general fate of LPS at febrile temperatures. Bypassing notorious pathogens, I tested 82 of the approximately 150 different

FIGURE 2
THE GRAM-NEGATIVE CELL ENVELOPE



This is a schematic of the cell envelope of Gram-negative bacteria. These bacteria have two membranes with a middle peptidoglycan (cell wall) layer. The cytoplasm is at the bottom of the drawing, with the surface of the bacteria at the top. Lipopolysaccharide makes up most of the outer leaflet of the outer membrane. Note the lipid-A portion of the molecule is embedded in the membrane and the O-antigen extends into the environment.

FIGURE 3
THE LIPOPOLYSACCHARIDE MOLECULE



The lipopolysaccharide molecule consists of three components, lipid-A, core and O-antigen. Lipid-A is embedded in the outer surface of the outer membrane of Gram-negative bacteria. It is connected to core by a unique eight carbon sugar, KDO. The O-antigen differs for each genetically distinct strain in its structure and composition. It is this portion of the molecule which is of concern in this study.

known O-types of E.coli. In addition I tested six different common enterobacterial relatives, which also exhibited LPS, and are thus denominated as "smooth" according to terminology derived from Griffith's milestone pneumococcal work in both the field of pathology and microbiology (Griffith, 1928). Analysis of the LPS was made possible by subjecting lysed bacterial cells to detergent augmented polyacrylamide gel electrophoresis (SDS-PAGE). The gels were then visualized using an LPS-specific silver stain. This stain differs from that commonly employed in silver staining proteins. With the substitution of per-iodic acid, instead of chromic acid, as the activator, the stain becomes highly specific for oligo- and polysaccharides, of which LPS constitutes at least 99% in the bacterial cell (Jann, 1983). The stain is based upon periodate's specific ability to partially oxidize vicinal carbons that are at intermediary oxidation states. Most glycosides, such as polysaccharides, have at least one pair of vicinal alcoholic carbons. In addition, the lipid moieties in LPS have unusual alpha-keto structures. Both of these are sensitive to neutral-pH periodate oxidation yielding a plethora of aldehydes, which can then, in turn, reduce Ag^{+} to Ag_{o}. The Ag_{o} then forms a focus upon which more Ag_{o} can aggregate forming visible granules upon development (Tsai et al, 1982).

Charles Tsai's group, which was then at the National Institutes of Health, first examined the specificity of this

stain using autoradiography. The core oligosaccharide domain contains an unusual eight-carbon sugar that is unique in nature to Gram-negative bacteria, 2-keto-3-deoxymononulosaetonic acid (KDO) (Figure 3) (Orskov, et al, 1977). Assimilated radioactive KDO tags LPS and results in autoradiograms of gels that are identical in every detail but one to those that have been silver stained with periodate modification. The only difference is that the lipid-A bands is not tagged in the autoradiograms.

Later, members of Penny Hitchcock's lab used standard antisera produced by Copenhagen's International Escherichia Center in a modified western blot procedure (Hitchcock, et al, 1983). Electroblots of the LPS from SDS-PAGE were visualized by ELISA. The results reproduced all the higher molecular weight ladder-like bands seen on Tsai's gels. Of course, due to the fact that the primary antibodies could only attach to the epitopes on the O-antigen side-chains, neither lipid-A nor core bands were seen or expected.

Further elucidation of the side-chain structure of the various LPSs were identified by Klaus Jann of the Immunology and Biochemistry section of the Max Planck Institute in Freiburg, Germany. His group lifted bands from SDS-PAGE gels and found that each successively higher molecular weight band differed only by having an added "repeating unit" of a discrete and constant oligosaccharide structure. Thus only the molecular weights of lipid-A, of core, and of a single

repeating unit need be known to calculate the molecular weight on any specific band in the gel (Jann, 1983).

The whole O-antigen LPS molecule is a thermostable surface antigen found on, by definition, all smooth or S-form Enterobacteriaceae (Orskov, et al, 1977). The covalent bonds within the entire LPS molecule are so stable that boiling is one of the common steps in the isolation and preparation of LPS for analysis (Goldman, et al, 1984). Furthermore LPS is so firmly anchored in the outer membrane that none is released spontaneously into the surrounding medium in the form of membrane blurbs at temperatures lower than 48°C (Lüderitz, et al, 1966), which is 3°C above the maximum viable temperature tolerated by these bacteria. This is quite the contrary to the freely released capsular K-antigens (Vermeulen, et al, 1988). As expected, I found that no detectible LPS was found in the cell-free spent media of bacteria grown at 43°C. The sensitivity of my detection was below 10^{-3} of the normal, low temperature grown cells.

I was thus able to determine which portions of the LPS molecule were affected by experimental manipulation. Not only were variations imposed by environmental stresses on the molecular weight within an O-type readily apparent, but also semi-quantitation was attainable without the use of radioisotopes.

Variation in the structure and composition of LPS as a function of growth temperature has been reported previously,

but these studies were conducted at growth temperatures between 19°C and 37°C (Cadieux, et al, 1983; McConnell, et al, 1979). To continue with Wilson's findings, my study here concentrates on the phenotypic differences between bacteria grown at 37°C and those at 43°C.

After I established that 90% of the E.coli strains tested showed variation in LPS at febrile temperature, I investigated how this might affect the biology of the bacteria. One of the known functions of the LPS layer is protection from serum complement killing by a host (Goldman, et al, 1984). This is because the O-antigen of the many molecules of LPS protrude into the environment, creating a layer which the proteins of serum complement cannot physically penetrate. Studies have been done with point-mutant enterobacteria that are devoid of O-antigen and are called rough or R-forms (Orskov, et al, 1977). It was found that cells lacking O-antigen are in many cases killed directly by serum complement in the absence of specific antibodies, but the prototypic parent-types of these mutants are resistant (Orskov, et al, 1977) due to the failure of the C5b-9 complex of serum complement to insert into the cell membrane (Goldman, et al, 1984). Without the insertion of C5b-9, serum complement cannot target, perforate and kill the bacterium (Goldman, et al, 1984). It was thus demonstrated that cells lacking O-antigen were in many cases killed directly by complement in the absence of specific antibodies (Goldman, et al, 1984). Due to the fact that I was

seeing perturbation of the O-antigen, I felt the serum sensitivities of the strains grown at febrile temperatures should be tested.

My results showed a relationship between reduction in LPS O-antigen and increased serum complement sensitivity. These results were notable in that they demonstrated that fever temperatures can weaken the defensive capabilities of invading bacteria and thus boost the effectiveness of the host's extremely important non-specific immune activities. I have included speculations on the future impact of this finding in the Discussion.

Finally, I was curious about the mechanism by which LPS apparently loses its O-antigen at increased growth temperature. My experiments were not intended to solidly answer this question, but merely to propose a logical hypothesis to lay the groundwork for future study. After elimination of a number of possibilities, I have evidence that a reversibly temperature-sensitive enzyme in the LPS production pathway is the cause for what Wilson termed the "Fever Effect" on LPS, which renders the cells more serum sensitive.

