MONTANA STATE UNIVERSITY

Physiological conditions influencing attachment of Salmonella typhimurium to epithelial cells in vitro
by Shaun Jensen Gillis

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:
Three different assays were used to examine the attachment of Salmonella typhimurium to epithelial cells in vitro. The assays were able to isolate attachment as a separate, measurable process of bacteria-cell interactions. The chamber slide assay provided visual evidence of attachment, while the cold attachment and the resuscitation attachment assays provided quantitative evidence of bacterial attachment. These assays were used to study the physiological conditions that may influence cell attachment by S. typhimurium. Anaerobically-grown bacteria in the logarithmic growth phase were 100-fold more adherent than aerobically-grown, stationary phase bacteria. Temperature, pH, and osmotic conditions also affected the attachment of S. typhimurium to cultured epithelial cells, but to a much lesser extent than growth phase and oxygen. Previously described mediators of attachment were evaluated in this study. There was evidence that proteins were involved in attachment, while type-1 fimbriae, mannose-resistant hemagglutinins, hydrophobicity, and the 100 kb virulence plasmid did not contribute to attachment. A non-flagellated strain of S. typhimurium did not attach, yet shearing the flagella from a flagellated strain did not decrease attachment. In addition, polyclonal antibodies against the flagella did not inhibit attachment. Thus, the role of the flagella in attachment of S. typhimurium to epithelial cells in vitro remains unclear.

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by

Shaun Jensen Gillis

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TABLE OF CONTENTS

Page

LIST OF TABLES vii

LIST OF FIGURES viii

ABSTRACT x

INTRODUCTION 1
Salmonella typhimurium 1
The Pathogenicity of *S. typhimurium* 3
S. typhimurium in Mice 3
S. typhimurium in Primates 4
Initial Events in Salmonellosis 4
Virulence Factors 5
Mediators of Attachment 7
Lipopolysaccharide 8
Hemagglutinin 9
Flagella 10
Other Factors Influencing Adhesion 11
Statement of Research Problem 13

MATERIALS AND METHODS 14
Chemicals 14
Media and Reagents 15
Equipment and Supplies 16
Bacterial Strains 17
Storage of Cultures 18
Preparation and Handling 18
Cell Cultures 19
Cell Lines 19
Preparation of Cell Monolayers for Assays 20
Antibodies 20
Indirect Fluorescent Antibody Assay 21
Derivation of Non-motile Mutant 22
2-Aminopurine Mutagenesis 22
Enrichment 22
Screening of Mutants 23
Cloning the Mutants 23

TABLE OF CONTENTS (Continued)

Page

Chamber Slide Assay 23
The Enzyme Linked Immunosorbant Assay 25
Attachment Assays 26
Hydrophobicity Assay 27
Hemagglutination Assay 28
Shearing the Flagella off Bacteria 29
Statistical Analyses 30

RESULTS 31
Effect of Growth Phase on Attachment 31
Chamber Slide Assay 31
ELISA 31
Cold Attachment Assay 34
Attachment Assay 34
Choice of Cell Line and Multiplicity 37
Choice of Cell Line 37
Multiplicity 37
Validity of Method for Measuring Attachment 40
Physiological Conditions Influencing Attachment 40
Temperature 40
pH 42
Osmolarity 42
Washing the Bacteria 45
Bacterial Growth Media 45
Oxygen Availability 48
Cell Involvement in Attachment 50
Antibody Versus Cells 50
Endocytic Inhibitors 50
Bacterial Competition in Attachment 52
Mediators of Attachment 53
Protein(s): Treatment with Proteases 53
Protein(s): Treatment with Inhibitors 54
Protein(s): Heat Treatment 54
Protein(s): Alkylation 56
Hydrophobic Interactions 60
Hemagglutinins 60
Flagella: Motility and Chemotaxis 62
Flagella: Physical Structure 64

TABLE OF CONTENTS (Continued)

Page

DISCUSSION 66
Attachment of *S. typhimurium* to Epithelial Cells 66
Mediators of Attachment 70
Conclusions 75
APPENDIX 76

LITERATURE CITED 77

LIST OF TABLES

Table — Page

1. Putative mediators of attachment of *Salmonella typhimurium* to host epithelial cells. 8

2. Attachment of *S. typhimurium* to fixed monolayers 32

3. Attachment of *S. typhimurium* to unfixed monolayers 32

4. Attachment of strain SL3201 to cultured cell monolayers with and without gentamicin 42

5. Effect of oxygen and growth phase on bacterial attachment to cultured cell monolayers 48

6. Competitive attachment to cultured cell monolayers with *S. typhimurium* and *S. senftenberg* 53

7. Hemagglutination of sheep erythrocytes by *S. typhimurium* 62

8. Growth and attachment after incubation in DME at 37°C (raw data to accompany Figure 2) 76

LIST OF FIGURES

Figure Page

1. *S. typhimurium* attachment after incubation with monolayers in DME with 0.5% FBS 33

2. *S. typhimurium* growth and attachment after anaerobic incubation in DME at 37°C 35

3. *S. typhimurium* attachment after resuscitation in DME at 37°C 36

4. *S. typhimurium* attached after 4 h infection to I-407 cells and MDCK cells 38

5. *S. typhimurium* attachment after 4 h resuscitation in DME at 37°C 39

6. *S. typhimurium* attachment after resuscitation in DME for 5 h at 22°C, 4 h at 37°C and 4.5 h at 42°C 41

7. *S. typhimurium* attachment after 4 h resuscitation at 37°C in PBDME at pH 6.2, 6.7, 7.2, and 7.7 43

8. *S. typhimurium* attachment after 4 h resuscitation at 37°C in DME containing 0.0, 0.06, 0.18, and 0.30 M NaCl 44

9. *S. typhimurium* attachment after 5 h resuscitation at 25°C in PBDME with and without 0.18 M NaCl and 4 h resuscitation at 37°C in PBDME with and without 0.18 M NaCl 46

10. *S. typhimurium* attachment after 5 h resuscitation at 25°C in PBDME with or without 0.18 M NaCl, and after resuspension of recovered bacteria in PBDME with or without 0.18 M NaCl 47

11. *S. typhimurium* attachment after resuscitation in DME, M56 or osmotically-balanced DME buffer for 4 h at 37°C 49

12. *S. typhimurium* attachment after aerobic or anaerobic resuscitation in PBDME at 37°C 51

LIST OF FIGURES (Continued)

Figure Page

13. *S. typhimurium* attachment after resuscitation in DME alone and with nalidixic acid or chloramphenicol for 4 h at 37°C 55

14. *S. typhimurium* inoculum and attachment after 3.5 h resuscitation in DME, dilution in DME with 0.5% FBS, holding in 54°C water bath for 2, 4, and 6 min, and cooling on ice 57

15. *S. typhimurium* attachment after 3.5 h resuscitation in DME, addition of potential inhibitors, incubation for 0.5 h, washing, and dilution in DME . 58

16. *S. typhimurium* attachment measured after 3.5 h resuscitation in DME, addition of iodoacetic acid (2 mM) and varying concentrations of cysteine, incubation for 0.5 h, washing, and dilution in DME 59

17. *S. typhimurium* SL3201 and St39 and *Y. enterocolitica* E641 in the stationary phase or log phase (strain SL3201 and St39 resuscitated 4 h, strain E641 resuscitated 4.5 h) 61

18. Comparison of attachment by *S. typhimurium* SL3201, SL3201NM, St_x3337 and St39 63

19. Attachment of *S. typhimurium* stationary or resuscitated in PBDME for 3.5 h and incubated for 30 min with anti-flagellar antibody 65

20. Physiological stairway to attachment 69

ABSTRACT

Three different assays were used to examine the attachment of *Salmonella typhimurium* to epithelial cells *in vitro*. The assays were able to isolate attachment as a separate, measurable process of bacteria-cell interactions. The chamber slide assay provided visual evidence of attachment, while the cold attachment and the resuscitation attachment assays provided quantitative evidence of bacterial attachment. These assays were used to study the physiological conditions that may influence cell attachment by *S. typhimurium*. Anaerobically-grown bacteria in the logarithmic growth phase were 100-fold more adherent than aerobically-grown, stationary phase bacteria. Temperature, pH, and osmotic conditions also affected the attachment of *S. typhimurium* to cultured epithelial cells, but to a much lesser extent than growth phase and oxygen. Previously described mediators of attachment were evaluated in this study. There was evidence that proteins were involved in attachment, while type-1 fimbriae, mannose-resistant hemagglutinins, hydrophobicity, and the 100 kb virulence plasmid did not contribute to attachment. A non-flagellated strain of *S. typhimurium* did not attach, yet shearing the flagella from a flagellated strain did not decrease attachment. In addition, polyclonal antibodies against the flagella did not inhibit attachment. Thus, the role of the flagella in attachment of *S. typhimurium* to epithelial cells *in vitro* remains unclear.

INTRODUCTION

Salmonella typhimurium

Salmonella typhimurium is a member of the genus *Salmonella* in the family Enterobacteriaceae, which includes bacteria that are Gram negative, facultative anaerobic rods with, when motile, peritrichous flagella. *Salmonella* produce gas from glucose, utilize citrate as their sole carbon source, usually produce hydrogen sulfide gas, decarboxylate lysine and ornithine, are urease-negative, and do not produce indole or ferment lactose.

In 1988, there were 2,250 recognized serotypes of *Salmonella* as described by the serotyping scheme of Kauffmann and White (61). This classification scheme recognizes each antigenically distinguishable type of *Salmonella* by its possession of particular O (polysaccharide portion of LPS) or H (flagellar) antigens (61), and assigns species status to each serovar.

S. typhimurium is the most common human pathogen found in foods (11). *Salmonella* can cause infections which may present as any of three distinct clinical entities: (i) salmonellosis, which is a self-limiting gastroenteritis; (ii) enteric fever; and (iii) septicemia, which may lead to secondary problems such as osteomyelitis, pneumonia, pulmonary abscesses, meningitis, or endocarditis (80). Salmonellosis represents a major communicable disease problem in the United States (80). An average

of 40,000 cases are reported annually, however, this number likely represents only a small fraction of the true incidence. Extrapolations from outbreak surveillance data by the Centers for Disease Control (CDC) estimate the "true" incidence of salmonellosis to be from 400,000 to 4,000,000 cases annually with 1000 fatal infections (64). *S. typhimurium*, *Salmonella heidelberg*, and *Salmonella enteritidis* are the serotypes present in 53% of reported cases, with *S. typhimurium* being the most common at 26% (37).

Humans usually acquire *Salmonella* by ingestion of contaminated food or water (1). Many foods have been implicated as vehicles of *Salmonella*, with poultry and other types of raw meat products being the most common (8, 52, 64). Salmonellosis from consumption of dairy products has generally been restricted to raw or improperly pasteurized fluid milk, ice cream, and cheese (80). The single largest foodborne outbreak ever reported was caused by *S. typhimurium* in 1985 (9). The outbreak was associated with 2% low-fat pasteurized milk produced by a dairy plant in Chicago (9, 52). More recently, undercooked or uncooked egg products have been implicated as a major source of a very severe form of salmonellosis (11).

Medical expenses and loss of productivity costs due to salmonellosis are estimated to be anywhere from 983 million to 1.4 billion dollars annually (64). This dollar amount does not include pain and suffering, reduction of leisure time choices, other individual costs, industry costs of reduced animal performance or product recall, plant cleanup, or the cost of the public health surveillance system (64).

The Pathogenicity of *S. typhimurium*

Salmonella are capable of causing different syndromes in different hosts. The contribution of virulence factors varies with the serotype causing the infection and the host species involved (80).

S. typhimurium in Mice

S. typhimurium causes a lethal infection in susceptible strains of mice. The systemic infection that occurs in mice with *S. typhimurium* demonstrates many of the hallmarks of the human disease caused by *Salmonella typhi*, including anorexia, dissemination through the reticuloendothelial system (RES), and splenomegaly (56). *S. typhimurium* invades the intestinal epithelium and is transported through the cells in membrane bound vacuoles (25). The bacteria replicate and appear to remain within these vacuoles following this replication (18). Once the bacteria have penetrated the intestinal barrier, they pass through the lamina propria and into the gut-associated lymphoid tissue (GALT). *S. typhimurium* colonizes the Peyer's patches, enters draining mesenteric lymph nodes, and spreads through the lymphatic system (25). The macrophage is the target cell for *S. typhimurium* in the murine model. The ability to survive in macrophages is believed to be due to the production of bacterial proteins that enable the organism to withstand both the oxygen-dependent and oxygen-independent killing mechanisms of these "professional" phagocytic cells (80). *S. typhimurium* is also resistant to the bactericidal activity of serum complement (66). *S. typhimurium's* ability to survive in the lymphatic system allows it to spread to the liver and spleen and a disseminated infection follows (25).

S. typhimurium in Primates

In humans, *S. typhimurium* causes gastroenteritis, or salmonellosis, rather than a typhoid-like disease. The organism penetrates the distal ileum and proliferates in the intestinal epithelial cells (7, 38). Salmonellosis is characterized by fever, diarrhea, and abdominal pain (80). The symptoms usually last for two to five days, but can persist for several weeks. Dehydration and electrolyte loss constitute the major threats in severe cases, in infants, and in the elderly (80). Immunosuppressed patients are at a larger risk when bacteria from the intestine reach the blood and lymph systems. These bacteria can disseminate and cause focal lesions in the tissue. Osteomyelitis via this secondary route of infection poses a grave problem, especially in AIDS patients (66).

Monkeys infected with *S. typhimurium* demonstrate fluid secretion in the jejunum and the ileum. Diarrhea is manifested when the colon is unable to absorb fluid entering it from the small intestine (25). Histologic alterations in the jejunum are not observed (25).

Bacteria are cleared from the system via an inflammatory response at the submucosal level (11). Treatment of enterocolitis with antimicrobics does not significantly alter the clinical course and may even cause prolonged excretion of the organism (66).

Initial Events in Salmonellosis

Takeuchi (73) used electron microscopy to observe the entry and passage of *Salmonella* through guinea pig intestinal epithelial cells. This work was limited to morphological observations, yet it provided the first understanding of *Salmonella*

invasion. The initial site of *Salmonella* infection is the distal ileum, where bacteria associate with the epithelial lining (7, 38). When bacteria lay close to the microvilli, the brush border remains intact. As the bacillus comes within a critical proximity to the brush border (<350 Å from the surface) the microvilli begin to degenerate. The usual portal of entry is through the brush border, but often can be via the intercellular junction between epithelial cells. These tight junctions are impermeable to ions and have a high transepithelial electrical resistance. However, when *Salmonella choleraesuis* or *S. typhimurium* are added to the apical surface, the epithelial cells lose their electrical resistance within four hours (19). As the bacterium advances further through the brush border, the apical cytoplasm in proximity to the bacterium begins to degenerate. A cavity is formed around the penetrating organism as it moves through the brush border and into the apical cytoplasm. As the bacterium advances into the host cell, the cavity in which it is located expands, accumulating additional degenerated microvilli and parts of the host cytoplasm in the form of blebs or vesicles. *Salmonella* are internalized individually, and vacuoles containing these bacteria appear to coalesce. The membrane bound vacuole containing the bacteria eventually becomes separated from the luminal plasmalemma (73). The bacteria within these vacuoles go through a lag period of several hours and then multiply extensively (19).

Virulence Factors

The polysaccharide antigens (O antigens) have been described as virulence factors of *Salmonella* (70, 74). Small qualitative differences in the O antigen structure, or loss of polysaccharide, can reduce the virulence of *S. typhimurium* in mice (31, 74).

Flagella have also been described as virulence factors of *Salmonella*. One group of investigators showed that the presence of flagella helped *S. typhimurium* survive within macrophages of the RES system (78). Another group of investigators showed that a flagellated strain was considerably more virulent than its isogenic, non-flagellated partner (6). Once the flagella were restored, virulence was regained.

A 100 kb plasmid has been found in many strains of *S. typhimurium*, and loss of the autonomous form of this plasmid has been correlated with an avirulent phenotype, incapable of translocation (25, 36, 45). Reintroduction of the autonomous plasmid into the cured strain restored the translocative property of this strain back to the levels originally observed for the wild-type parental strain (45). The plasmid was not necessary for colonization of Peyer's patches, but was found to be involved in infection of the spleen and mesenteric lymph nodes after oral inoculation of mice (36). *Salmonella* containing the plasmid showed much higher multiplication and survival in the spleen than those without the plasmid (36).

Certain genes, termed *inv*, have been found to be responsible for invasion of cultured cells by *S. typhimurium* (27, 28). At least five genes have been found to be involved with the invasive phenotype: *InvA*, *B*, *C*, *D*, and *E* encode for a 54, 64, 47, 30, and 43 kDa protein, respectively (27, 32). *InvA, B, and C* are sequentially arranged in the same transcriptional unit while the *invD* is located downstream of this cluster in an independent transcriptional unit (27). *InvE* is located immediately upstream of *invA* (32). Highly virulent strains of *S. typhimurium* carrying *inv* mutations were found to be defective for entry into cultured intestinal-407 (I-407) cells (28) These genes were

cloned into a non-invasive strain of *S. typhimurium* and enabled this strain to enter cultured cells (27). However, *inv* genes cloned into a non-invasive strain of *Escherichia coli* did not enable that strain to invade, suggesting that other mechanisms are involved (27).