MATERIALS AND METHODS

BACTERIOLOGY. The strains of enterobacteria were obtained from local hospital control stocks. The E.coli strains were acquired from three sources: The Walter Reed Army Institute of Research in Washington D.C., the E.coli Typing Center at the Pennsylvania State University and the International Escherichia Center - World Health Organization, Copenhagen, Denmark. The strain list is included in Table 4, in the results. The various growth conditions used are given in appropriate points in the methods that follow.

LPS ANALYSIS. Strains were grown aerobically overnight at 30°C in autoclaved L-broth (0.3% w/v glucose, 0.7% w/v tryptone, trace of thiamine). Each overnight sample was then diluted 50-fold in three test tubes of 5 ml L-broth. These were incubated at 30°C, 37°C and 43°C until the first trace of turbidity appeared to the unaided eye. Optical densities were taken and recorded for use in adjusting sample volumes for electrophoresis. The cells were then centrifuged at 10,000 rpm for 1 to 2 min. at 4°C. The supernates were saved for analysis (below) and the bacterial pellet was resuspended in 0.4 ml double-distilled water. The samples were transferred to microcentrifuge tubes and 0.4 ml of 2X-lysing buffer (400 ml 1.5 M Tris (pH 8.8), 20 g SDS, 100 ml glycerol and a trace

of Bromophenol Blue) was added in preparation for electrophoresis (Peterson, et al, 1985). Samples were boiled for 5 min. to denature the DNA thereby reudcing viscosity. Non-boiled controls were tested for the effects of boiling on LPS structure. The previously mentioned supernates were similarly analyzed for LPS that may have sloughed off the growing cells. For this, 0.4 ml samples of the supernates were added to 0.4 ml of lysing buffer, briefly boiled and submitted for electrophoresis analysis.

ELECTROPHORESIS. All gels were 1.5 mm thick 12% SDS-polyacrylamide, the components of which are in Table 1 (Laemmli, et al, 1970). Samples were loaded into wells in amounts adjusted for the optical density (600 nm) of the aqueous resuspended cells subsequent to centrifuging. The gels were immersed in electrode buffer, Table 2, in a SE 600 Vertical Slab Gel Unit (Hoefer Scientific Instruments). Gels were run at 200 volts until the loading dye was 2 cm from the bottom of the gel, taking approximately 4 hours.

LPS SPECIFIC SILVER STAIN. All LPS gels were stained by the procedure in Table 3 (Hitchcock, et al, 1983; Kropinski, et al, 1986; Tsai, et al, 1982). In brief, the staining procedure differed from that used for protein analysis in that the chromic acid activator was substituted with the more mild, saccharide-specific per-iodic acid. This stain revealed LPS by virtue of the fact that per-iodate oxidation activated the lipid-A components to macromolecular aldehydes, which later

TABLE 1
12% SDS-PAGE COMPONENTS

Component	Volume for bottom gel	Volume for top gel
A/B (29.2% w/v acrylamide;0.8% w/v bis-acrylamide)	28.0ml	1.3ml
TRIS (1.5M, pH8.8)	17.5ml	0.5ml
ddH_2O	23.7ml	8.2ml
10% SDS	0.7ml	0.1ml
TEMED	0.35ml	0.06ml
10% AmPerSulf	1.75ml	0.5ml

The above components are combined in order. The bottom gel is poured between the glass plates of a 1.5mm slab gel set-up. It is topped with butanol to allow the gel to harden anaerobically. Once set the remaining liquid is poured off and the top gel mixture is poured between the glass plates. A well comb is placed in the top gel. Once set, the well comb is removed and the gel is ready for electrophoresis.

TABLE 2
ELECTRODE BUFFER COMPONENTS

Component	Amount	Molarity
Tris base	52g	0.25M
Glycine	250g	1.92M
SDS	26g	0.05M
ddH_2O	1500ml	

Add HCl to pH 8.3
Add ddH_2O to final volume 1736ml
Dilute 5-fold for use in top and bottom buffer chambers.

This is the recommended buffer for use with any Hoeffer Scientific Instruments slab gel apparatus.

TABLE 3
LPS-SPECIFIC SILVER STAIN

For one pair of 1.5mm gels.

		ml	time
1.	Fix I (200ml ddH_2O & 160ml EtOH & 40ml HOAc)	400	1 hr.
2.	Fix II (680ml ddH_2O & 80ml EtOH & 40ml HOAc)	400	30 min.
3.	Fix II	400	30 min.
4.	Oxidizer	400+	10 min.
	(220ml ddH_2O & 160ml EtOH & 20ml HOAc & 2.8g per-iodic acid)		
5.	ddH_2O	400	10 min.
6.	ddH_2O	400	10 min.
7.	ddH_2O	400	10 min.
8.	Silver Rgt (20ml BioRad Silver & 180ml ddH_2O)	200	30 min.
9.	ddH_2O	400	2 min.
10.	Developer(11g BioRad Developer & 600ml ddH_2O)	200	2 min.
11.	Developer	400	to done
12.	Stop (400ml ddH_2O & 20ml HOAc)	400	5 min.
13.	Drying (200ml EtOH & 200 ml ddH_2O)	400	O/N
14.	Place in Cellophane Dryers		

This procedure stains the lipid-A of LPS on SDS-PAGE producing two bands at the bottom of the gel for lipid-A and core, as well as ladder-like bands up the gel which are lipid-A bound O-antigen.

reduced the applied silver ions for subsequent development and visualization. Therefore all molecules incorporated into the mature lipopolysaccharide molecules, which consist in part of lipid-A, are readily detected. The specificity of this protocol has been confirmed through both immuno-chemical and radiolabelling techniques (Hitchcock, et al, 1983). Ladder-like bands up the gel represent increasing lengths of O-antigen which are bound to lipid-A (Peterson, et al, 1985). This was determined by chemical analysis of molecules lifted from these bands (Jann, 1983). Core and lipid-A are found in the fast-moving bands at the bottom of the gel, as labelled in Figure 1 of the introduction (Peterson, et al, 1985).

Analysis of the gels were made by comparing the placement and intensities of the O-antigen bands. Such visual semi-quantitative interpretation was possible due to the fact that samples were loaded based on normalized optical density, as mentioned earlier.

STAIN SENSITIVITY. The sensitivity of the LPS Specific Silver Stain was determined using $1.5x10^{10}$ cells pelleted from a 5 ml overnight L-broth culture. As in the protocol above, these cells were suspended in 0.4 ml of double-distilled water, and then lysed in 0.4 ml 2X-lysing buffer to give a 1 ml lysate of $1.5x10^{10}$ cells. The lysate was then serially diluted with 1X-lysing buffer, and electrophoresed as above. Band positions and intensities were noted.

SERUM SENSITIVITY TESTS. Standard serum killing trials were conducted (Mackowiak et al, 1983; Sabag, et al, 1977). Fresh, non-immune blood from healthy human donors was defibrinated by moderate swirling with glass beads at 37°C. Blood samples were mixed 20:1 with bacterial samples which were growing in L-broth at various temperatures. The reacting mixtures were swirled continuously at 37°C or 43°C for 15 min. Because serocidally sensitive cells are rapidly killed, zero time results were obtained by substituting normal saline (sterile 0.15 M NaCl) for the blood. All timed samples from either saline or defibrinated blood were enumerated by conventional serial dilution and plating on fresh MacConkey agar plates. Colonies were counted after 24 hours and calculations were made based on the percentage of killing between zero time and 15 min. exposure to blood. Analysis involved comparing the rates of killing between bacteria grown at 37°C and 43°C.

THERMAL GRADIENT. The experiment was run in the same manner as the LPS analysis with the exception that many more temperatures were used. This was facilitated by the use of a 7 x 7 x 70 cm^3 aluminum bar, in which there were a series of wells. The bar was suspended between two waterbaths, and the fins at each end of the bar were immersed into the baths. Culture tubes in the wells quickly became thermally equilibrated to temperature corresponding to the linear distance between the baths. The temperatures of the baths had

been pre-adjusted such that the wells at each end of the bar settled at 37°C and 43°C.

DOUBLING EXPERIMENTS. A 40 ml L-broth culture of log-phase bacteria was grown at 37°C to an OD (600 nm) = 0.30, at which time 20 ml was removed and killed by addition of 1 ml of 30% formalin. The remainder of the culture was abruptly upshifted to 43°C by the addition of 20 ml of 50°C broth. The culture was allowed to continue growing at 43°C. When it had reached OD = 0.30, a 20 ml "first generation" sample was taken and formalin killed. 20 ml of fresh pre-adjusted 43°C broth was added to the remainder of the culture and the culture was allowed to grow to OD = 0.30. At that time the "second generation" sample was taken, and the cycles of adding fresh 43°C broth and taking samples continued. In this way, by definition, the use of halving the cell concentrations and allowing then to grow back to OD = 0.30 saw the passage of generations.

STREPTOMYCIN TEST. Bacteria were grown overnight in 10 ml aerated L-broth at 43°C, and then diluted 50-fold with 490 ml of fresh 43°C medium for continued growth at 43°C. Once the culture reached OD (600 nm) = 0.15, a 10 ml "before" sample was taken and immediately killed with the addition of 1 ml formalin. 12 ml of 1% filter-sterilized streptomycin was added to the remaining 490 ml of 43°C culture. After allowing 5 min. for the streptomycin to penetrate the cells, the culture was quickly downshifted to approximately 37°C by rapid

addition of 500 ml of 30°C L-broth. At timed intervals, 20 ml samples were taken and killed by addition of 2 ml formalin. The samples were prepared for electrophoresis by the method described earlier. A control was run by the same method, in the absence of streptomycin.

LAC-OPERON INDUCTION TEST. The efficacy of the above streptomycin method was tested on its ability to prevent the de novo synthesis of beta-galactosidase upon inducing the lac-operon. Genetically inducible lac^+ strains were grown to early log-phase in 0.7% tryptone broth at both 37°C and 43°C. At 5 min. prior to induction, streptomycin was added as above. Then the lac-operon induction was attempted by the addition of isopropyl-thiogalactopyranoside (IPTG) to 0.03 mM (Pardee, et al, 1959; Zabin, et al, 1970; Miller, 1972). In brief, this long-used, standard procedure required that, samples of 0.5 ml were taken each min., and killed immediately by swirling with an equal volume of antioxidant buffer containing SDS and chloroform. Beta-galactosidase was assayed by its ability to convert colorless o-nitrophenyl-beta-galactopyranoside to yellow o-nitrophenol. This color change was determined by the unaided eye.

ETHANOL TEST. Cultures of bacteria were grown to log phase at 30°C and 43°C in L-broth. A third sample was grown at 30°C, in L-broth which was made to 1% with filter-sterilized ethanol. Samples were then taken and both prepared for electrophoresis, as above, as well as for lac-operon

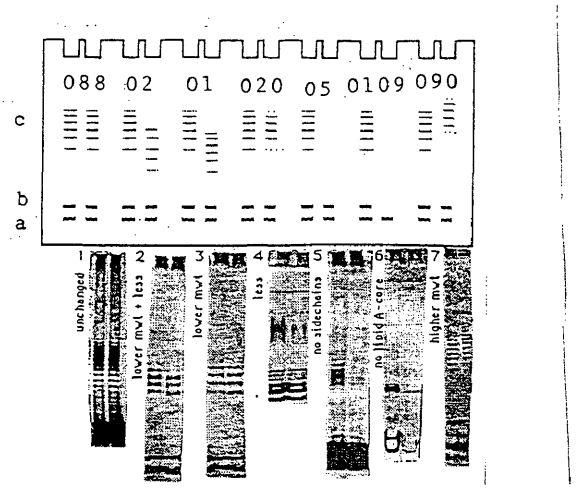
induction studies.

RESULTS

LPS ANALYSIS. Examples of the kinds of results obtained can be seen in Figure 4. Each schematic is accompanied by copies of actual LPS-specific silver stained SDS-PAGE lanes. The first of each pair is growth at 37°C, while the second is 43°C incubation. I observed seven different results by comparison of 43°C grown strains to 37°C. The first group had LPS bands which remained unchanged between the two temperatures. Next are two groups with O-antigen bands which fell lower on the gel. The first of these groups also had O-antigen bands which faded between 37°C and 43°C, as did the forth group. O-antigen bands were not evident at all in both group five and group six, although the later also had no lipid-A-core bands. The O-antigen bands of the final group did not travel as far down the gel at 43°C compared to 37°C.

The electrophoretic results of all the 82 E.coli strains and the tested Gram-negative relatives grown at 30°C, 37°C and 43°C are shown in Table 4. The characteristics of the bands on SDS-PAGE were classified into one of the seven above categories, and labelled such in the table. Approximately 90% of the strains exhibited some perturbation on LPS-specific silver stained SDS-PAGE. These perturbations are the kinds of results seen in groups two to seven on Figure 4. Only about

FIGURE 4
EXAMPLES OF LPS PERTURBATION



This is a drawing of a 12% SDS-PAGE gel visualized by the LPS-specific silver stain. The lanes below are the pictures of the actual gel. Lipid-A (a) and core (b) bands can be seen at the bottom of the gel. The ladder-like bands are LPS O-antigen (c). The changes in these bands as a result of growth at 43°C, divided the strains tested into seven categories. These strains are examples from each of the categories. For each sample, the pair of lanes shows results from growth at 37°C (left) and growth at 43°C (right). Samples were loaded based on optical density adjustments.
O88: the O-antigen bands in the lanes are similar thus O-antigen is unchanged from 37°C to 43°C.
O2: the 43°C O-antigen bands are lower and lighter compared to 37°C bands thus the O-antigen has decreased both in molecular weight and in amount per cell.
O1: the 43°C O-antigen bands are lower on the gel compared to 37°C bands thus the O-antigen has decreased in molecular weight but not in amount per cell as OX1.
O20: the 43°C O-antigen bands are lighter compared to 37°C bands thus the O-antigen has decreased in amount per cell but not in molecular weight as OX1.
O5: there are no O-antigen bands at 43°C thus the cells have no detectable O-antigen at 43°C.
O109: there are no O-antigen, lipid-A or core bands at 43°C thus the cells have no detectable LPS components at 43°C.
O90: the 43°C O-antigen bands are higher on the gel and lighter compared to 37°C bands thus the O-antigen increases in molecular weight and decreases in amount per cell at 43°C.