Mediators of Attachment

Attachment of pathogenic bacteria to mammalian mucosal surfaces is an essential step in the pathogenesis of many bacterial diseases (60). In recent years, the study of the adherence of both Gram positive and Gram negative bacteria to host tissue has received a great deal of attention. The interest has stemmed from the hope that preventing attachment might prevent infection (17). Most reported studies on invasion have not clearly separated the processes of attachment and penetration, which would be helpful for understanding the initial events that occur during infection and colonization of the host.

The use of cultured animal cells as a model for quantitative study of the interactions between *Salmonella* and eukaryotic cells was first described with HeLa cells by Giannella *et al.* (31) in 1973. Since then, many investigators have taken advantage of this *in vitro* model for studying the interactions of *Salmonella* with eukaryotic cells (2, 3, 12, 18, 20, 21, 22, 23, 24, 26, 30, 41, 46, 47, 48, 49, 50, 53, 55, 62, 68, 75, 76). Recently, Finlay and Falkow (19) showed that the sequence of events described by Takeuchi (73) using guinea pig intestinal epithelial cells held true when using cultured cells. Using various *in vitro* assays, investigators have shown that attachment of *S. typhimurium* to cultured cell monolayers proceeds through two phases (47, 54). The

first phase is reversible attachment, in which continual fluid displacement will remove the bacteria from the eukaryotic cell surface. The second phase is irreversible attachment, in which continual fluid displacement does not remove the bacteria from the cells. The period of reversible attachment seems to be a prerequisite for irreversible attachment by providing the time necessary for a putative bacterial adhesin to bind to the cell surface (47). Despite considerable research efforts, the factors which mediate the attachment of *Salmonella* to mammalian cells remain largely unknown, although several factors have been suggested (Table 1).

Table 1. Putative mediators of attachment of *Salmonella typhimurium* to host epithelial cells.

Mediator	Reference
Lipopolysaccharide	2, 48, 50, 58, 60
Hydrophobicity	2, 50, 58, 63
Mannose sensitive hemagglutinin (MSHA) (type 1 fimbriae)	1, 2, 15, 34, 41 54, 60, 62, 75
Mannose resistant hemagglutinin (MRHA)	46, 60, 62, 75
Flagella (including motility and chemotaxis)	39, 44, 47, 51, 55 60, 62, 70, 74, 75 76, 79

Lipopolysaccharide

Lipopolysaccharide (LPS) extracted from Gram-negative bacteria have an affinity for animal cells (48). It has been postulated that LPS may promote bacterial colonization at mucosal surfaces (48, 2). However, investigators have shown in competitive inhibition

experiments that LPS did not inhibit attachment of *S. typhimurium* to cultured cells (60). Supporting the idea that LPS is not important for attachment is the work by Magnusson *et al.* (58) and Kihlstrom and Edebo (50). These investigators showed that interactions with cultured cells are increased with LPS defective, or rough, strains of bacteria. This has been attributed to the fact that rough strains are more hydrophobic than smooth strains, or strains with intact LPS. Hydrophobicity and negative charge have also been shown to play a role in the association of *Salmonella* with mammalian cells (2, 50, 58, 63).

Hemagglutinins

Fimbriated strains of *S. typhimurium* have been reported to cause significantly more infections and deaths than non-fimbriated strains in orally infected mice (15). The debate over whether or not fimbriae are attachment factors for *S. typhimurium* is still unresolved. The majority of reported evidence supports the hypothesis that type 1 fimbriae (MSHA) are involved in attachment (2, 34, 42, 54, 62, 75). One group of investigators, looking at the adhesion of *S. typhimurium* to porcine intestinal epithelial surfaces, reported that bacteria of the adhesive phenotype had fimbriae that resembled type 1, while none of the non-adhesive bacteria produced fimbriae (42). One conflicting result from this group, as well as from others (34), was that D-mannose, which type 1 fimbriae are sensitive to, did not inhibit adhesion. However, other investigators have shown that D-mannose did inhibit adhesion (1, 41, 75). Another piece of evidence supporting the possibility of a MSHA as an adhesin was found using antibodies against type-1 fimbriae, which protected orally infected mice (1).

In conflict with the hypothesis that type 1 fimbriae are involved in attachment are results from groups that have used *S. typhimurium* of the fimbriae-negative biotype (FIRN). These workers have concluded that the ability of non-fimbriate strains to attach to HeLa cells demonstrates that type 1 fimbriae are not responsible for the attachment of all *S. typhimurium* strains (46, 60). They found, instead, that the mannose-resistant nature of both fimbriate and non-fimbriate strains implicated an adhesive mechanism other than that provided by the type 1 fimbriae. A mannose resistant hemagglutinin (MRHA) was demonstrated to be responsible for the attachment that these groups observed (46, 60).

Flagella

Flagella have been described as mediators of attachment in terms of motility, chemotaxis, and physical contact. Motility was found to greatly increase the number of bacteria that adhered to cultured cells, while non-motile strains showed significantly less association *in vitro* (75, 79). Motile strains have been shown to colonize 100 to 1000 times more efficiently than non-motile strains (70). One group of investigators found that the efficiency of invasion by wild-type *S. typhimurium* was much higher than that of isogenic non-motile mutants, yet they did not present any evidence that the flagellar structures were acting as adhesins (51). Other investigators have shown that motility only promotes the contact between bacteria and animal cells, and can be negated by centrifugation (47). In contrast with most of the reported studies, one group of investigators found that flagella were not necessary for association of *S. typhimurium* with the mucosal surface of the mouse ileum (74).

Chemotactic bacteria have been reported to adhere much better to cultured cells than tumbly non-chemotactic mutants (44, 76). This might be attributed to a higher number of collisions by chemotactic strains with the epithelial cells (76). Another explanation may be that, once damaged, the mammalian cells release chemoattractants and that chemotactic strains would have a greater advantage over the non-chemotactic mutants (44). In support of the hypothesis that flagella, motility, and chemotaxis are important for attachment, anti-flagellar antibodies have been reported to prevent the adherence of *Salmonella* to cultured cells (39). Scanning electron microscopic examination of *Salmonella* associated with the mucosa has revealed various structures that appear to emanate from the surface of the bacteria. These have included flagella-like processes, which appeared to extend from the bacteria to the mucosa, and shorter appendages which may have been the remains of broken flagella (79).

Other Factors Influencing Adhesion

Other factors that influence attachment have recently been described. Bacterial adherence is an active process, requiring both RNA and protein synthesis (22, 23). Bacteria adhering to fixed MDCK cell monolayers made at least six new proteins that were not synthesized by non-adhering bacteria in the supernatant of the same sample (21, 23). In addition, synthesis of at least seven proteins was apparently shut off in the bound bacteria (23). Anaerobic bacteria attach to a much greater extent than aerobic bacteria (16, 53). One group of investigators described an increased invasion by *S. typhimurium in vitro* when the bacteria were grown under anaerobic conditions, which was accompanied by the repression of at least one major outer membrane protein (68).

Growth phase also appears to influence association with cultured cells. The highest rate of invasion was obtained with cells from the logarithmic phase of growth, and the bacteria became less invasive as the culture progressed into the stationary phase (16). It is becoming increasingly clear that environmental signals may play an important role in the attachment and invasion processes of *S. typhimurium*.

Statement of Research Problem

This study was undertaken to examine the attachment of *S. typhimurium* to cultured epithelial cells, and had three main objectives: (i) to develop an assay that would measure cell attachment only and not invasion of cultured cells; (ii) to identify physiological conditions that influence cell attachment *in vitro*; and (iii) to determine whether the attachment observed with the experimental assay was mediated by any of the putative adhesins described by other investigators.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from Sigma Chemical Co., St. Louis, MO: 2-aminopurine; N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonicacid] (HEPES); bovine serum albumin (BSA); bromoacetic acid; chloramphenicol; chloroacetic acid; cycloheximide; cytochalasin D; L-cysteine-HCl-H_2O; 2,4-dinitrophenol (DNP); ethylenediaminetetraacetic acid (EDTA); gelatin; gentamicin sulfate; D-(+)-glucose; glutaraldehyde; glycerol; heparin; n-hexadecane; hydrogen peroxide; iodoacetic acid; N-laurylsarcosine (sarkosyl); D-(+)-mannose; nalidixic acid; paraformaldehyde (pHCHO); penicillin G; phenylmethyl-sulfonyl fluoride (PMSF); potassium chloride; sodium acetate; sodium azide; sodium bicarbonate; sodium chloride; sodium phosphate, dibasic; sodium phosphate, monobasic; streptomycin sulfate; triphenyl tetrazolium chloride (TTC); trypan blue; trypsin; trypsin inhibitor type 11-5; and yeast extract.

The following chemicals were obtained from J. T. Baker Chemical Company, Phillipsburg, NJ: ammonium sulfate; calcium nitrate 4-hydrate; chloroform; dimethyl sulfoxide (DMSO); ferrous sulfate 7-hydrate; formalin; magnesium sulfate 7-hydrate; methanol; potassium iodide; and potassium phosphate monobasic.

Polyxyethylene sorbitan monolaurate (Tween 20) and Tris [hydroxymethyl] aminomethane (Tris) were obtained from BioRad Laboratories, Hercules, CA.

Proteinase K was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Fetal bovine serum (FBS) came from HyClone Laboratories, Inc., Logan, UT. Agar was from Oxoid Limited, Basingstoke, Hampshire, England. Ethanol (95%) was from Chemistry Stores, Montana State University (MSU) Bozeman, MT. Tetramethylbenzidine Microwell peroxidase substrate was obtained from Kirkegaard & Perry Laboratories, Inc., Gaithesburg, MD. Sheep erythrocytes were obtained from the Veterinary Molecular Research Laboratory, MSU, Bozeman, MT. Guinea pig erythrocytes were collected by Dr. Warren Frost from the Animal Resource Center, MSU, Bozeman, MT.

Media and Reagents

The following media were obtained from Sigma Chemical Co., St. Louis, MO: basal medium Eagle (BME); Dulbecco's modified Eagle (DME); and Dulbecco's phosphate buffered saline (DPBS).

The following media were also used in this study: brain heart infusion (BHI) and tryptone (Difco Laboratories, Detroit, MI); DEAE affi-gel blue (BioRad Laboratories, Hercules, CA); and trypticase soy agar (Becton Dickinson Microbiology Systems, Cockeysville, MD).

Reagents used in this study included: DME buffer solution containing 6.5075 g HEPES/L and 3.7 g $NaHCO_3$/L in deionized water with pH adjusted to 7.2 and filter sterilized (0.22 μm pore size). The osmolality was adjusted to be equal to that of DME (400 mmol/kg).

Minimal growth medium (M56) contained (g/L): $NaHPO_4$-$7H_2O$ (8.2), KH_2PO_4 (7.2), $(NH_4)_2SO_4$ (1.0), and 0.25mg $FeSO_4$-$7H_2O$/L in deionized water. The solution was autoclaved for 15 min, 0.1 g $MgSO_4$-$7H_2O$/L, 5 mg $Ca(NO_3)_2$/L, and 2 g glucose/L was added, and the solution was filter sterilized.

The protease buffer contained 20 mM tris and 1.0 mM $CaCl_2$ in distilled water, pH 7.5.

Equipment and Supplies

The following supplies were obtained from Corning Inc., Corning, NY: 96-well flat bottom polystyrene tissue culture plates; 24-well polystyrene tissue culture plates; 16 x 125mm culture tubes; and 75cm^2 polystyrene tissue culture flasks.

The following supplies were obtained from Becton Dickinson Microbiology Systems, Cockeysville, MD: BBL GasPak hydrogen and carbon dioxide generator; Bio-Bag environmental chamber type A; Falcon 24-well polystyrene tissue culture plates; BBL GasPak disposable anaerobic indicator (methylene blue).

Fuchs-Rosenthal and Petroff-Hausser counting chambers came from Hausser Scientific, Blue Bell, PA.

The Acrocap 0.2 μm filter units were from Gelman Sciences, Inc., Ann Arbor, MI.

The following items of equipment were used in this study: Benchtop centrifuge (Sorvall model T6000B, DuPont Company Biomedical Products Division, Wilmington, DE); refrigerated superspeed centrifuge (Sorvall model RC-5B, DuPont); Lab-Tek culture chamber slides (Nunc, Inc. Naperville, IL); Psychrotherm controlled environment

incubator shaker (model G-26 381194, New Brunswick Scientific Company, Inc., Edison, NJ); Waterjacketed carbon dioxide incubator (Forma Scientific, Inc., Marietta, OH); Spectrophotometer (model DMS 80, Varian); Olympus BH-2 microscope and UV lamp (Olympus Optical Company LTD., Tokyo, Japan); Blender (model 31BL92, Waring Products Division Dynamics Corporation of America, New Hartford, CT); Microplate (ELISA) reader (model 450, BioRad Laboratories, Hercules, CA).

Bacterial Strains

The main bacterial strain used in this assay was *S. typhimurium* SL3201. This strain was obtained from B. A. D. Stocker, Stanford University School of Medicine, Stanford, CA. This strain is representative of the FIRN biotype, which indicates that it is fimbriae negative, and does not ferment inositol or rhamnose (13). The lack of fimbriae was confirmed during this study by non-hemagglutination of guinea pig erythrocytes in a rocked tile hemagglutination assay described by Duguid *et al.* (14). This strain is motile and did not cause non-specific agglutination, thereby demonstrating that it has intact lipopolysaccharide (LPS). This strain also carries the 100 kb virulence plasmid and is highly virulent in BALB/c mice (68).

SL3201NM is a flagellated, but non-motile mutant derived during this study from SL3201 by 2-aminopurine mutagenesis (described later).

S. typhimurium strain St39 was a gift from M. Carsiotis, University of Cincinnati, College of Medicine, Cincinnati, OH. This strain is a non-flagellated mutant. The mutation was constructed by the introduction of the transposon *zcd*-907::*Tn*10 into gene *zcd* of *S. typhimurium* strain SL3201.

S. typhimurium strain St_x3337 was obtained from R. Curtiss III, Washington University, St. Louis, MO. This strain lacks the 100 kb virulence plasmid.

S. typhimurium strain S141 and *Salmonella senftenberg* strain S144 were obtained from the National Veterinary Services Laboratory, U.S. Department of Agriculture, Ames, IA. S141 produces type 1 fimbriae as demonstrated in this study by mannose sensitive hemagglutination (MSHA) of guinea pig erythrocytes in a rocked tile test.

Yersinia enterocolitica strain E641 was obtained from S. Toma, Canadian National Reference Service for *Yersinia*, Toronto, Ontario, Canada.

Storage of Cultures

Stock cultures were stored in a solution of 1.0% peptone and 40% glycerol at -20 (primary stock) and -70°C (backup or long-term stock). All experimental subcultures were taken from the -20°C stock to avoid any changes in the strain due to repeated subculturing.

Preparation and Handling

Stationary phase cultures were prepared by inoculating 4 ml of BHI with the primary stock culture. The broth was incubated statically overnight (18 h) at 37°C until it reached the stationary phase. This was called the static-stationary bacterial preparation.

Bacteria from the static-stationary culture were swabbed onto an agar plate (40 g TSA/L with 0.6% yeast extract) (TSY) and incubated in an anaerobe jar containing a GasPak envelope and a methylene blue indicator strip for ca. 18 h at 37°C.

Bacterial cultures were also prepared by inoculating 5 ml of BHI with the primary stock culture. The broth culture was incubated for ca. 18 h in an anaerobe jar with a GasPak and indicator strip at 37°C. This was called the anaerobic-stationary bacterial preparation.

Bacteria cultured for the production of fimbriae were prepared by inoculating 4 ml of BHI with the primary stock culture. The culture was incubated statically for 48 h at 37°C, after which 10 μl were transferred from the top of the tube to 4 ml of fresh BHI, which was held static at 37°C for 48 h.

Cell Cultures

Cell Lines

The epithelial cell lines used in this study were obtained from the American *Type Culture* Collection (ATCC), Rockville, MD: HeLa, strain CCL2; Intestinal 407 (I-407), strain CCL6; HEp-2, strain CCL23; and Madin Darby Canine Kidney (MDCK), strain CCL34. Cells were stored in liquid nitrogen.

HeLa, I-407, and HEp-2 cells were maintained in BME culture medium (BME with 2.2 g $NaHCO_3$/L and 25 mM HEPES, pH 7.2) and MDCK cells in DME culture medium (DME with 3.7 g $NaHCO_3$/L plus 25 mM HEPES, pH 7.2) supplemented with 10% FBS, 50 U penicillin G and streptomycin/ml, and 2.5 μg amphotericin B/ml. Cell cultures were maintained at 37°C under 5% CO_2 with regular subcultures, and were not used after 20 passages.

Preparation of Cell Monolayers for Assays

Cells were harvested the day before an assay by decanting the medium from a confluent monolayer, washing the monolayer twice with 10 ml of calcium-free DPBS, and adding 5 ml of cell detachment buffer (0.05% trypsin and 0.02% EDTA in calcium-free DPBS, filter sterilized). The detached cells were centrifuged at 460xg for 5 min at room temperature on a benchtop centrifuge, resuspended in 5 ml of DME or BME, 50 μl were diluted in 450 μl trypan blue (0.4% in DPBS, filter sterilized), and counted with a Petroff-Hausser counting chamber.

Depending on the experiment, the cells were diluted to different densities in DME or BME with 10% FBS (no antibiotics). Monolayers for the chamber slide assay were prepared by seeding $2x10^5$ cells per chamber. Three chambers were seeded for each sample to be tested. For the ELISA procedure, a 96-well tissue culture plate was seeded with $4x10^4$ cells per well. Monolayers for the attachment assays were prepared by seeding $2x10^5$ cells per well of a tissue culture plate. The cells were incubated for 24 ± 2 h at 37°C under 5% CO_2.

Antibodies

Antibodies used in this study included: (i) Rabbit (Rb) antibody #07251, prepared by D. A. Schiemann. This antibody was prepared using washed, formalized strain SL3201 as the antigen which was injected iv into two rabbits. The sera collected from both rabbits were composited and the immunoglobulins were purified by DEAE affi-gel blue. (ii) Goat (Gt) anti-Rb IgG-fluoroscein-isothiocyanate (FITC) conjugate (Sigma Chemical Co.). (iii) Gt anti-Rb IgG-horseradish peroxidase conjugate (Sigma Chemical

Co.). (iv) *Salmonella* H antiserum poly a-z (Difco Laboratories). (v) *Salmonella* H antiserum 1 complex (Difco Laboratories). (vi) Anti-flagellar Fab fragments prepared by D. A. Schiemann from Rb antibody #07251 adsorbed with non-flagellated *S. typhimurium* strain St39. (vii) *Salmonella* O antiserum poly A (Difco Laboratories). (viii) *Salmonella* O antiserum poly B (Difco Laboratories). (ix) Rb antibody #09092 $F(ab')_2$, prepared by D. A. Schiemann. This antibody was prepared by injecting MDCK cell membrane im with TiterMax™ adjuvant into a rabbit, boosting with whole cells 4 wk later, and bleeding 10 d later. $F(ab')_2$ fragments were prepared using ImmunoPure® $F(ab')_2$ Preparation Kit (Pierce, Rockford, IL) according to manufacturer's instructions.

Indirect Fluorescent Antibody Assay

The indirect fluorescent antibody assay was used to visualize flagella. The procedure was adopted from the U.S. Food and Drug Administration Bacterial Analytical Manual (69) with some modifications. Bacteria were mixed with 1% glutaraldehyde in saline and held for 30 min at 37°C. This pretreatment was developed during this study as a way to fix the flagella on the bacteria, as they had a tendency to break off without the treatment. This treatment also promoted attachment of the bacteria to the glass slide. These prefixed bacteria were added to a slide and air dried. Rb antibody #07251 was diluted 1:100 in PBS (0.2586 g KH_2PO_4/L, 0.9720 g Na_2HPO_4/L, 8.0 g NaCl/L, and 0.2 g KCl/L in deionized H_2O, pH 7.2, filter sterilized), added to the bacterial smear, and held for 30 min at room temperature in a moisture chamber. The slide was rinsed with PBS and then Gt anti-Rb IgG-FITC diluted 1:80 in PBS was added to the bacterial smear. The slide was held for 30 min at room temperature in a moisture chamber,

rinsed, and air dried. A drop of glycerol-saline solution (69) was added to each smear and the slide was examined under ultraviolet light (490 nm).

Derivation of Non-motile Mutant

2-Aminopurine Mutagenesis

S. typhimurium strain SL3201 was subcultured from the primary stock culture and incubated for 18 h. These stationary phase bacteria were diluted to give 100 bacteria in 5 ml of Luria-Bertani (LB) (1% tryptone, 0.5% yeast extract, and 0.06 M NaCl in deionized H_2O) broth containing 600 μg of 2-aminopurine/ml. The culture was incubated at 37°C for about 18 h. The mutations induced by 2-aminopurine are primarily transition mutations (59).

Enrichment

Mutated bacteria (20 μl) were inoculated into the center of a culture plate containing semisolid medium (37 g BHI/L and 0.2% agar in deionized water) and incubated for 18 h at 37°C. The center of the semisolid medium was transferred to a fresh plate of semisolid medium using a Pasteur pipet and the plate was incubated for 18 h at 37°C. This transfer procedure was repeated for 7 d, for a total of 10 transfers. After the last transfer, a plug of medium was removed from the center of the semisolid medium and added to 1 ml saline. Dilutions were made in saline to yield approximately 100 colony forming units per TSY agar plate after incubation for 18 h at 37°C.

Screening of Mutants

Two thousand colonies were screened for motility using a rapid screening procedure developed during this study. Tissue culture plates (96-well) were filled with semisolid medium supplemented with 0.001% TTC. Isolated colonies from the TSY agar plates were stabbed into the wells with toothpicks (1 colony/well). The 96-well tissue culture plates were incubated for 18 h at 37°C. Each well was examined and a solid red stab line indicated non-motility, while red medium throughout the well indicated motility. The medium from each well that appeared to have non-motile bacteria was added to BHI and grown at 37°C to the log phase (6 h).

Cloning the Mutants

Each non-motile mutant in the log phase was streaked for isolation on TSY agar plates and incubated for 18 h at 37°C. One colony was chosen and streaked for isolation. This was repeated once again and after the third cloning, one colony was grown to log phase in BHI. A microscopic examination was performed, and of the 2000 colonies initially screened, three clones were found to be non-motile. An indirect fluorescent antibody assay was performed on the non-motile mutants. All three mutants produced flagella. One of the mutants was shown to have defective LPS by agglutination in non-specific sera, one of the mutants reverted to about 20% motility, and one mutant was acceptable for the attachment assay. This non-motile mutant was called SL3201NM.

Chamber Slide Assay

The chamber slide assay was a method used for visualizing the bacteria attached to the cells. It was known that they were attached and not intracellular since antibody

cannot penetrate live cells, and the cells were not fixed until after the primary antibody treatment.

Bacteria were recovered from a TSY plate into 10 ml cold DPBS and centrifuged at 11,220xg for 20 min at 4°C. The supernatant was poured off and the pellet was resuspended in 10 ml cold DPBS. Bacteria were diluted 1:100 in DPBS with 0.5% formalin, enumerated in a Petroff-Hausser chamber, and diluted in BME or DME with 10% FBS.

For assays requiring fixed monolayers, 2.5% glutaraldehyde in DPBS was added to the chambers and held for 1 h prior to the addition of bacteria. The monolayers were prewashed twice with 0.5 ml DPBS to remove dead and floating cells, and 0.2 ml of bacteria at $2x10^7$/ml was added to each chamber. The chamber slides were incubated at 37°C for varying lengths of time then each chamber was washed five times with 0.5 ml of DPBS. During each wash, the chamber slide was put on a shaker for 2 min and vigorously shaken to remove any reversibly-attached bacteria. Rb antibody #07251, diluted 1:100 in BME or DME (0.2 ml), was added to each chamber. The chamber slide was incubated for 1 h at 37°C, then 1.0 ml of DPBS was added to each chamber and held for 10 min. This was removed and 0.2 ml of Gt anti-Rb IgG-FITC, diluted 1:80 in BME or DME, was added to each chamber. The chamber slide was incubated for 1 h at 37°C. Each chamber was rinsed twice with 0.5 ml DPBS and then 0.2 ml of 0.25% glutaraldehyde in DPBS was added to each chamber to fix the cells and the bacteria. This solution was held in the chambers for 10 min at 4°C and then the chambers were rinsed twice with 0.5 ml DPBS. The chamber divider was removed, the

slide was air dried, and a drop of glycerol saline solution (69) and a coverslip were added. The number of attached bacteria was determined by examining the cells under phase microscopy to locate cells that were well separated and easily countable. The light source was then switched to ultraviolet (490 nm) and the number of fluorescent bacteria on the cell was counted. If the bacteria were layered, clumped, or >50 per cell, then a "C", for clumped, was recorded for that cell. Results were expressed as the number of cells with bacteria.

The Enzyme Linked Immunosorbant Assay

The ELISA was used as another method for following attachment over time, confirming the results from the chamber slide assay. Monolayers in a 96-well tissue culture plate were pretreated with 1 μg cytochalasin D/ml in DMSO and incubated for 37°C for 1 h prior to the addition of bacteria, to prevent the bacteria from invading the cells. The bacteria were recovered from the TSY plate (see Chamber Slide Assay) and diluted 1:100 in DME with 0.5% FBS. The monolayers were rinsed once with 0.2 ml DPBS to remove dead and floating cells and 0.1 ml of the bacterial suspension was added to each well. Control wells received 0.1 ml of DME with 0.5% FBS (no bacteria). Cytochalasin D (1 μg/ml) was again added to all of the wells. The 96-well tissue culture plates containing bacteria and monolayers were incubated for varying lengths of time.

The wells were each washed five times with 0.3 ml of DPBS. During each wash, the tissue culture plate was put on a shaker for 2 min and vigorously shaken to remove any reversibly attached bacteria. The monolayers and bacteria were fixed by adding 0.2 ml of 3% pHCHO in DPBS to each well and holding for 10 min at room

temperature. Each well was rinsed once with 0.3 ml PBST (PBS with 0.05% Tween 20) and 0.2 ml of Rb antibody #07251, diluted 1:500 in ELISA reagent diluent (PBS with 0.1% BSA and 0.02% NaN_3), was added to each well. Control wells received 0.2 ml of ELISA reagent diluent (no antibody). The 96-well tissue culture plate was incubated for 1.5 h on a rocker at room temperature. Each well was washed four times with 0.2 ml PBST, then 0.2 ml of Gt anti-Rb IgG-horseradish peroxidase (diluted 1:2000 in ELISA reagent diluent) was added to each well. The plate was incubated for 1 h on a rocker at room temperature. Each well was washed four times with 0.2 ml PBST. The substrate (0.1 ml) containing equal volumes of horseradish peroxidase substrate and H_2O_2, was added to each well. The plate was held at room temperature for 1 h and then 0.1 ml of 1 M H_3PO_4 was added to each well to stop the enzyme reaction. The liquid from each well (0.1 ml) was transferred to a new well and the plate was read on an ELISA reader at 450 nm.

Attachment Assays

Bacteria from an anaerobic stationary culture were diluted 1:100 in DME or PBDME (DME with 20 mM NaH_2PO_4, pH 6.6) in a tissue culture tube. This was considered to be time zero, or stationary phase. The number of bacteria was determined by preparing dilutions in saline and spreading on TSY agar plates. Depending on the experiment, the cultures were incubated in anaerobic bags, aerobically at 240 rpm, or on a roller at 25 or 37°C for varying lengths of time. After the desired time had elapsed, the bacteria were diluted in DME or PBDME until the medium was barely turbid, providing approximately $1x10^7$/ml. Dilutions of this suspension were prepared in saline

and spread on TSY agar plates. This count was used to calculate the number of bacteria going on the monolayer, or the inoculum. The monolayers were each rinsed once with 0.5 ml DPBS and 0.2 ml of bacteria in DME or PBDME was added to each well. For the cold attachment assay, the plate was held on ice for 5 min, centrifuged at 460xg for 5 min, then held at 4°C for 55 min. For the attachment assay, the plate was centrifuged at 460xg for 30 min. The wells were each washed 5 times with 0.5 ml DPBS. During each wash, the 24-well plate was vigorously shaken on a rotary shaker to remove any reversibly attached bacteria. After the last wash, 1.0 ml of sarkosyl (0.5% sarkosyl in saline) was added to each well and the plate was vigorously shaken for 5 min. To enumerate the number of attached bacteria, 0.5 ml from each of three wells was composited in 13.5 ml of saline, dilutions were made and spread on TSY agar. The TSY plates were incubated overnight at 37°C. Percent attachment was the number of bacteria attached per monolayer, divided by the number of bacteria that went on the cells, multiplied by 100.

$$\% \text{ attached} = \frac{\text{number of bacteria added}}{\text{number of bacteria attached}} \times 100$$

Hydrophobicity Assay

The hydrophobicity assay was adopted from Rosenberg *et al.* (65). *Y. enterocolitica* E641 was used as a positive control (67). Bacteria in the stationary phase were recovered by centrifugation at 5,000xg for 10 min at 4°C. The pellet was

suspended in DME and then 1:100 dilutions were made in DME. The bacteria were incubated at 37°C for 4 to 4.5 h, depending on the bacterial strain and an initial reading of light absorbance was measured with a spectrophotometer (400 nm). Bacteria (4.8 ml) were put in round bottom test tubes and 0.8 ml of n-hexadecane was added to each tube. The tubes were incubated for 10 min at 37°C. The mixtures were then agitated uniformly for 2 min using a vortex mixer then left to stand for 15 min to allow the hexadecane layer to rise completely. The aqueous phase was carefully removed with a Pasteur pipet and the light absorbance measured at 400 nm. The percent decrease in turbidity was measured by taking the initial reading (i), minus the reading after mixing with n-hexadecane (f), divided by the initial reading, multiplied by 100.

$$\% \text{ decrease in turbidity} = \frac{i-f}{i} \times 100$$

Hemagglutination Assay

The static settling hemagglutination assay was adopted from Tavendale *et al.* (75). A 1.0 ml aliquot from each of the following cultures was removed and set aside prior to centrifugation: (i) bacteria from a static stationary culture; (ii) bacteria specially cultured for fimbriae production; and (iii) bacteria from an anaerobic stationary culture, diluted 1:100 in DME and incubated on a roller for 37°C. Cultures were centrifuged at 5000xg for 20 min at 4°C. The supernatant was removed and set aside. The pellet was suspended in saline to a density of approximately $5x10^{10}$/ml. Bacteria grown on TSY

agar were recovered in 7 ml saline. Therefore, there were four populations of each bacterial strain assayed: (i) uncentrifuged suspension in BHI or DME containing bacteria and the extracellular products; (ii) bacteria-free supernatant obtained by centrifugation; (iii) bacteria washed by centrifugation, suspended in saline, and (iv) agar grown bacteria suspended in saline.

Whole sheep blood containing 0.1% thimerosal and 5 U heparin/ml was washed three times by centrifuging at 460xg for 8 min at room temperature, removing the supernatant with a Pasteur pipet, and resuspending in saline. After the final wash, the volume of packed cells was measured and 99 volumes of saline or saline with 0.5% D-mannose were added to the packed cells.

Two-fold serial dilutions, from 2 to 128, of the bacteria were made in plastic tubes. To 0.4 ml of each dilution, an equal volume of a 1% suspension of erythrocytes in saline or saline with 0.5% D-mannose was added. The tubes were swirled gently and placed in an ice bath for 2 h and then transferred to 4°C overnight. A dispersed pattern of the deposited cells indicated hemagglutination while a compact button at the bottom of the tube indicated absence of hemagglutination. All tubes that appeared to have hemagglutination were checked microscopically.

Shearing the Flagella off Bacteria

Stationary or resuscitated bacteria (50 ml) were put in a prechilled blender jar. The bacteria went through three cycles of being blended on high speed for 90 sec and then being cooled in an ice bath for 5 min. Control bacteria were not blended, but held on ice during the blending procedure.

Statistical Analyses

The InStat computer program version 1.13 by GraphPAD software was used to statistically analyze the data. The statistical methods used were the multiple means one way analysis of variance (ANOVA) and the two-tailed unpaired t test.

RESULTS

Effect of Growth Phase on Attachment

Chamber Slide Assay

It has been previously shown that stationary phase *Salmonella* do not invade cultured cells, but do have invasive capabilities in the logarithmic phase (53). The question of whether the growth phase affected attachment was examined using a variety of methods. The chamber slide method was used to check the number of bacteria attached to fixed and unfixed monolayers. The unfixed cells incubated with bacteria for 5 and 6 h, upon microscopic examination, showed signs of rounding up and detaching from the slide. The cells were damaged enough that they would not have withstood the washing, therefore, the antibody detection was not done on the 5 and 6 h unfixed cell monolayers. Attachment to both fixed and unfixed cells increased with time and then leveled off (Table 2 and 3). This suggested that something occurred during incubation with the cells that promoted attachment.