TABLE 4
ELECTROPHORESIS RESULTS
of Selected Enterobacteria Which are Smooth at 30°C.
A comparison of 37°C and 43°C LPS profiles with those of 30°C.

E.coli strains & relatives	37°C	43°C
O1	L & L	lower
O2	L & L	L & L
O4	lower	unchanged
O5	L & L	none
O6	L & L	L & L
O7	lower	lower
O8	unchanged	unchanged
O9	unchanged	unchanged
O10	none	none
O17	lower	none
O18ab	L & L	L & L
O18ac	lower	L & L
BORT	L & L	L & L
O19	lower	L & L
O20	less	less
O21	lower	less
O22	less	less
O24	none	none
O25	lower	lower
O27	lower	less
O30	lower	less
O41	lower	less
O43	lower	lower
O44	unchanged	L & L
O46	lower	lower
O48	lower	L & L
O50	unchanged	L & L
O51	lower	L & L
O55	L & L	L & L
O58	less	less
O63	less	less
O64	unchanged	less
O65	none	none
O70	lower	lower
O71	lower	lower
O74	lower	lower
O75	less	less
O76	lower	L & L
O77	L & L	lower

TABLE 4 (CONT.)
ELECTROPHORESIS RESULTS

E.coli strains & relatives	37°C	43°C
O79	lower	L & L
O80	unchanged	L & L
O83	less	less
O85	less	less
O86	lower	L & L
O88	lower	unchanged
O90	H & L	H & L
O93	less	L & L
O98	lower	lower
O99	lower	lower
O103	lower	lower
O104	less	lower
O109	lower	no l-A
O110	unchanged	H & L
O111	less	less
O112ac	less	less
O113	lower	lower
O115	L & L	L & L
O116	L & L	L & L
O117	L & L	L & L
O119	L & L	L & L
O120	L & L	L & L
O121	less	less
O123	less	L & L
O125	L & L	less
O126	none	no l-A
O127	L & L	less
O128	unchanged	less
O129	less	less
O132	L & L	unchanged
O133	less	no l-A
O134	L & L	L & L
O135	unchanged	unchanged
O138	less	less
O139	L & L	L & L
O140	L & L	less
O142	none	none
O143	less	less
O145	L & L	L & L
O146	L & L	less
O147	none	none
O150	less	less
O154	unchanged	less
C.freundii	less in a range from 32.5°C to 39.5°C	
E.aerogenes	less	less
P.mirabilis	lower	none
P.vulgaris	unchanged	less

TABLE 4 (CONT.)
ELECTROPHORESIS RESULTS

E.coli strains & relatives	37°C	43°C
S.enteritidis	less	less
S.marcesens	H & L in a range from 32°C to 40°C	

These are the results of interpretation of LPS-specific silver stained SDS-PAGE. Bacteria samples were grown at each of the three temperatures, unless otherwise noted. The O-antigen bands were analyzed according to the following:

lower- LPS O-antigen is of a lower molecular weight on average, O-antigen bands migrated farther in the gel

less- there appears to be less O-antigen, fading bands of O-antigen on the gel

L & L- the O-antigen is lower in average molecular weight and less of it is bound to lipid-A

unchanged- no significant change in O-antigen bands is visible between the temperatures

none- no O-antigen bands are visible at this temperature, bands for lipid-A and core do appear

no l-A- not enough lipid-A-core is visible at the bottom of the gel to get reliable results

H & L- the O-antigen is higher in average molecular weight and less is bound to lipid-A, fading bands higher on the gel

10% of the strains had no change in LPS on these gels, as in the first group of Figure 4.

Gel lanes loaded with samples of cell-free spent media exhibited no banding, while LPS^+ standard cell lysates showed well defined banding.

STAIN SENSITIVITY. Bands for lipid-A, core and O-antigen side-chains could be seen at 10^{-3} dilution of the standard LPS^+ cell lysate. Since 0.02 ml of undiluted lysate corresponded to $2.6x10^8$ cells, the lower resolution of this test was approximately the LPS complement contained by $3x10^5$ smooth cells.

SERUM SENSITIVITY TESTS. Based on colony counts done within 24 hours, all strains tested, except O88, showed an increase in serum complement sensitivity when grown at 43°C compared to 37°C. A relationship was seen between serum sensitivity increase and the O-antigen gel bands at 43°C. Table 5 illustrates this clearly. The strains in the table are ordered by increasing serum sensitivity to illustrate the correlation between 43°C bands and sensitivity. Those strains which had LPS that appeared unchanged by elevated temperature, showed the least increase in serum sensitivity. Sensitivity increased notably as the LPS O-antigen levels decreased and lowered in molecular weight. Those with no O-antigen bands visible at 43°C had the greatest increase in sensitivity.

THERMAL GRADIENT. Cultures between 37°C and 43°C showed four modes of perturbation on LPS-specific silver stained SDS-

TABLE 5
SERUM SENSITIVITY RESULTS
(43°C vs 37°C)

Strain	Relative Serum Sensitivity	O-antigen
O88	1 (unchanged)	unchanged
O4	4	unchanged
O9	6	unchanged
O18ac	10	L & L
O6	10	L & L
O2	15	L & L
O1	20	lower
S.enteritidis	40	less
E.aerogenes	60	less
O111	80	less
S.marcesens	80	H & L
P.vulgaris	100	less
O24	125	none
O138	250	less
O10	300	none
O20	300	less
O93	400	less
O5	800	none
P.mirabilis	3000	none