ELISA

An ELISA method was used to confirm that attachment of *S. typhimurium* did increase in the logarithmic phase of growth. Monolayers were pretreated with cytochalasin D, which inhibits actin polymerization and thereby blocks bacterial invasion of the cells (18). Attachment reached a peak absorbance at 5 h (Figure 1).

Table 2. Attachment of *S. typhimurium* to fixed monolayers

Time (h)	% of Cells with Bacteria[a]
2	33.3 ± 2.40[b]
3	56.0 ± 3.21
4	61.0 ± 2.65
5	69.7 ± 6.01
6	69.7 ± 3.76

[a] *S. typhimurium* strain SL3201 incubated with cell monolayers for various times and enumerated with indirect fluorescent Ab assay.

[b] Results expressed as the mean ± standard error of the mean. One way ANOVA showed significant difference in attachment over time ($p=0.0003$).

Table 3. Attachment of *S. typhimurium* to unfixed monolayers

Time (h)	% of Cells with Bacteria[a]
2	10.7 ± 1.86[b]
3	31.3 ± 5.90
4	74.3 ± 3.18

[a] *S. typhimurium* strain SL3201 incubated with cell monolayers for various times and enumerated with indirect fluorescent Ab assay.

[b] Results expressed as the mean ± standard error of the mean. One way ANOVA showed significant difference in attachment over time ($p<0.0001$).

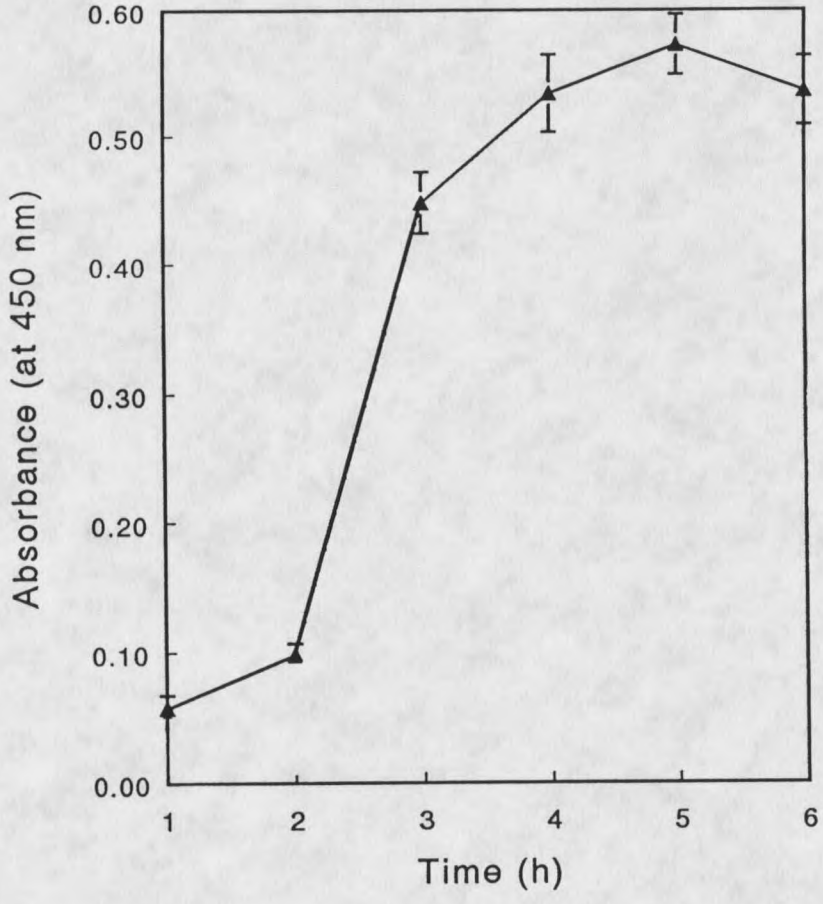


Figure 1. *S. typhimurium* attachment after incubation with monolayers in DME with 0.5% FBS. Results expressed as the mean ± standard error of the mean for three replicate monolayers. One way ANOVA showed a significant difference in attachment over time ($p < 0.0001$).

Cold Attachment Assay

An experiment was done to determine whether incubation of the bacteria with cells was necessary to induce attachment. Strain SL3201 was incubated anaerobically in DME for 0 to 12 h. At different times the bacteria were added to precooled monolayers, centrifuged at 460xg for 5 min, then held for 55 min at 4°C. Cold temperatures inhibit invasion (22, 53), therefore, this method measured only attached bacteria. Even though *S. typhimurium* was still in the logarithmic phase at 12 h, the number of bacteria that attached decreased dramatically with the greatest attachment occurring at 3 to 5 h, or during the early logarithmic phase (Figure 2). At this point, the term "resuscitation" was first applied, referring to bacteria grown after dilution in fresh medium in a way that induced attachment without any prior exposure to the cells.

Attachment Assay

The attachment assay was also used to measure the effect of growth phase on attachment. Strain SL3201 was grown in DME and tested for attachment to cultured MDCK cells at various times. Once again it was apparent that attachment was low in the stationary phase and increased during the early logarithmic phase of growth (Figure 3). This attachment assay became the standard attachment assay for all further experiments, since the percent of attachment was more than 10 times higher than that measured with the cold attachment assay.

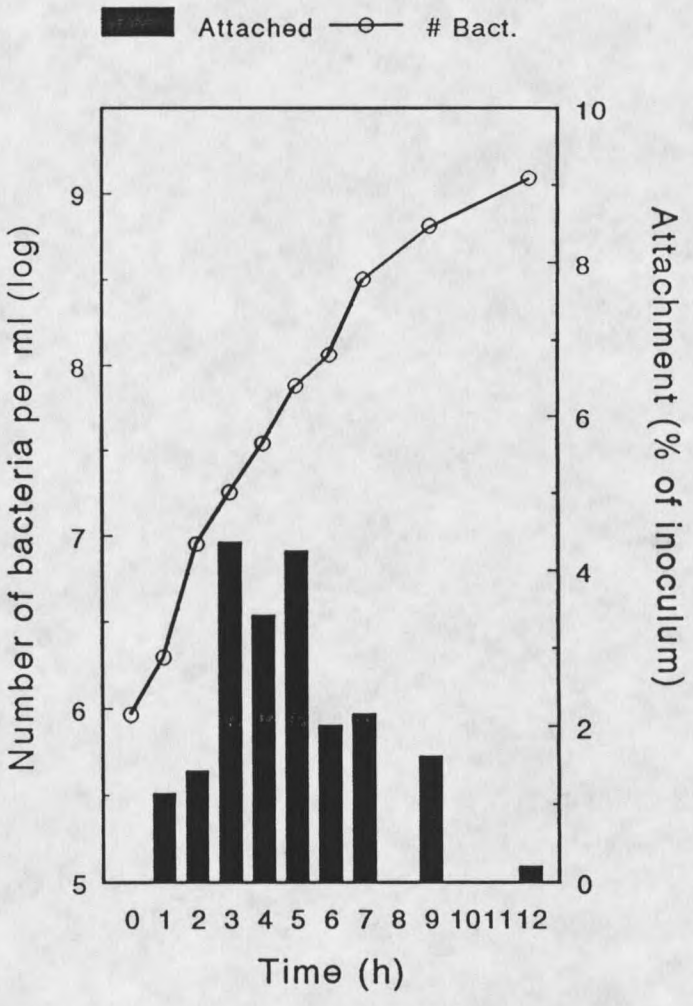


Figure 2. *S. typhimurium* growth and attachment after anaerobic incubation in DME at 37°C. Graph composited from three cold attachment assays (see Appendix for raw data).

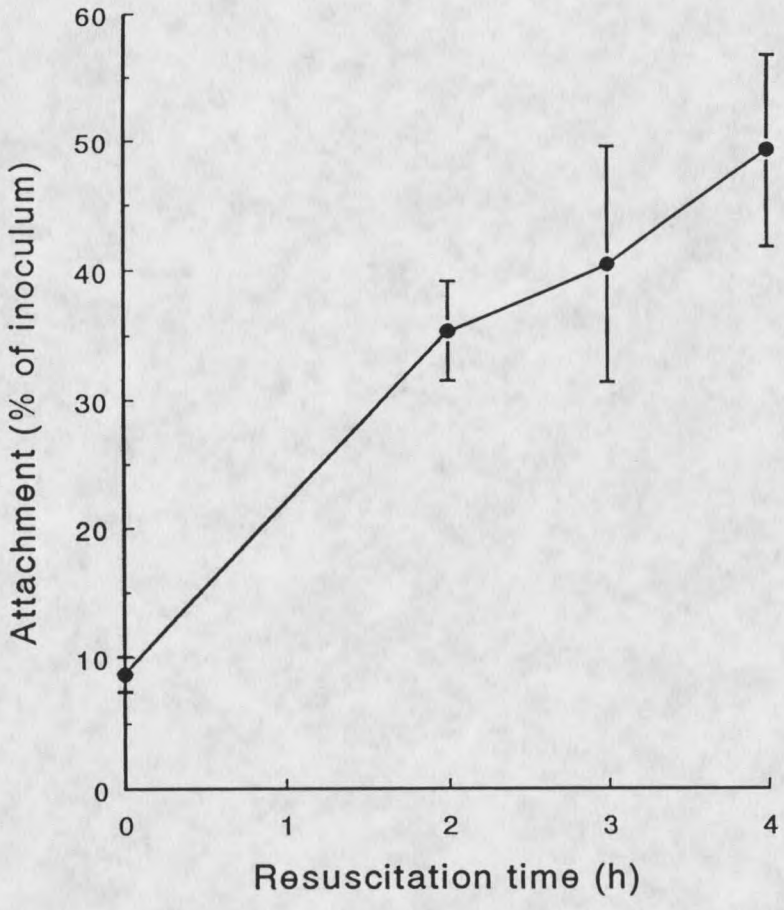


Figure 3. *S. typhimurium* attachment after resuscitation in DME at 37°C. Results expressed as the mean ± the standard error of the mean for four experiments, each representing a composite of three monolayers. One way ANOVA showed a significant difference in attachment over time ($p=0.0036$).

Choice of Cell Line and Multiplicity

Choice of Cell Line

The chamber slide assay provided a way of visualizing the bacteria attached to the cells (Figure 4). The chamber slide assay was used to determine which tissue culture cell line would be most suitable for the assays. I-407, HEp-2, HeLa, and MDCK cells were compared for their ability to adhere to the chamber slide during the washing procedure, bacterial attachment to the cells, and ease of differentiating the bacteria from the cells during counting.

MDCK cells were chosen for use in all further experiments because they adhered to the chamber slide much better than the other three cell lines during the wash steps. The pattern of bacterial adherence to the cells also influenced the decision of which strain to use. Strain SL3201 attached in a chain-like pattern to the I-407, HeLa, and HEp-2 cells. This made it difficult to tell which bacteria were actually attached and which might be progeny of attached bacteria. Strain SL3201 attached to the intercellular junctions of the MDCK cells and it was not difficult to determine which bacteria were attached (Figure 4).

Multiplicity

The influence of bacteria to cell ratio (multiplicity) was examined by resuscitating strain SL3201 in DME and then diluting to different densities in DME. Multiplicity had little influence on attachment , which varied between 20 and 30% (Figure 5). A multiplicity of approximately 10 was chosen for use in all further assays.

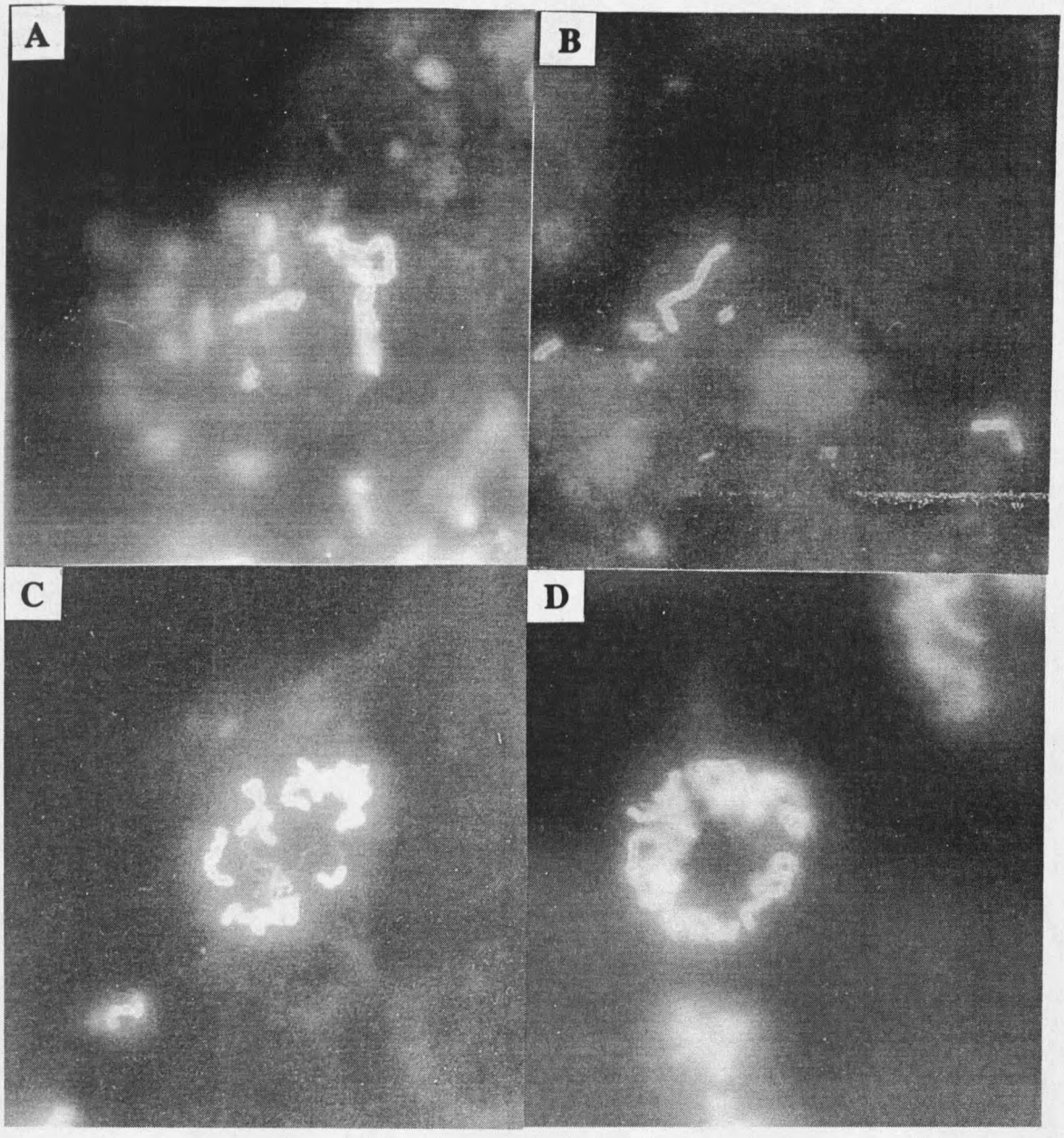


Figure 4. *S. typhimurium* attached after 4 h infection to I-407 cells (A and B) and MDCK cells (C and D). Bacteria visualized by fluorescent antibody. Notice the chain-like attachment of the bacteria to the I-407 cells, and the attachment of the bacteria to the border of MDCK cells.

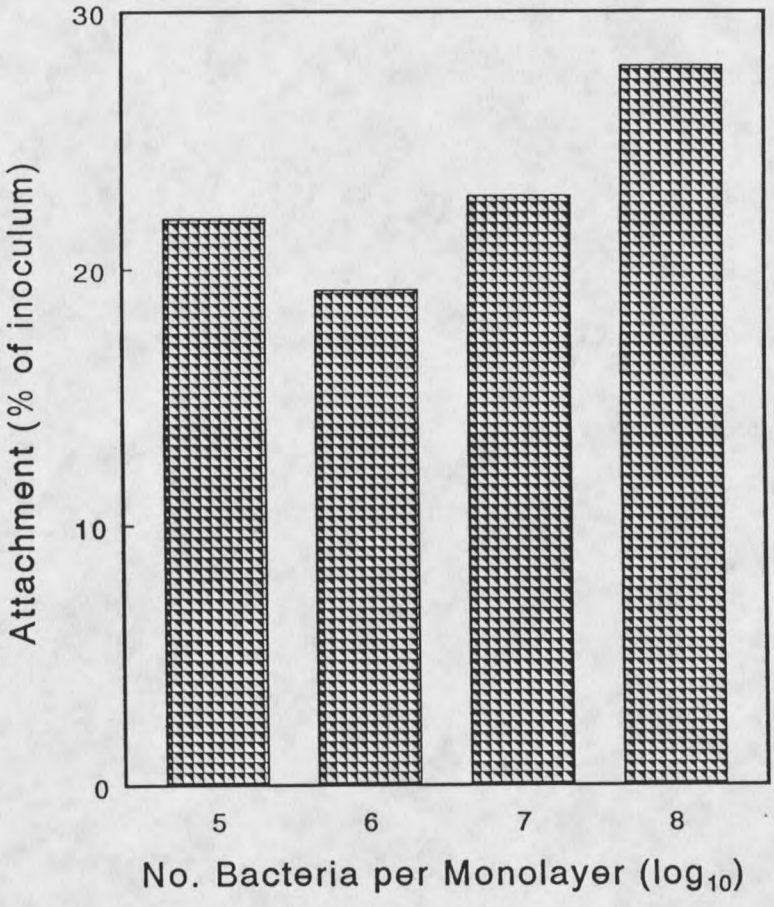


Figure 5. *S. typhimurium* attachment after 4 h resuscitation in DME at 37°C. Bacteria counted in Petroff-Hausser chamber and diluted to desired densities. Results represent a composite of three monolayers from an attachment assay.