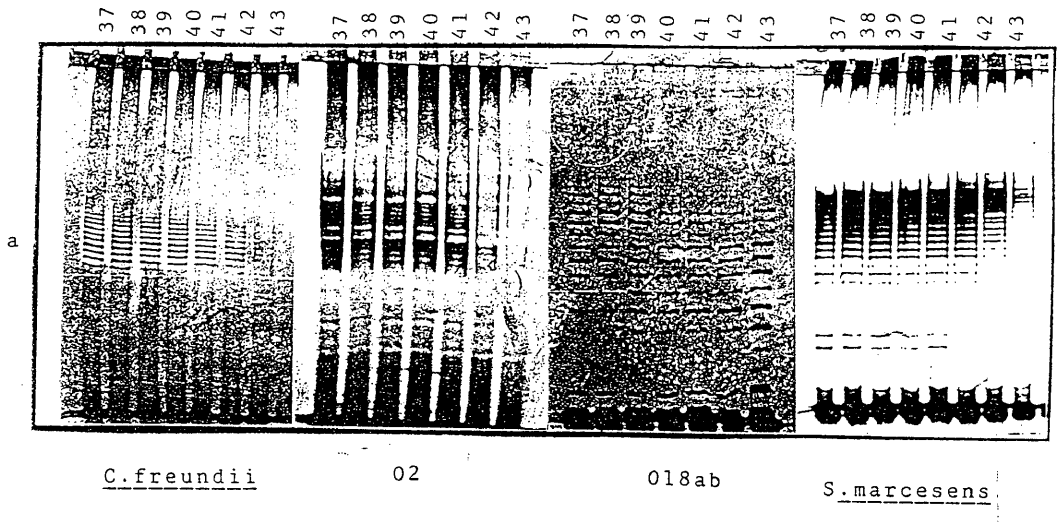
Abbreviations used in the "O-antigen" column are the same as those in Table 5. This data was placed in order of increasing serum complement sensitivity, up to an increase of 3000-fold in bacteria grown at 43°C, versus those of the same strain grown at 37°C. Results were obtained by comparison of the fraction of surviving bacteria (via plate counts) after timed exposure to normal saline. Then this data for each growth temperature was compared to determine the increase with increased temperature. This data suggests a correlation between the LPS O-antigen bands seen on LPS-specific silver stained SDS-PAGE and serum sensitivity. Strains with no visible perturbation of LPS has little increase in serum sensitivity while strains with no O-antigen bands visible on the gels at 43°C, increased greatly in serum complement sensitivity.

PAGE, as seen in Figure 5. Those grouped as Mode One exhibited O-antigen bands that gradually faded with increased temperature. Those in Mode Two had O-antigen bands that also gradually faded, but there was additionally a critical temperature above which there were no O-antigen bands. In Mode Three, the average molecular weights of the O-antigen molecules decreased gradually. This can be seen in the third gel of Figure 5 as bands which migrated farther down the gel. Bacteria belonging to Mode Four gradually increased in average O-antigen molecular weight (higher bands in gel four, Figure 5) and then reached a critical temperature above which no O-antigen bands appeared.

DOUBLING EXPERIMENTS. In these abruptly upshifted cells, the O-antigen electrophoretic bands faded for each passing generation, as in Figure 6. The amount of O-antigen in each passing generation appeared to be half as much as that in the previous generation. This can be seen in the significantly fading bands of the first five lanes of the gel. When the samples were added in sequentially doubled volumes for each passing generation, the O-antigen levels appeared similar from lane to lane, as in the second half of the gel.

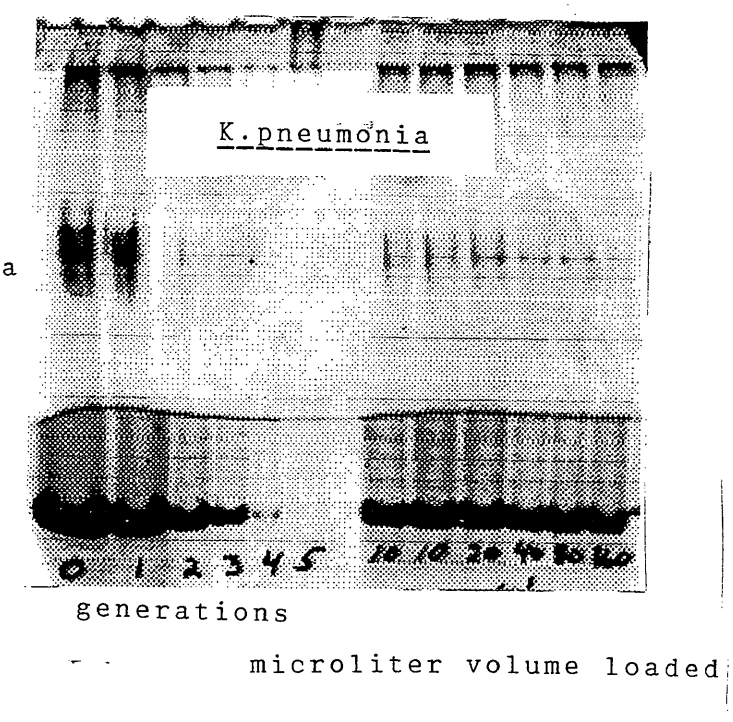
STREPTOMYCIN TEST. The sample of a selected Mode Two strain, that was taken before down-shift from 43°C, had no O-antigen bands, as in lane one of the gel in Figure 7. Both with and without the addition of streptomycin prior to abrupt thermal downshift to 37°C, O-antigen bands began to appear at

FIGURE 5
EXAMPLES OF THERMAL GRADIENT MODES



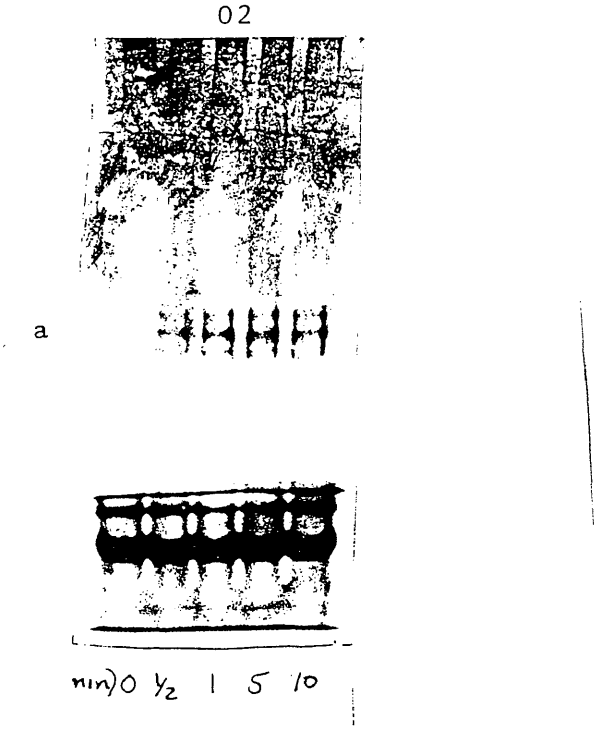
These are four 12% SDS-PAGE gels visualized by the LPS-specific silver stain. The bacteria were grown across a thermal gradient from 37°C to 43°C. The results of the strains tested fell into four modes. These are examples of each of the modes.
Mode 1: the O-antigen bands (a) gradually fade with increasing temperature.
Mode 2: the O-antigen bands gradually fade, additionally there is a critical temperature above which there are no O-antigen bands.
Mode 3: the O-antigen bands become lower on the gel as the temperature increases thus the average molecular weight of the O-antigen molecules decreases gradually as temperature increases.
Mode 4: the O-antigen bands become higher on the gels as the temperature increases thus the average molecular weight of the O-antigen molecules increases gradually as temperature increases.

FIGURE 6
EXAMPLE OF DOUBLING EXPERIMENTS



This is a 12% SDS-PAGE gel visualized by the LPS-specific silver stain. Samples were taken at the passing of each generation of bacterial growth at 43°C. Each of lanes 1-6 represent successive generations of cells. The O-antigen bands (a) in these lanes fade rapidly, with each lane apparently showing half as much as the previous lane. Lanes 8-13 are the same samples as lanes 1-6, loaded in doubling volumes for each passing generation. The O-antigen bands now appear similar, although each lane represents twice as many cells. These results suggest that no new LPS is produced at 43°C and that the LPS already present is being evenly distributed to the daughter cells of the next generation.

FIGURE 7
STREPTOMYCIN EXPERIMENT



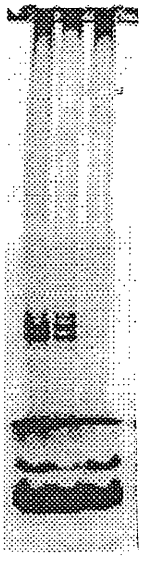
This is a 12% SDS-PAGE gel visualized by the LPS-specific silver stain. The bacteria, grown to log at 43°C, were exposed to streptomycin, to inhibit transcription and translation, then quickly downshifted to 30°C. The sample taken before shift (lane 1) has no O-antigen bands (a). These bands begin to appear at 30 seconds after downshift (lane 2). Results obtained without the addition of the antibiotic were similar. These results show that, although they do not produce complete LPS molecules at 43°C, the production pathway enzymes are present at 43°C, and once again begin to function when the cell returns to permissive temperature. This supports a temperature sensitive enzyme model over a genetic inhibition model to explain LPS perturbation at increased temperature.

30 seconds, as in lane two. The bands increased in intensity reaching normal levels within 5 min, as in lane 4, Figure 7.

LAC-OPERON TEST. Addition of streptomycin 5 min. prior to addition of IPTG inhibited induction of beta-galactosidase at both 37°C and 43°C, as evident by a lack of color change. Controls to which no antibiotic was added both exhibited induction within 5 to 10 min. The 43°C culture showed much lower levels of beta-galactosidase than the 37°C culture, indicated by the degree of color change observed by the unaided eye.

ETHANOL TEST. Growth of Mode Two strains in 1% ethanol produced similar O-antigen bands as similar strains without alcohol in 30°C cultures. In Figure 8, the first two lanes were loaded with samples grown at 30°C, but the growth media of the second lane contained 1% ethanol. These lanes are similar, while the third lane, with the sample grown at 43°C, has no O-antigen bands. It was shown, however, that 1% ethanol grown cultures were sluggish in their induction of the lac-operon.

FIGURE 8
EXAMPLE OF ETHANOL TEST



O5

This is a 12% SDS-PAGE gel visualized by the LPS-specific silver stain. The samples were grown to log phase at 30°C (lane 1), 30°C with 1% EtOH (lane 2), and 43°C (lane 3). An identical shock response is often created by exposure to heat and ethanol (Weiner, et al, 1994). Such responses can inhibit normal cellular processes at the genetic level. If such a stress response were at work here, lanes 2 and 3 would appear similar. Instead, lanes 1 and 2 appear similar, supporting the streptomycin experiment, in favor of a temperature sensitive enzyme model to explain LPS perturbation at increased temperature.

DISCUSSION

All strains were initially grown at 30°C to screen for those cells having the ability to produce LPS. Growth at 37°C and at 43°C in enriched medium was then conducted to ascertain the characteristics of their LPS under conditions mimicking average endothermic and fever conditions, respectively. Although 43°C may be a high febrile temperature for humans, I was not anthropocentric in my choice of temperature. Reference to Table 6 shows that fevers of 43°C are easily reached in many animals such as rabbits, which are the common experimental animal model in fever studies, and is close to the normal body temperatures for small birds.

I concluded that since LPS was neither being lost into the medium, nor being destroyed, the temperature must be affecting its synthesis. To get a better idea of what happens to the LPS over a range of temperatures, to see if critical thresholds, for example, exist, I set up a thermal gradient. In this way I could examine the SDS-PAGE profiles of gradually increasing temperature. The results of electrophoresis clearly divided the bacteria strains into four modes consisting of seven different categories. When comparing 43°C gel profiles with those of 37°C:

TABLE 6
HEALTHY ANIMAL BODY TEMPERATURES

Animal	°C	°F	Fevers °F
Elephant	36.1	97	
Prairie dog	36.7	98	
Humans	37.0	98.6	
Cattle	38.6	101.5	
Horse	38.6	101.5	
Cat	38.6	101.5	106.5
Dog	38.9	102	107
Pig	38.9	102	
Goat	38.9	102	
Rabbit	38.9	102	107.5
Sheep	39.4	103	
Chicken	41.1	106	
Small birds	43.3	110	

These are the average body temperatures of healthy animals.

Mode One (refractory)

1. 9% were unchanged

Mode Two (diminished molecular weight)

2. 31% were lowered in both average molecular weight and amount per cell
3. 17% were only lowered in average molecular weight

Mode Three (diminished amount)

4. 29% had less LPS per cell
5. 10% had become completely devoid of O-antigen LPS side-chains
6. 4% had become devoid of all domains of LPS including core and lipid-A

Mode Four (increased molecular weight)

7. 2% had higher molecular weight and less LPS per cell

To further investigate the fate of LPS, I looked at what happened to pre-existing O-antigen over several generations subsequent to the culture's being elevated to a temperature impermissive to synthesis, yet still permitting cell proliferation (i.e.: 43°C). Samples were taken for each new generation, the O-antigen bands of which faded significantly from those of the generation before. Since each generation is a doubling of of the number of bacterial cells in the sample, I decided to load a gel with serially doubled volumes of sample for each passing generation. The O-antigen bands on this gel appeared rather similar, leading me to conclude that

no new complete LPS molecules were produced at 43°C, but instead those molecules already present were dispersed among the daughter cells. Although I do not know as a certainty that those molecules I see on the gel in subsequent generations are the same molecules which are in the previous lane, I suspect that they are, due to the inherent stabilities of the LPS. This could be verified by using radiolabelled LPS in a future study.

The pervasiveness of the temperature-effect on LPS then prompted me to see whether or not it correlated with increased sensitivity to killing by serum complement. Healthy non-immune human blood was used, thereby eliminating the effects of antibodies as in the classical pathway of complement activation. I was interested in whether fever would be able to indirectly augment the non-specific immune function termed the alternative pathway to complement activation where the complement proteins alone can perforate and kill susceptible invading bacteria. Such research is especially pertenant in light of the recent demise of antibiotics.