Validity of Method for Measuring Attachment

An experiment was designed to demonstrate that *S. typhimurium* were attached to the cell monolayers, but not intracellular. Gentamicin (100 μg/ml) was used to kill extracellular bacteria (49), while iodoacetic acid (1.0 mM) and DNP (1.0 mM) were used to inhibit internalization of the bacteria during incubation with gentamicin. The solution (0.2 ml) of gentamicin and inhibitors was prewarmed to 37°C and added to the bacteria/monolayers immediately after the centrifugation and washing steps. The 24-well tissue culture plate was held at 37°C for 1 h, the cells were lysed and bacteria were suspended in sarkosyl-saline and plated on TSY. The control had no gentamicin and was washed and plated immediately after centrifugation. The results shown in Table 4 indicate that 94% of the bacteria were killed by the gentamicin, and, therefore, were extracellular. These extracellular bacteria represent bacteria attached to cells since gentamicin does not penetrate live eukaryotic cells (49). It was previously shown by Schiemann and Shope (68) that bacterial adherence to the plastic plate is not significant.

Physiological Conditions Influencing Attachment

Resuscitation conditions for later experiments were established after studying the effect of temperature, pH, osmotic conditions, and oxygen availability on attachment.

Temperature

The temperatures examined for resuscitation were 22, 37, and 42°C (Figure 6). Strain SL3201 showed higher attachment when resuscitated at 22 and 37 than at 42°C, therefore 22 or 37°C was used in all further assays.

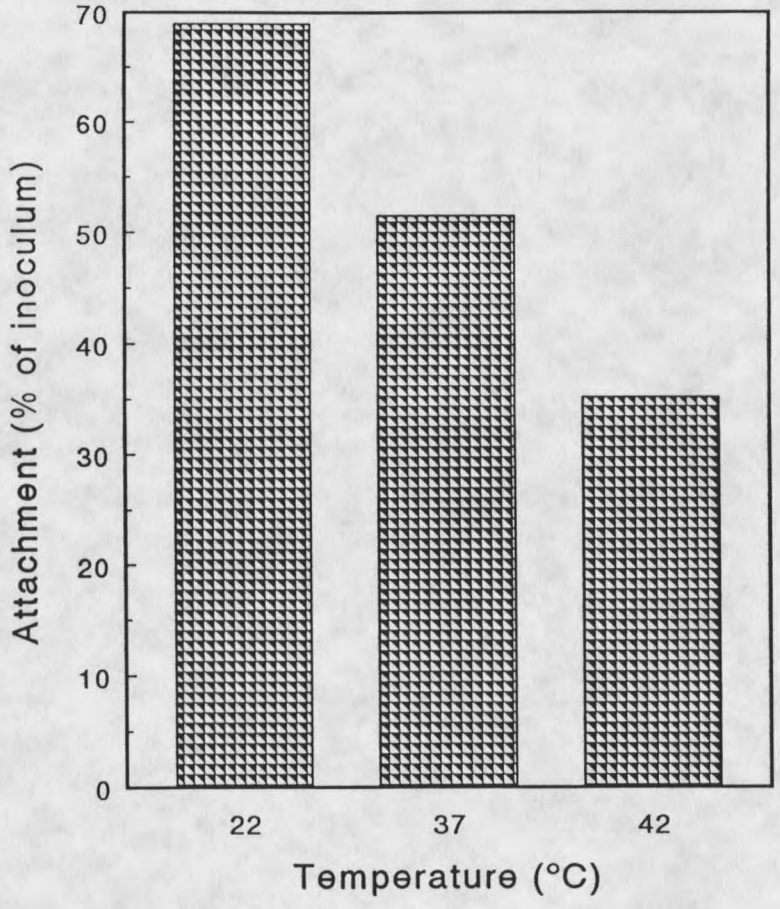


Figure 6. *S. typhimurium* attachment after resuscitation in DME for 5 h at 22°C, 4 h at 37°C and 4.5 h at 42°C. Results represent a composite of three monolayers from an attachment assay.

Table 4. Attachment of strain SL3201 to cultured cell monolayers with and without gentamicin

Treatment	% Attachment[a]
Control	34.0 ± 6.57[b]
Gentamicin	1.0 ± 0.45

[a] Percent attachment quantified as the % of inoculum that remained on the cells after five washes and sarkosyl treatment.

[b] Results expressed as mean ± standard error of the mean for three experiments, each representing composite of three monolayers. Two-tailed unpaired t test showed significant difference between untreated and gentamicin treated bacteria ($p<0.05$). Percent kill was 94.3 ± 1.77.

pH

The pH of the DME during resuscitation was tested at 6.2, 6.7, 7.2, and 7.7. The bacteria resuscitated in media adjusted to lower pH showed slightly better attachment (Figure 7). A pH of 6.6 was chosen for resuscitation of bacteria in all further experiments.

Osmolarity

Salmonella were resuscitated in DME containing no NaCl, or with 0.06, 0.18, or 0.30 M NaCl. The attachment of strain SL3201 was slightly greater with a small amount of NaCl added to the medium (Figure 8).

A problem arose when resuscitation conditions established individually were combined. Strain SL3201 was resuscitated in DME at pH 6.6, with or without 0.18 M

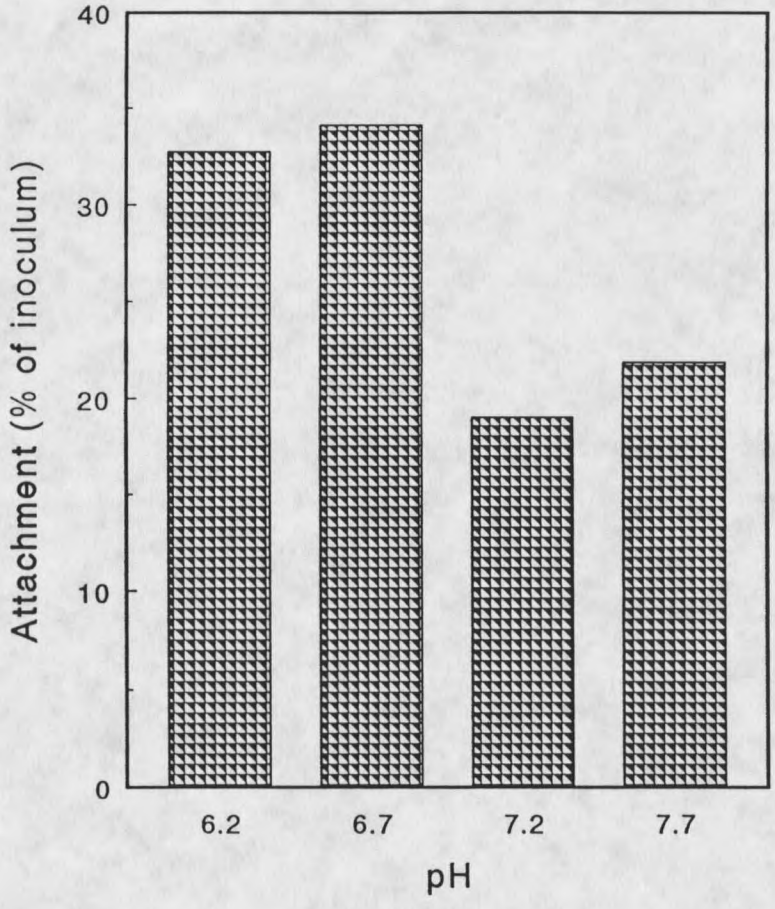


Figure 7. *S. typhimurium* attachment after 4 h resuscitation at 37°C in PBDME at pH 6.2, 6.7, 7.2, and 7.7. Results represent a composite of three monolayers from an attachment assay.

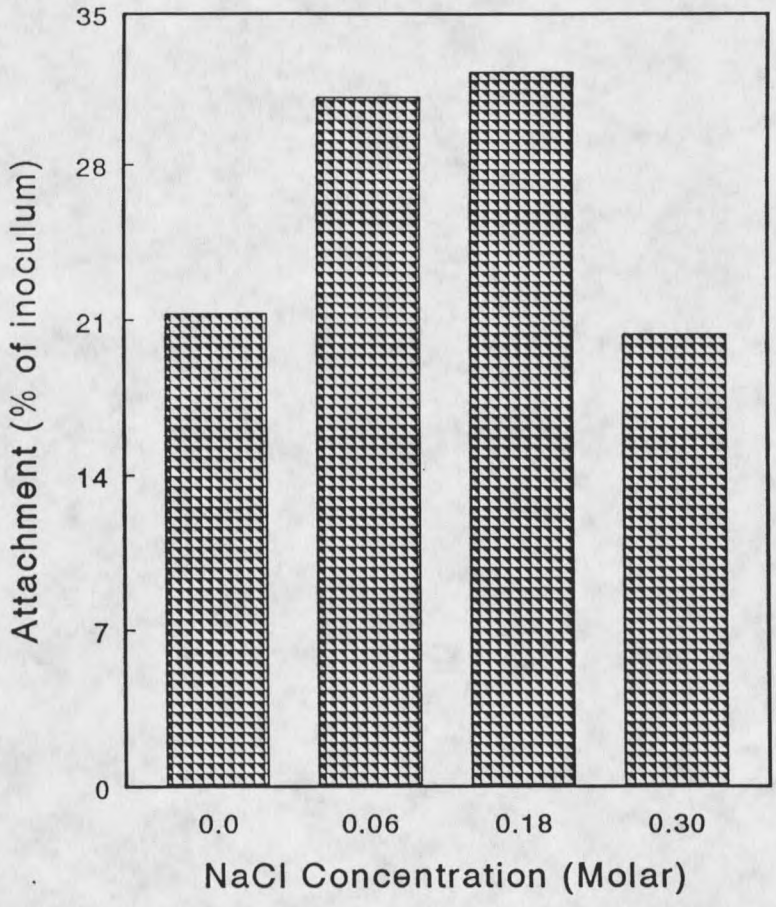


Figure 8. *S. typhimurium* attachment after 4 h resuscitation at 37°C in DME containing 0.0, 0.06, 0.18, and 0.30 M NaCl. Results represent a composite of three monolayers from an attachment assay.

NaCl, and incubated at 25 or 37°C. There was inhibition of attachment of *Salmonella* resuscitated in 0.18 M NaCl at 25°C, but not when resuscitated at 37°C (Figure 9). To determine if the inhibition was occurring during resuscitation or when the bacteria were added to the cells, strain SL3201 was resuscitated in DME with and without 0.18 M NaCl. The bacteria were washed after resuscitation by centrifugation at 5000xg for 20 min at 25°C. The supernatant was removed and the bacteria that had been resuscitated in medium with no NaCl were resuspended in medium with NaCl. The bacteria that had been resuscitated in medium with NaCl were resuspended in DME with no NaCl. Control bacteria were resuscitated and resuspended in DME without NaCl. The greatest amount of inhibition by NaCl was found to occur during resuscitation (Figure 10).

Washing the Bacteria

During the above experiments it was discovered that resuspending strain SL3201 in distilled water for 5 min after resuscitation and washing caused a two-fold decrease in attachment. Therefore, if bacteria were washed after resuscitation they were resuspended in saline or DPBS, which did not cause a decrease in attachment (data not shown).

Bacterial Growth Media

DME was used as the standard growth medium for all of the attachment assays. Once it became apparent that growth was necessary for attachment, DME was compared with M56 minimal media and DME buffer, which was osmotically balanced against DME. Measurable growth occurred in M56 during the 4 h, while the density of the

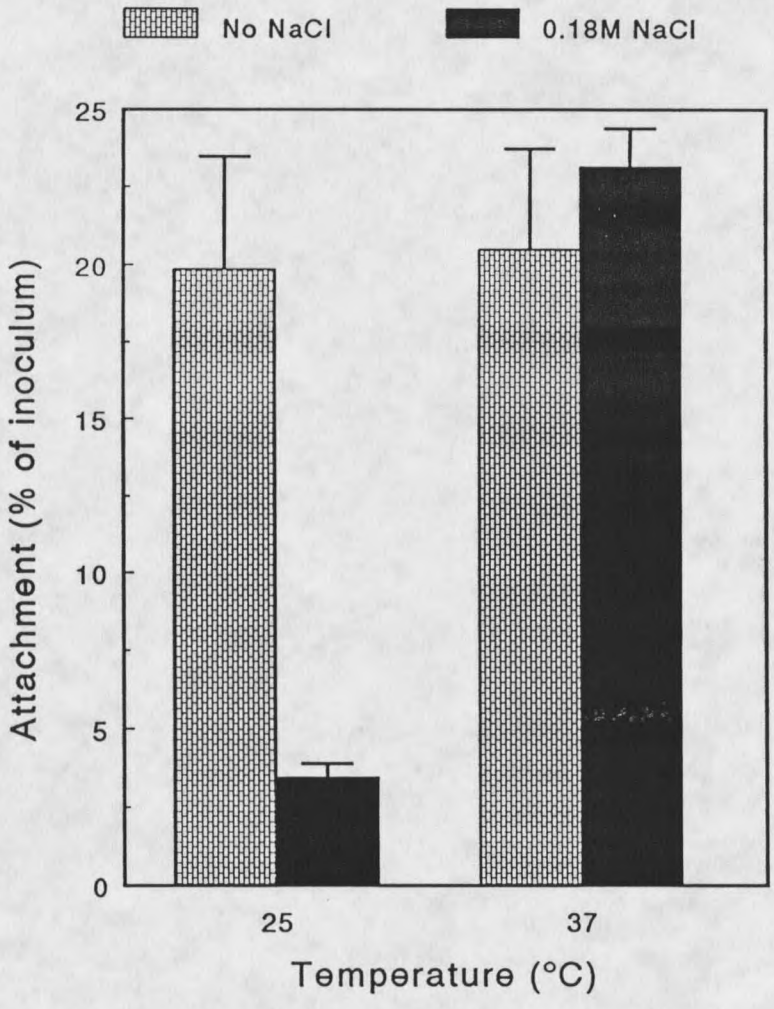


Figure 9. *S. typhimurium* attachment after 5 h resuscitation at 25°C in PBDME with and without 0.18 M NaCl and 4 h resuscitation at 37°C in PBDME with and without 0.18 M NaCl. Results expressed as the mean ± standard error of the mean for three experiments, each representing a composite of three monolayers. One way ANOVA showed significant differences between no NaCl and 0.18 M NaCl at 25°C ($p=0.0099$) and between 0.18 M NaCl at 25 and 37°C ($P=0.0052$).

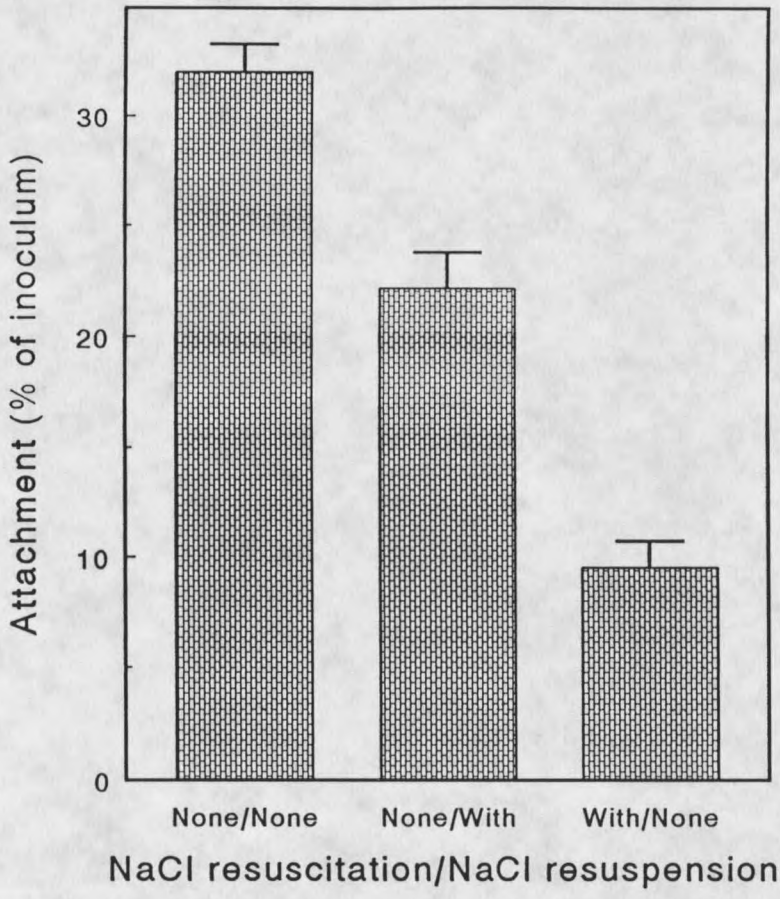


Figure 10. *S. typhimurium* attachment after 5 h resuscitation at 25°C in PBDME with or without 0.18 M NaCl, and after resuspension of recovered bacteria in PBDME with or without 0.18 M NaCl. Results expressed as the mean ± standard error of the mean for three experiments, each representing a composite of three monolayers. One way ANOVA showed significant differences among all three treatments ($p < 0.0001$).

bacteria in the DME buffer did not increase. These results confirmed that growth was necessary for attachment (Figure 11).