Sensitivity was determined by noting the proportion of the initial number of bacteria able to withstand 15 min. of exposure to 37°C blood. The defibrinated blood, of course, contained a host of professional phagocytic lymphocytes, which, in themselves were able to kill the test bacteria. But it is known that phagocytosis is several orders of magnitude slower than simple complement killing via the alternative

pathway (Moll, et al, 1979; Vermeulen, et al, 1988; Weiss, et al, 1982). Commonly serum-free phagocytic assays are run for several hours with a loss of less than 90% of especially sensitive strains of bacteria. On the other hand, cell-free serocidal assays show killing rates on the order of three logs within 5 minutes. I thus felt that the added procedures needed to produce phagocyte-free assays were unnecessary. This reasoning became especially valid in later assays, which of necessity had to be performed at 43°C so as to prevent the possibility of the cells rapidly reconstituting their LPS layers and recovering serum resistance. This recovery, as noted in the results, took only seconds, which was far too little time for any phagocytic effects to be noticeable.

In short, all strains tested except E.coli O88, which is refractory Mode One, had some increase in serum sensitivity with increased growth temperature. Many strains exhibited very marked increases in serum sensitivity with higher growth temperatures. Indeed, 43°C-grown Proteus mirabilis, which has no visible O-antigen bands on SDS-PAGE, increased 3000-fold in serum sensitivity. This kind of association was an important finding that held true for all strains tested. Bacteria which retained O-antigen bands had little to no increase in sensitivity, while cells with some perturbation of LPS increased in sensitivity commensurate with LPS changes. Those bacteria which produced no LPS at elevated temperature showed the greatest increases in sensitivity. Apparently the amount

and structure of O-antigen present on the surface of the cell, determines the degree of serum resistance of the bacteria. Since the O-antigen physically prevents the insertion of the serum complement proteins into the membrane, it is logical that a critcal concentration of complete LPS on the surface is necessary to confir serum resistance. As the amount of O-antigen per cell decreases, thus the serum resistance becomes reduced. I therefore concluded that in most Gram-negative bacteria febrile temperatures perturb lipopolysaccharide O-antigen leading to increased serum sensitivity.

This general finding gave me license to begin looking at possible mechanisms. Because of the generality of this "fever effect" a first suggested mechanism was of a global nature - that heat triggered something in the cells causing their LPS genes to be repressed. This type of mechanism would include the heat-shock, or stress protein induction pathways, which do indeed shut down many genetic expressions in bacteria. Only upon return to permissive temperature do those genes become operative and become transcribed and translated.

I quickly disproved this heat-induced genetic inhibition model, which depended upon de novo protein synthesis activity to make new LPS-synthatases once the cells returned to permissive temperature after a long tenure at impermissive elevated temperature. The classical, definitive test for involvement of de novo protein synthesis is that a translation

inhibitor such as streptomycin blocks the making of new enzyme molecules. Streptomycin is an antibiotic which interferes with protein synthesis at the level of initiation by interacting with prokaryotic ribosomal RNA (von-Ahsen, et al, 1991; Honore, et al, 1994). I found that streptomycin, while effective in blocking the induction of the lac-operon, was not effective in blocking LPS synthesis. Within seconds after temperature downshifting, LPS was synthesized.

This conclusion was supported by some other less-definitive studies. The heat shock response is known also to be triggered by ethanol (Weiner, et al, 1994). I found that LPS was produced normally in cells grown at 30°C in ethanol, while the lac-operon was repressed. The electrophoretic lanes for 30°C and 30°C with 1% ethanol were similar, whereas the 43°C cells showed essentially no O-antigen bands.

Also in support against the heat-shock model is merely the time in which cells were able to recover LPS coats after downshifting. In the control for the streptomycin experiment, in less than 30 seconds, the cells recovered what appears to be complete LPS layers. It is known that activation at the genetic level, such as turning on the lac operon, takes at least 3 minutes to begin getting the first traces of enzymatic activity, by expression of a final product (Pardee, et al, 1959). Therefore, for this to be genetic activation, it must be at a rate never before seen.

A second mechanism was one more on the enzymatic level. It posed that all the strains could exhibit such a spectrum of effects due to differences in the various synthetic pathways needed to produce the many different varieties of LPS. It seemed reasonable that each unique pathway might be affected differently by temperature. The primary support for this model also came from the streptomycin experiment. No new protein synthesis was needed by the bacteria to recover upon abrupt thermal downshifts. Hence, at elevated temperature the synthetases must already be present. But they must be in an inactive state since they don't produce LPS. Upon being downshifted to permissive temperatures LPS was almost instantly produced. This implies two things. First, that the inactive synthetases instantly <u>en mass</u> regain activity and begin making LPS in bulk. And, second, it implies that all during growth at elevated temperature the inactive forms of the synthetases were being synthesized. Otherwise, the cells would find themselves devoid of significant quantities of synthetase upon the downshift, and thus unable to commence the massive synthesis of LPS as the data indicate.

Other possible mechanisms were disproved early on, when the methods were investiagted closely. One idea proposed that the LPS changes could be merely a matter of display. If the complete molecules were produced but were not transported to the outer membrane, the molecules would still be visible on the gels. This is due to the lysing method used before

electrophoresis. Therefore it is known that all LPS on and within the cell is being shown on the gels. This model could not then explain the changes seen on the gel.

Another model suggested the bands changed due to conformational change of the LPS molecule. LPS treated before electrophoresis by boiling produced the same bands as samples which were not boiled. If conformational changes occurred they would be seen by this method.

Since the cells were dividing at approximately the same rate at both temperatures, other models involving restricted growth were eliminated.

Hence, the mechanistic portion of this thesis has narrowed the search to the enzymatic level, where, in the pathways of each O-type, there exist one or several synthetases that are reversibly thermolabile. Depending on which portion of the pathway this enzyme is found, different manifestations are exhibited in the electrophoretic gels, giving rise to the various Modes. It is now but a matter of time and effort by successors before the identities of these enzymes are found.

Before any of this knowledge can be applied to the benefit of humans, much more research must be done. These preliminary data suggest that fever temperatures could sufficiently alter invading bacteria, which would be killed more readily by serum complement. To determine what would occur <u>in vivo</u>, experiments should be done on an animal model

such as the rabbit, which is commonly used for fever research. Fever has many effects on the body which may enhance the serum killing results seen here. This line of investigation could lead to renewed interest in fever therapy to combat infection.

Also much more needs to be understood about the nature of the change in LPS. We need to identify, on a molecular level, how increased temperature is effecting the cells phenotypically, and how resistant strains are able to retain the ability to produce complete LPS. Such resistance may be easily transferable, possibly via plasmid, as with antibiotic resistance. If this is the case we will eventually end up no better off than with our failing antibiotics. I believe, however, that due to the complexity and heterogeneity of the LPS synthesis pathway, resistance could not be readily transfered between different strains.