Oxygen Availability

Investigators have recently shown that the ability of *Salmonella* to invade cultured cells was inhibited when the bacteria were grown aerobically (16, 53). The effect of oxygen availability on attachment was determined using the cold attachment assay. Strain SL3201 was incubated at 37°C aerobically and anaerobically to the early logarithmic and stationary phase. Bacteria in the aerobic logarithmic phase attached 100 times greater than those in the aerobic stationary phase, while bacteria in the anaerobic logarithmic phase attached only 7 times greater than those in the anaerobic stationary phase (Table 5). These data demonstrated that both growth phase and oxygen availability played important roles in the attachment of *S. typhimurium* to cultured cells.

Table 5. Effect of oxygen and growth phase on bacterial attachment to cultured cell monolayers

Oxygen	Growth Phase	Time(h)	Attachment[a]
aerobic	stationary	3.5	0.03[b]
	logarithmic	18	2.77
anaerobic	stationary	3.5	0.46
	logarithmic	18	3.28

[a] Attachment expressed as % of inoculum recovered after five washes, cell lysis and detachment with sarkosyl-saline.
[b] Based on composite of three monolayers from 24-well tissue culture assay plate.

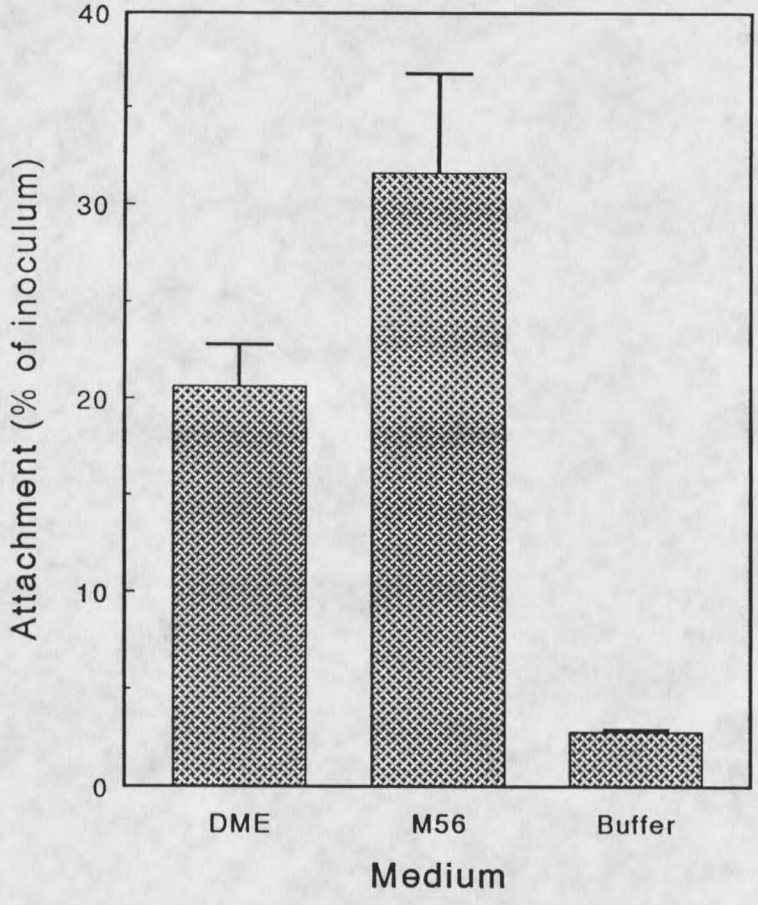


Figure 11. *S. typhimurium* attachment after resuscitation in DME, M56 or osmotically-balanced DME buffer for 4 h at 37°C. Results expressed as mean ± standard error of the mean for three experiments, each representing a composite of three monolayers. One way ANOVA showed a significant difference between DME and DME buffer ($p < 0.05$) and M56 and DME buffer ($p < 0.05$).

The attachment assay was also used to determine the effect of oxygen availability on attachment. Strain SL3201 was resuscitated in PBDME aerobically and anaerobically for 3 to 5 h and attachment to MDCK cells was measured. There was a 3.5 fold difference in attachment of aerobically- versus anaerobically-resuscitated bacteria (Figure 12).

A question was raised whether anaerobiosis or reduced conditions were causing increased attachment. Cysteine-HCl is a reducing agent (10). Strain SL3201 was incubated aerobically in DME with and without 0.025% cysteine. No difference in attachment of bacteria grown in medium with or without cysteine was observed (data not shown).

Cell Involvement in Attachment

Antibody Versus Cells

The question was raised whether the cells were actively involved in the bacterial attachment that was being observed. Attachment of strain SL3201 to monolayers pretreated with $F(ab')_2$ fragments prepared from a polyclonal Rb antibody against MDCK cells did not show any decrease in attachment (data not shown).

Endocytic Inhibitors

One group of investigators used endocytic inhibitors to block cellular activity and inhibit attachment and internalization of *Brucella abortis* in Vero cells (12). Experiments were designed to test the hypothesis that inhibition of cellular activity would inhibit

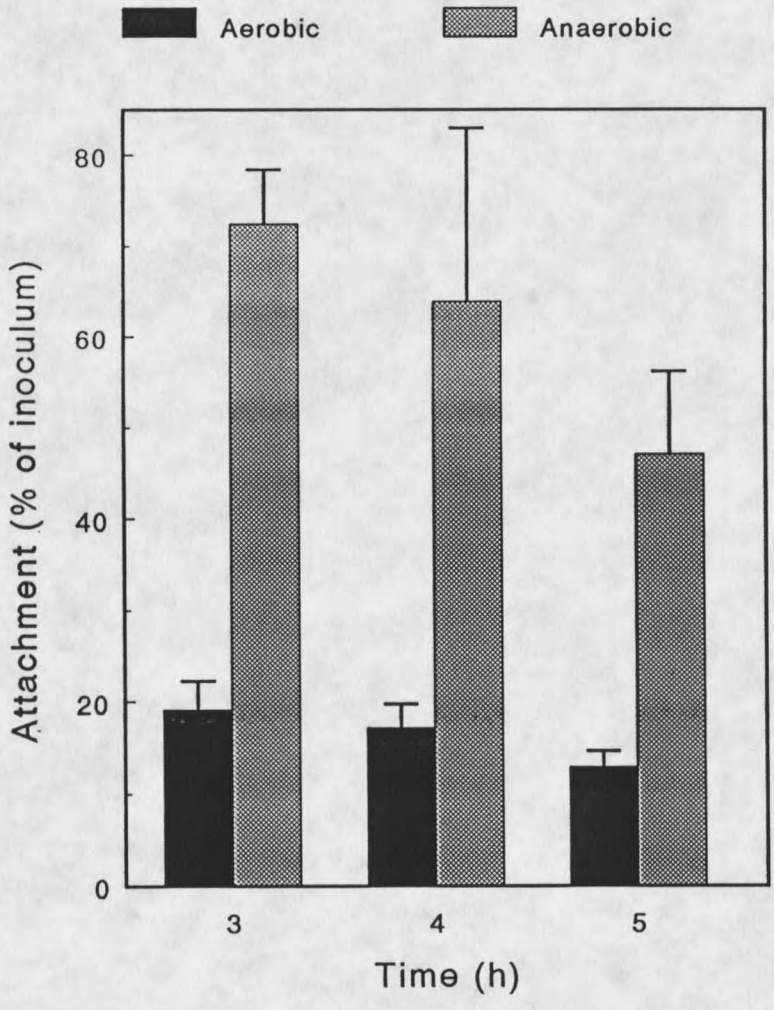


Figure 12. *S. typhimurium* attachment after aerobic or anaerobic resuscitation in PBDME at 37°C. Results expressed as the mean ± the standard error of the mean for three experiments, each representing a composite of three monolayers. Two-tailed unpaired t test showed a significant difference between aerobic and anaerobic at 3 and 5 h ($p=0.0014$ and $p=0.021$, respectively). At 4 h $p=0.0715$.

attachment of *S. typhimurium*. Monolayers were pretreated for 3 h with 1.0 mM iodoacetic acid, 1.0 mM DNP (0.5 N NaOH added until DNP dissolved), or cycloheximide (50 μg/ml) in DPBS with 1% FBS. The control had no inhibitor. The inhibitors were present during the time that the bacteria were on the cells. Attachment of bacteria to cells treated with DNP or cycloheximide was not decreased. The presence of iodoacetic acid caused a 5-fold decrease in attachment (data not shown). A follow up experiment was done to determine if the inhibition by iodoacetic acid was due to an effect on the cells or the bacteria. Monolayers were pretreated for 1 h with 2 mM iodoacetic acid in DPBS with 1% FBS. After pretreatment, the monolayers were washed once with 0.5 ml DPBS. The control had no pretreatment with inhibitor. The bacteria were added to the cells with no iodoacetic acid present. The results showed no difference in bacterial attachment to pretreated cells compared with that of the control (data not shown), indicating that the effect of the iodoacetic acid was on the bacteria.

Bacterial Competition in Attachment

Competition experiments were done to determine if the *Salmonella* were attaching to specific receptors on the cell. The chamber slide assay was used to determine if *S. senftenberg* could compete with *S. typhimurium* for attachment sites on the cells. *S. senftenberg* was not visualized because the first antibody was specific for *S. typhimurium*, therefore, only *S. typhimurium* were counted. The results indicated that attachment of strain SL3201 was competitively inhibited by *S. senftenberg* (Table 6).

Table 6. Competitive attachment to cultured cell monolayers with *S. typhimurium* and *S. senftenberg*

Ratio of *S. senftenberg* to *S. typhimurium*	Attachment of strain SL3201 [a] (% of cells with bacteria)
0 : 1	35.0 ± 1.53[b]
1 : 2	28.4 ± 2.19
2 : 1	15.0 ± 1.00

[a] *S. typhimurium* strain SL3201 multiplicity held constant at 1:100. Incubated with monolayers for 2 h, and enumerated with indirect fluorescent Ab assay.

[b] Results expressed as the mean ± standard error of the mean. One way ANOVA showed significant reduction of attachment in presence of *S. senftenberg* ($p=0.0004$).

Mediators of Attachment

Protein(s): Treatment with Proteases

The hypothesis that the attachment factor was a protein was tested using trypsin and proteinase K, which are proteolytic enzymes (4). *S. typhimurium* was resuscitated for 3 h at 37°C. After resuscitation, bacteria were recovered by centrifugation (11,220xg, 15 min, 4°C) and resuspended in protease buffer (see Materials and Methods). Four aliquots were prepared: Tube 1 received trypsin (0.25 mg/ml); Tube 2 received trypsin (0.25 mg/ml) and trypsin inhibitor (0.25 mg/ml); Tube 3 (control) received gelatin (0.25 mg/ml); Tube 4 (control) received gelatin (0.25 mg/ml) and trypsin inhibitor (0.25 mg/ml). All four tubes were incubated at 37°C for 1 h, then tubes 1 and 3 received trypsin inhibitor (0.25 mg/ml). For proteinase K experiments, proteinase K (0.25 mg/ml) was substituted for trypsin and 2 mM PMSF for trypsin

inhibitor. The bacteria were tested for their ability to attach. No difference in attachment was observed among any of the treatments (data not shown). It appeared that the bacteria held in protease buffer may have lost their ability to attach, therefore, the experiments were repeated except that after resuscitation the four bacterial aliquots were prepared and the proteases were added directly to the resuscitation medium. Once again, no difference in attachment among any of the treatments was observed (data not shown).

Protein(s): Treatment with Inhibitors

Other researchers studying bacterial interactions with cells have evaluated the effect of protein synthesis inhibitors or DNA synthesis inhibitors on attachment and invasion (16, 23). Experiments were designed to determine if these inhibitors would block attachment of *S. typhimurium* to cultured cells. Strain SL3201 was resuscitated in medium containing chloramphenicol (50 μg/ml), which inhibits protein synthesis (72); nalidixic acid (10 μg/ml), which inhibits DNA gyrase (35); or DME with no inhibitors present. Nalidixic acid had no effect on attachment, whereas chloramphenicol almost completely inhibited attachment (Figure 13).

Protein(s): Heat Treatment

It has been previously shown that heat treatment caused a decrease in ability of *S. typhimurium* to invade (17). The hypothesis that heat inactivation would inhibit attachment of *S. typhimurium* was tested. Bacteria were resuscitated, diluted in DME with 0.5% FBS, held in a 54°C water bath for 2, 4, and 6 minutes, then immediately

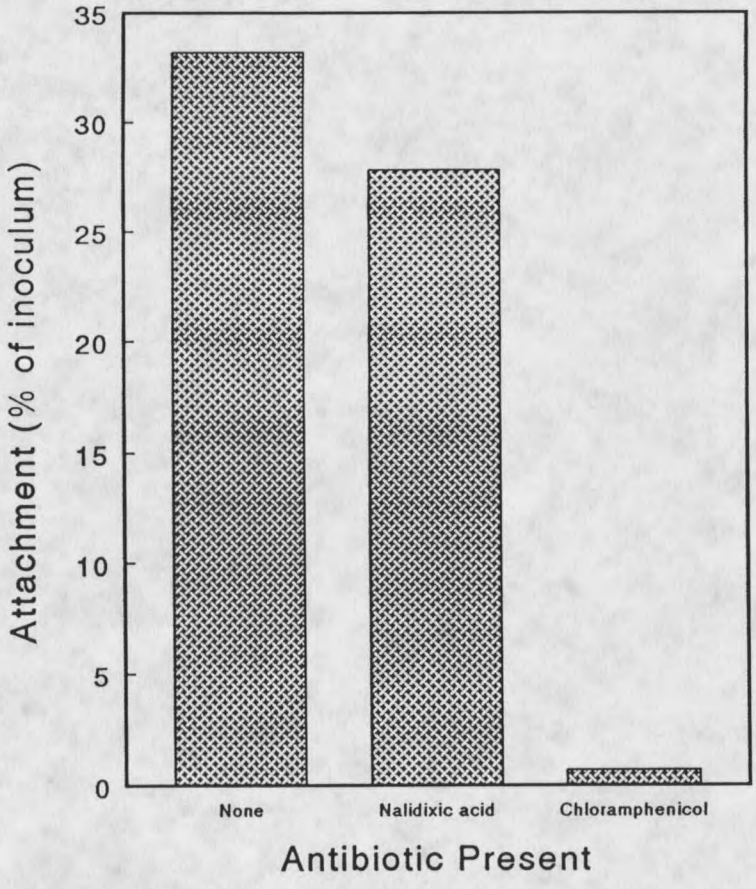


Figure 13. *S. typhimurium* attachment after resuscitation in DME alone and with nalidixic acid or chloramphenicol for 4 h at 37°C. Results represent a composite of three monolayers from an attachment assay.

placed in an ice bath for 5 min. The bacteria were tested for their ability to attach to cell monolayers. The results demonstrated that even though the decrease in the number of viable bacteria from 4 to 6 min was less than 2 fold, the decrease in the attachment from 4 to 6 min was greater than 10 fold (Figure 14). Therefore, heat affected the attachment ability of viable bacteria.

Protein(s): Alkylation

It was determined during the experiments with cell involvement in attachment that the decreased attachment caused by iodoacetic acid was due to the effect on the bacteria and not on the cells. In order to determine what part of the iodoacetic molecule was causing inhibition, 2 mM sodium acetate in DPBS with 1% FBS was incubated with the bacteria and then attachment was compared with a control with no sodium acetate. No difference was observed in attachment (data not shown). In a similar manner, attachment was measured after treatment of the bacteria with (2 mM) KI, iodoacetic acid, bromoacetic acid, or chloroacetic acid. Bromoacetic acid slightly inhibited attachment and chloroacetic acid and KI did not affect attachment (Figure 15). Iodoacetic acid alkylates sulfhydryl groups on proteins (71, 77). Thus, it was hypothesized that the inhibition was due to alkylation of a necessary protein(s). Competitive experiments were done using cysteine, which is also alkylated by iodoacetic acid (10). Cysteine (at 2, 4, and 8 mM) was added to the medium at the same time as the iodoacetic acid. The inhibitory effect of iodoacetic acid was competitively reversed by 8 mM cysteine (Figure 16).