It is also possible that a chemotherapeutic means may be found to produce the same effect in bacteria as fever without the need for elevated and delirious temperature.

Although much more needs to be done, I feel privileged to have been able to perform ground-breaking research in this field.

NOTES

Cadieux, J.E., J. Kuzio, F.H.M. Iazzo, A.M. Kropinski. Spontaneous Release of Lipopolysaccharide by Pseudomonas aeruginosa. J.Bact. **155:** 817-825, 1983.

Egan, W., L. Leive. ^{1}H-^{31}P Nuclear Magnetic Double Resonance Study of Escherichia coli Lipopolysaccharide Mobility. Biochim.Biophys.Acta **692:** 165-167, 1982.

Goldman, R.C., K. Joiner, L. Leive. Serum-Resistant Mutants of Escherichia coli O111 Contain Increased Lipopolysaccharide, Lack an O Antigen Containing Capsule and Cover More of Their Lipid A Core with O Antigen. J.Bact. **159:** 877-882, 1984.

Griffith, F. Significance of Pneumococcal Types. J.Hyg.Camb. **27:** 113-116, 1928.

Hitchcock, P.J., T.M. Brown. Morphological Heterogeneity among Lipopolysaccharide Chemotypes in Silver-Stained Polyacrylamide Gels. J.Bact. **154:** 269-271, 1983.

Hitchener, B.J., A.F. Egan. Outer Membrane Damage in Sublethally Heated Escherichia coli K-12. Can.J.Micro. **23:** 311-318, 1977.

Honore, N., S.T. Cole. Streptomycin Resistance in Mycobacteria. Antimicrob.Agents Chemother. **38:** 238-242, 1994.

Jann, K. Capsular Polysaccharide of Uropathogenic Escherichia coli in Bacterial Lipopolysaccharides: Structure, Synthesis, and Biological Activities. (K. Anderson,F.M. Unger, eds.) Amer.Chem.Soc., Washington, D.C. pp.171-191, 1983.

Kastowsky, M., T. Gutberlet, H. Bradaczek. Molecular Modelling of the Three-dimensional Structure and Conformational Flexibility of Bacterial Lipopolysaccharide. J.Bact. **174:** 4798-4806, 1992.

Kropinski, A.M., D. Berry, E.P. Greenberg. The Basis of Silver Staining for Lipopolysaccharide in Polyacrylamide Gels. Curr.Micro. **13:** 29-31, 1986.

Laemmli, U.K. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. Nature **227:** 680-685, 1970.

Lightfoot, J., J.S. Lam. Molecular Cloning of Genes Involved with Expression of A-Band Lipopolysaccharide, a Antigenically Conserved Form, in Pseudomonas aeruginosa. J.Bact. **173:** 5624-5630, 1991.

Lüderitz, O., A.M. Staub, O. Westphal. Immunochemistry of O and R Antigens of Salmonella and Related Enterobacteriaceae. Bact.Rev. **30:** 193-255, 1966.

Mackowiak, P.A., M. Marling-Cason. Hyperthermic Enhancement of Serum Antimicrobial Activity: Mechanism by which Fever Might Exert a Beneficial Effect on the Outcome of Gram-negative Sepsis. Inf.Imm. **39:** 38-42, 1983.

McConnell, M., A. Wright. Variation in the Structure and Bacteriophage-Inactivity Capacity of Salmonella anatom Lipopolysaccharide as a Function of Growth Temperature. J.Bact. **137:** 746-751, 1979.

Miller, J.H. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, pp. 352-355, 1972.

Moll, A., F. Cabello, K.N. Timmis. Rapid Assay for Determination of Bacterial Resistance to the Lethal Activity of Serum. FEMS Microbiol.Lett. **6:** 273-276, 1979.

Munford, R.S., C.L. Hall, P.D. Rick. Size Heterogeneity of Salmonella typhimurium Lipopolysaccharide in Outer Membranes and Culture Supernatant Membrane Fragments. J.Bact. **144:** 630-640, 1980.

Orskov, I., F. Orskov, B. Jann, K. Jann. Serology, Chemistry, and Genetics of O and K Antigens of Escherichia coli. Bact. Rev. **41:** 667-710, 1977.

Pardee, A.B., F. Jacob, J. Monod. The Genetic Control and Cytoplasmic Expression of "inducibility" in the Synthesis of beta-Galactosidase by E.coli. J.Mol.Biol. **1:** 165, 1959.

Peterson, A., E.J. McGroarty. High-Molecular-Weight Components in Lipopolysaccharides of Salmonella typhimurium, Salmonella minnesota and Escherichia coli. J.Bact. **162:** 738-745, 1985.

Sabag, J., W.P. Reed, R.C. Williams Jr. Effect of Temperature on Bacteria Killed by Serum and by Polymorphonuclear Leukocytes. Inf.Imm. **16:** 947-954, 1977.

Tanamoto, K., U. Zahringer, G.R. McKenzie, C. Galanos, E.T. Rietschel, O. Lüderitz, S. Kusumoto, T. Shiba. Biological Activity of Synthetic Lipid A Analogs: Pyrogenicity, Lethal Toxicity, Anticomplement Activity, and Induction of Gelation of Limulus Amoebocyte Lipate. Inf.Imm. **44:** 421-426, 1984.

Tsai, C.M., C.E. Frasch. A Sensitive Silver Stain for Detecting Lipopolysaccharide in Polyacrylamide Gels. Anal.Biochem. **119:** 115-119, 1982.

von-Ahsen, U., R. Schroeder. Streptomycin Inhibits Splicing of Group I Introns by Competition with the Guanosine Substrate. Nucleic Acids Res. **19:** 2261-2265, 1991.

Vermeulen, C.W., A.S. Cross, W.R. Byrne, W. Zollinger. Quantitative Relationship between Capsular Content and Killing of K1-Encapsulated Escherichia coli. Inf.Imm. **56:** 2723-2730, 1988.

Weiner, L., P. Model. Role of an Escherichia coli Stress-Response Operon in Stationary-Phase Survival. Proc.Natl. Acad.Sci.USA **91:** 2191-2195, 1994.

Weiss, J., M. Victor, A.S. Cross, P. Elsbach. Sensitivity of K1-Encapsulated Escherichia coli to Killing by the Bactericidal/Permeability- Increasing Protein of Rabbit and Human Neutrophils. Infect.Immun. **38:** 1149-1153, 1982.

Zabin, I., A. Fowler. beta-Galactosidase and Thiogalactoside Transacetylase. The Lactose Operon, Cold Spring Harbor Laboratory, p. 27, 1970.

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As a graduate student, the author has been a Teaching Assistant for approximately 200 undergraduates. Member of the William and Mary Fever Study Group and the American Society for Microbiology.

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