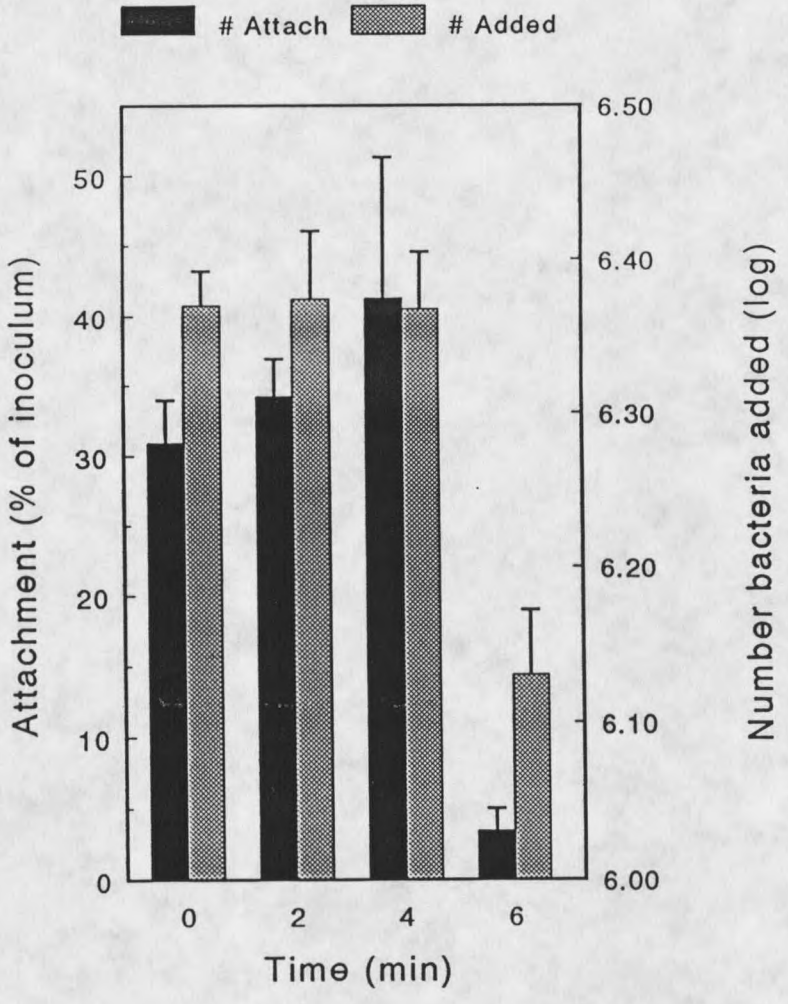


Figure 14. *S. typhimurium* inoculum and attachment after 3.5 h resuscitation in DME, dilution in DME with 0.5% FBS, holding in 54°C water bath for 2, 4, and 6 min, and cooling on ice. Results expressed as the mean $\pm$ the standard error of the mean for three experiments, each representing a composite of three monolayers. One way ANOVA showed significant differences between 0, 2, and 4 min versus 6 min ($p < 0.05$).

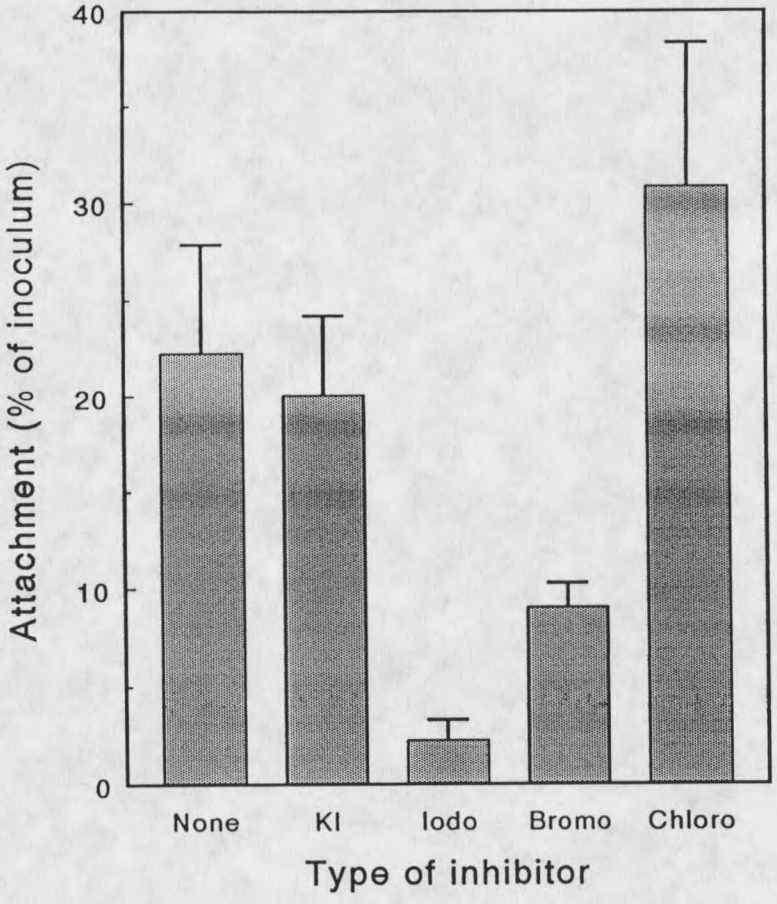


Figure 15. *S. typhimurium* attachment after 3.5 h resuscitation in DME, addition of potential inhibitors, incubation for 0.5 h, washing, and dilution in DME. Results expressed as the mean $\pm$ the standard error of the mean for three experiments, each representing a composite of three monolayers. One way ANOVA showed a significant difference among the groups ($p=0.0104$), specifically between control and iodoacetic acid ($p=0.0381$), iodoacetic acid and chloroacetic acid ($p=0.0121$), and bromoacetic acid and chloroacetic acid ($p=0.0292$).

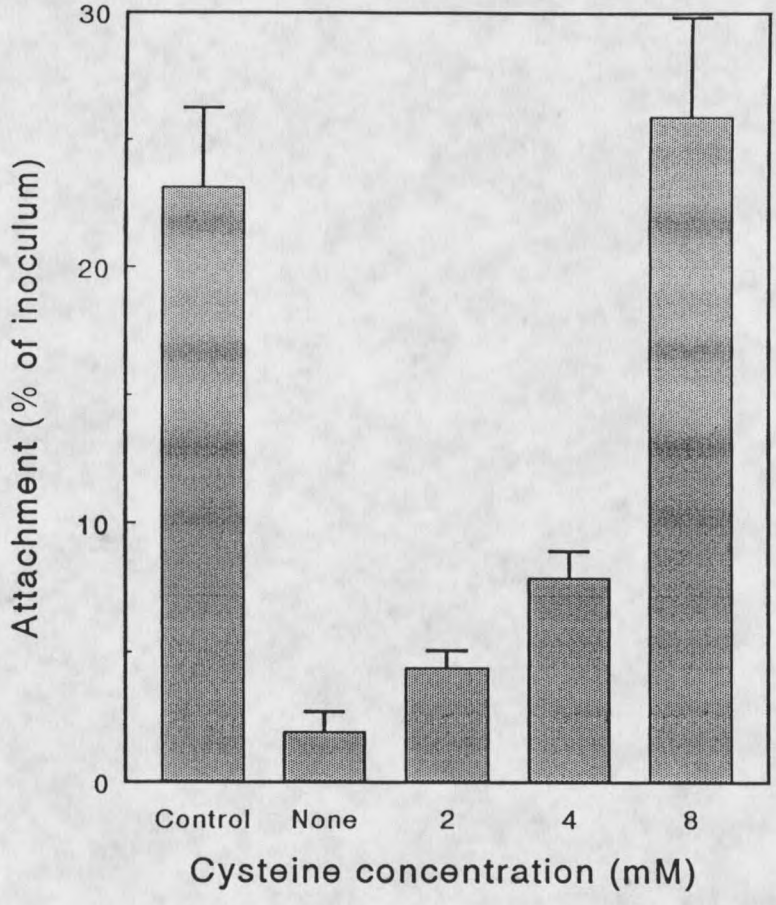


Figure 16. *S. typhimurium* attachment measured after 3.5 h resuscitation in DME, addition of iodoacetic acid (2 mM) and varying concentrations of cysteine, incubation for 0.5 h, washing, and dilution in DME. Results expressed as the mean $\pm$ the standard error of the mean for three experiments, each representing a composite of three monolayers. One way ANOVA showed a significant difference among the cysteine concentrations tested ($p < 0.0001$).

Hydrophobic Interactions

Hydrophobicity has been shown to play a role in the association of *Salmonella* with mammalian cells *in vitro* (2, 50, 58, 63). Adherence to hydrocarbons was used to demonstrate whether hydrophobicity was a factor in the observed attachment. The results indicated that strain SL3201 is not hydrophobic in the stationary or early logarithmic phase. *Y. enterocolitica* strain E641 was used as a control and showed a high degree of hydrophobicity. Hydrophobicity, therefore, did not account for the difference in attachment by *S. typhimurium* between stationary and log phase bacteria (Figure 17).

Hemagglutinins

MSHA and MRHA have been described as adhesins of *S. typhimurium* (2, 34, 42, 46, 54, 60, 62, 75). Strain SL3201 is a member of the FIRN biotype which does not produce type 1 fimbriae. This was further confirmed in this study by non-hemagglutination of guinea pig erythrocytes. Therefore, type 1 fimbriae could not be responsible for the observed attachment. *S. typhimurium* was tested for a MRHA by using sheep erythrocytes in a static settling test adopted from Tavendale *et al.* (75). The results shown in Table 7 show (-), indicating no hemagglutination, or (L), which indicated tubes that looked positive for hemagglutination, but, when examined microscopically, showed that lysis or partial lysis of the erythrocytes had occurred, giving a false positive reading. This suggested the presence of a cytotoxin, possibly a contact hemolysin, that, to my knowledge has not been previously described for *S. typhimurium*.

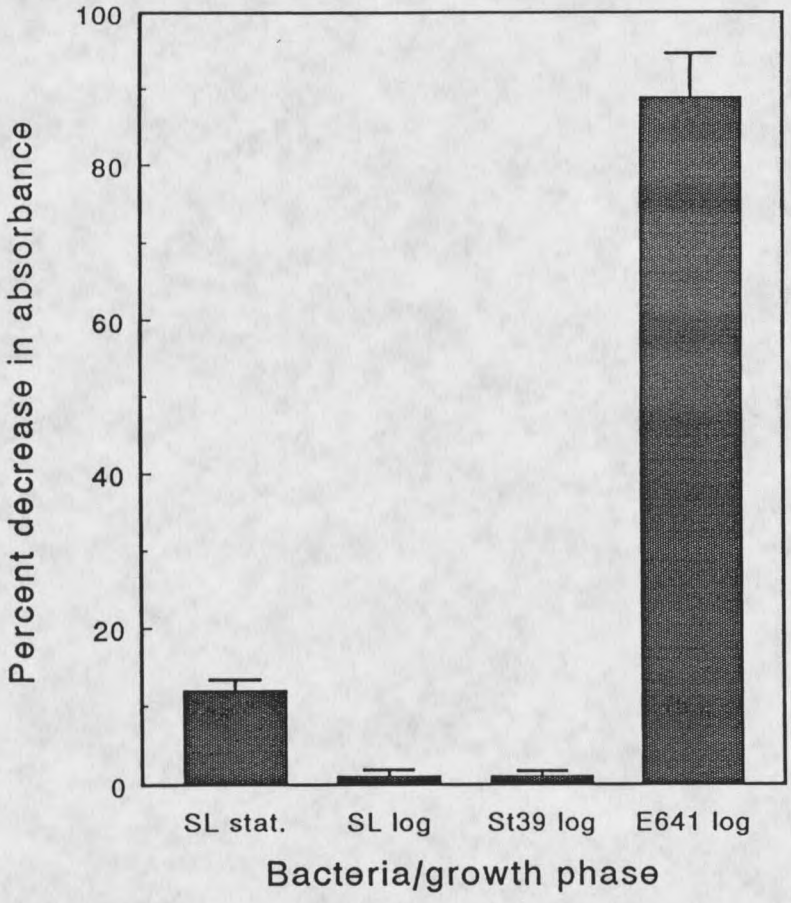


Figure 17. *S. typhimurium* SL3201 and St39 and *Y. enterocolitica* E641 in the stationary phase or log phase (strain SL3201 and St39 resuscitated 4 h, strain E641 resuscitated 4.5 h). Absorbance measured before and after mixing with n-hexadecane. Results expressed as the mean $\pm$ the standard error of the mean for three experiments. One way ANOVA showed a significant difference among the groups ($p < 0.0001$), specifically between strain E641 and each of the others tested ($p < 0.001$).

Table 7. Hemagglutination of sheep erythrocytes by *S. typhimurium*

Test Strain	Growth Phase[a]	Presence of D-Mannose (0.5%)	Hemagglutination at bacterial dilution of: 1:2	1:4	1:8	1:16
S141	stationary	-	-	-	-	-[b]
		+	L	-	-	-
	resuscitated	-	L	-	-	-
		+	L	L	L	-
SL3201	stationary	-	-	-	-	-
		+	L	L	L	-
	resuscitated	-	L	-	-	-
		+	L	L	-	-
	agar	-	-	-	-	-
		+	-	-	-	-
St39	stationary	-	-	-	-	-
		+	L	L	-	-
	resuscitated	-	L	-	-	-
		+	L	L	L	-
	agar	-	-	-	-	-
		+	-	-	-	-

[a] Bacteria were grown to the stationary phase and resuscitated in BHI. Strains SL3201 and St39 were also grown to the stationary phase on TSY agar. All bacteria were tested directly from the broth or the plate (in saline) at 2-fold dilutions of a suspension adjusted to $\approx 5x10^{10}$/ml.

[b] (-) = no agglutination; (L) = partial or complete lysis of erythrocytes.

Flagella: Motility and Chemotaxis

Flagella have been described as mediators of attachment in terms of motility, chemotaxis, and physical contact (44, 47, 51, 70, 75, 76, 79). The role of the flagella in the attachment of *S. typhimurium* was investigated during this study. Attachment of different strains of *S. typhimurium* was compared. Strain SL3201NM was a flagellated, non-motile mutant that was not impaired in its ability to attach (Figure 18). Strain

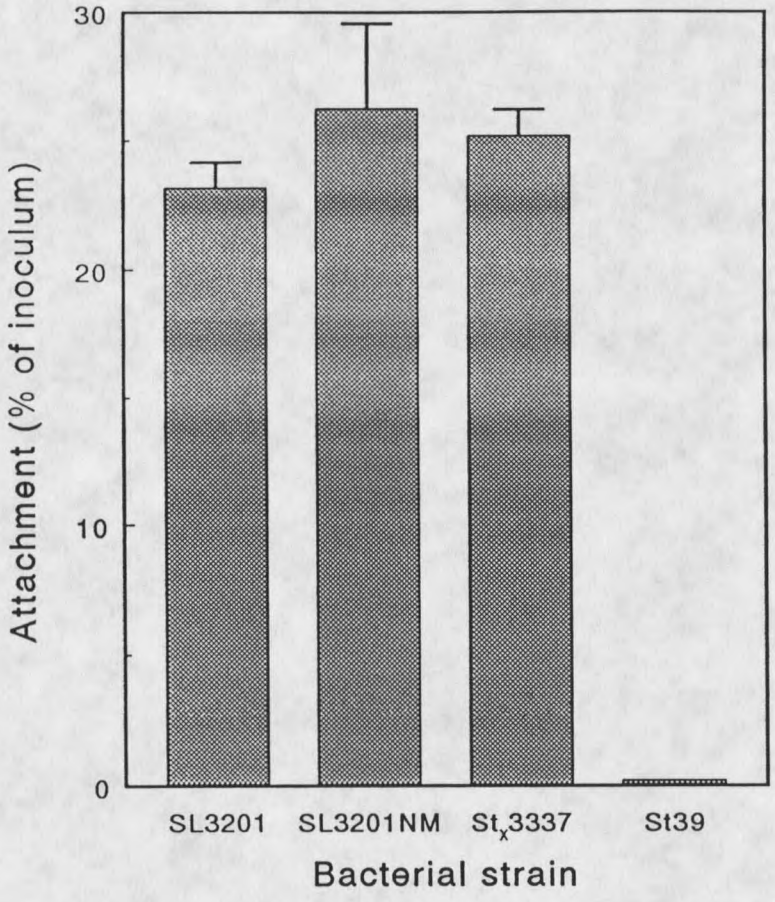


Figure 18. Comparison of attachment by *S. typhimurium* SL3201, SL3201NM, St$_x$3337 and St39. Results expressed as the mean $\pm$ the standard error of the mean for three experiments, each representing a composite of three monolayers. One way ANOVA showed a significant difference among the strains ($p < 0.0001$), specifically between strain St39 and each of the other strains ($p < 0.0005$).

St_x3337 was flagellated, motile, and lacked the 100 kb virulence plasmid. The lack of the virulence plasmid did not impair attachment by this strain. St39 was a non-flagellated mutant that was incapable of attaching. The results suggested that motility was not important, but that the physical structure of the flagella was somehow involved in attachment.

Flagella: Physical Structure

Physical removal of the flagella was considered as a way of examining their role in attachment. The flagella were sheared off of strain SL3201 in a blender after resuscitation. Microscopic examination of a wet mount showed that the sheared bacteria were not motile. The bacteria were tested for their ability to attach and the results showed that the control and the sheared bacteria attached equally well (data not shown).

Other investigators have examined the effect of anti-flagellar antibodies on attachment of *S. typhimurium* to cultured cells (39). Strain SL3201 was incubated with Fab fragments prepared from anti-flagellar antibodies for 30 min prior to being added to the cells. The antibody was unable to inhibit attachment (Figure 19).

S. typhimurium produces flagella in one of two antigenic phases, designated i (phase 1) and 1,2 (phase 2) (66). It was considered that a phase change might be occurring during resuscitation, thereby enhancing attachment. Stationary and resuscitated strain SL3201 were stabbed into a semisolid medium containing either anti-flagellar poly a-z or anti-flagellar I complex (Difco, diluted 1:500). The bacteria were found to be uniformly in phase 1. Therefore, a phase change was not responsible for the difference in attachment between the stationary and the resuscitated bacteria.

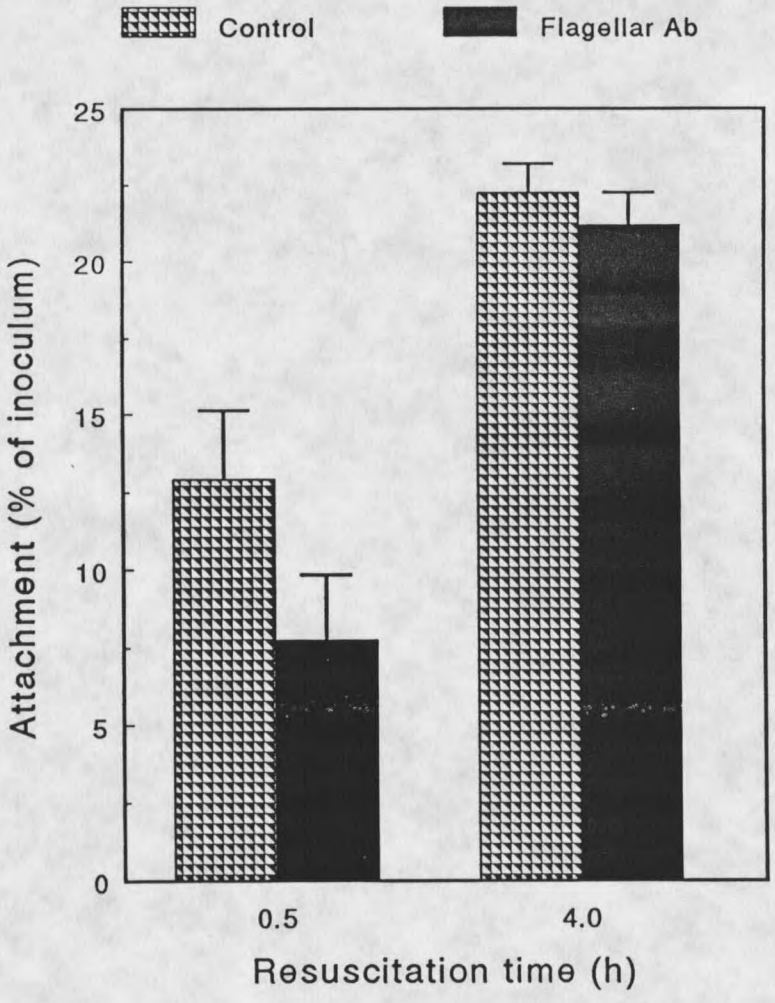


Figure 19. Attachment of *S. typhimurium* stationary or resuscitated in PBDME for 3.5 h and incubated for 30 min with anti-flagellar antibody. Results expressed as the mean $\pm$ the standard error of the mean for three experiments, each representing a composite of three monolayers. Two-tailed unpaired t test showed no significant differences at either time ($p=0.1679$ and $p=0.4917$, respectively).

DISCUSSION

Attachment of *S. typhimurium* to Epithelial Cells

Attachment to epithelial cells of an animal host by *S. typhimurium* is considered to be a prerequisite of invasion (19, 73). The accepted view of the process is that reversible attachment of bacteria to the cells is followed by irreversible attachment which leads to invasion. Many investigators have provided information on the mechanism of invasion, but have not concentrated their efforts on attachment. It has been hypothesized that preventing attachment could prevent infection (17), however, there are many unanswered questions about the attachment phenomenon. To begin to understand this process, an *in vitro* assay was developed to look at only attachment and not invasion by *S. typhimurium*. This assay was used to study various physiological conditions that might influence attachment. Studies were also done to test the hypothesis that the mediator of attachment was a protein. Many different mediators of attachment have been reported to be involved in the attachment process, and this report includes studies to determine if any of those previously described were responsible for the attachment measured by the experimental assay.

Different assays were initially examined for their ability to evaluate attachment. The chamber slide assay was helpful in identifying the patterns of attachment and in

selecting the most desirable cell line for further use. During this assay it became apparent that bacteria in the early logarithmic phase of growth attached better than those in the stationary phase (Tables 2 and 3). The ELISA was used as a more sensitive method to quantify the number of attached bacteria in a way that was not possible with the chamber slide assay. This assay confirmed that attachment of *S. typhimurium* reached its peak in the log phase, which agrees with reports by Ernst *et al.* (16) and Lee and Falkow (53). Finlay *et al.* (23) reported that incubation of *S. typhimurium* with epithelial cells induced the production of proteins necessary for attachment. More recently, however, Lee and Falkow (53) showed that incubation with the cells was not necessary to produce bacteria that would invade. This more recent report led to the development of the resuscitation procedure, which used bacteria grown in a way that did not require prior exposure to the cells. Bacteria were incubated without cells for varying amounts of time, then centrifuged onto precooled cells for 5 min. This cold attachment assay allowed quantification of attached bacteria only, since cold temperatures are known to block invasion (22, 53). However, the attachment was disappointingly low (Figure 2), suggesting that metabolic activity of the cells was required, or that the 5 min centrifugation was insufficient to promote maximum bacteria-cell collision. The results did confirm that a certain amount of bacterial growth was necessary for attachment to occur. The results also agreed with reports that attachment could occur without incubation on the cells (53).

A third attachment assay that included room temperature centrifugation for 30 min was used to determine physiological influences on attachment. Evidence was provided

that little invasion occurred during the 30 min that the bacteria were on the cells (Table 4). Consequently, the assay measured essentially attached bacteria.

Inhibitors of endocytosis and antibodies against the MDCK cell did not inhibit attachment. This provided evidence that a signal from the cells was not responsible for inducing the observed attachment. However, competition experiments with *S. typhimurium* and *S. senftenberg* showed that there must be sites, or receptors, on the cells specific for bacterial attachment (Table 6). The epidermal growth factor receptor was recently described by Galan *et al.* (30) as having a role in the attachment of *S. typhimurium* to epithelial cells *in vitro*.

Galan and Curtiss (29) reported that environmental stimuli such as osmotic conditions, temperature, and oxygen availability affect DNA supercoiling, which may influence the expression of certain genes, although they were not sure if the virulence genes of *S. typhimurium* were sensitive to supercoiling. The data from the NaCl experiments (Figures 9 and 10) indicated that temperature and osmotic conditions influence attachment. A temperature dependent ionic stimulus might have affected DNA supercoiling, thereby affecting genetic expression of attachment factors.

This work describes distinct conditions that affect the expression of *S. typhimurium* attachment to epithelial cells *in vitro*. By simply changing the growth conditions, the number of bacteria that attach can be controlled up to a certain level. It was also shown that it was growth, and not special nutrients in the DME that influenced attachment, since bacteria resuscitated in minimal medium (M56) attached as well as those in DME (Figure 11). The physiological stairway to attachment (Figure 20) is a

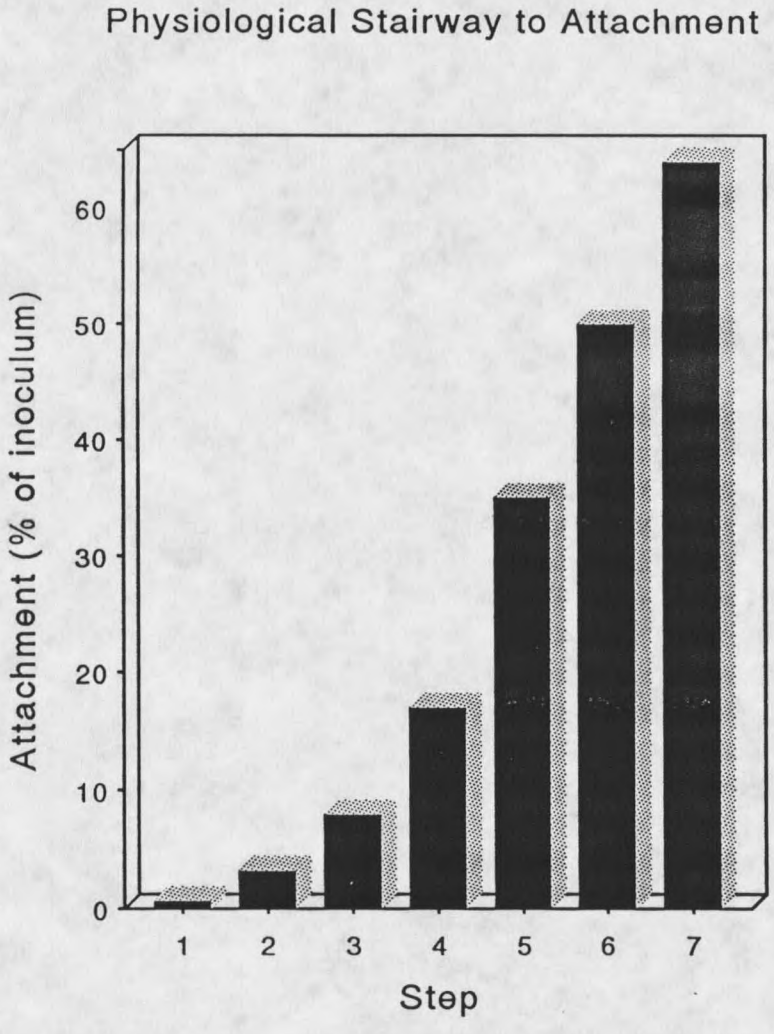


Figure 20. Physiological stairway to attachment.

Step 7: 4 h anaerobic resuscitation in PBDME, 37°C
Step 6: 4 h roller resuscitation in DME, 37°C
Step 5: 2 h roller resuscitation in DME, 37°C
Step 4: 4 h aerobic resuscitation in PBDME, 37°C
Step 3: anaerobic stationary in BHI, 37°C
Step 2: aerobic stationary in BHI, 37°C
Step 1: aerobic stationary on TSY, 37°C

schematic representation of how attachment was modulated by changes in growth condition. This study presents evidence that differences in oxygen availability, growth phase, pH, and osmolarity of resuscitation media influenced the degree of bacterial attachment. This information should be helpful for future researchers who want to focus their attention on attachment, which is presumed to be a prerequisite of host colonization and invasion.

Mediators of Attachment

Having shown that attachment could be separated from the invasion process, studies were undertaken to determine what structure or property of the bacterium was mediating attachment. Previous reports by others demonstrated that some proteins are synthesized or repressed in attached bacteria compared to those bacteria in the supernatant (21, 23, 68). It has also been shown that protein synthesis is required for the invasive properties of *S. typhimurium* (22, 23). The evidence from this study strongly suggested that a protein was responsible for the observed attachment, although definitive evidence was lacking. The results from an experiment with chloramphenicol and nalidixic acid agreed with reports that protein synthesis was necessary for attachment, although DNA synthesis was not (Figure 13). There seemed to be a basal level of attachment in the anaerobic stationary phase, perhaps demonstrating that small amounts of the attachment protein(s) are produced, but not enough for significant attachment. The aerobic stationary phase bacteria from the TSY agar plate might demonstrate the efficiency of the washing steps, or represent the background level of bacteria without attachment.

It has been shown that there are differences in protein profiles for *S. typhimurium* grown anaerobically and aerobically (16, 53, 68). The increased attachment in the anaerobic phase (Figure 12) agrees with reports of increased invasiveness under anaerobic conditions (16, 53, 68). Different proteins are produced under different conditions, and this could mean that the "attachment protein(s)" is not produced in the aerobic culture. Another suggested mechanism, adopted from Schiemann and Shope (68), is that anaerobic growth represses a protein(s) that might inhibit attachment. Therefore, when this protein(s) is not expressed, attachment can take place.

Heat treatment of the bacteria had a noticeable effect on the attachment by *S. typhimurium* (Figure 14). After 6 min at 54°C, half of the bacteria were killed (2.35×10^6 to 1.36×10^6), yet there was a 10-fold decrease in attachment of the remaining viable bacteria (35% to 3.4%). It is important to note that the decreased attachment is not related to the decreased inoculum, since it was shown that multiplicity can vary at least three logs with no effect on attachment, and the heat treated inocula varied no more than one log.

Experiments with iodoacetic acid provided the strongest evidence that attachment was mediated by a protein(s). Resuscitated bacteria, treated with iodoacetic acid, were unable to attach. The cultured cells were not affected by the iodoacetic acid, and treatment with sodium iodide demonstrated that the iodine part of the molecule was not responsible for the decrease in attachment. Iodoacetic acid is known to alkylate sulfhydryl groups on proteins (71, 77). It has been previously shown that bromoacetic acid will also alkylate proteins, but with a lower affinity than iodoacetic acid, while

chloracetic acid is unreactive except when used at very high concentrations and for a longer time (77). Results from this study agree with this relationship in that the bromoacetic acid inhibited attachment, although not as much as iodoacetic acid, and chloracetic acid had no effect (Figure 15). Cysteine, which competitively aklylates iodoacetic acid, was used in competitive experiments and it was determined that 8 mM cysteine could restore attachment to the level of the control (bacteria without iodoacetic acid or cysteine) in the presence of iodoacetic acid (Figure 16).

Although it was not done here, a way of providing further evidence that a protein might be involved in attachment would be protein extraction and electrophoresis. The data generated from this study suggest that the differences in the outer membrane protein composition worth examining would be: (i) strain SL3201 resuscitated aerobically vs. anaerobically, (ii) strain SL3201 resuscitated in PBDME with 0.18 M NaCl vs. those resuscitated in PBDME without NaCl at 25°C, (iii) strain SL3201 in PBDME with 0.18 M NaCl resuscitated at 25°C vs. those resuscitated at 37°C, and (iv) resuscitated strain SL3201 vs. resuscitated strain St39.

Hydrophobicity and defective LPS (rough) have been shown to mediate attachment (2, 50, 58, 63). The hydrophobicity experiment in this study showed that *S. typhimurium* strain SL3201 was not hydrophobic in the stationary or the logarithmic phase, and therefore, hydrophobicity could not have been responsible for the attachment observed in the experimental assay (Figure 17). Strain SL3201 is a smooth strain, meaning it is not deficient in LPS. Therefore, it was not "roughness" that causeed the bacteria to attach to the cells.

The experimental strain SL3201 is fimbriae negative, and therefore, type 1 fimbriae could not have been involved in the observed attachment. The static settling test, adopted from Tavendale *et al.* (75), was used to test for a MRHA. Upon microscopic examination the positive tests showed no hemagglutination but instead showed lysis of the erythrocytes, representing false positive results (Table 7). This leaves a question about the validity of the previous reports by Jones and Richardson (46) and Mintz *et al.* (60), who claimed they saw hemagglutination by this method. It is possible that what they observed was a phenomenon of contact hemolysis which occurred at higher concentrations of bacteria. To my knowledge, this has not been previously reported for *S. typhimurium*.

Motility and chemotaxis have been described as virulence factors by some (6, 78) and as mediators of attachment by others (51, 70, 75, 79). Jones *et al.* (47) reported that motility only increases bacterial contact with the cells and can be negated with centrifugation. The results obtained with strain SL3201NM, a non-motile flagellated strain produced in this study by 2-aminopurine mutation, showed that motility was not involved in attachment. Whether this was due to the centrifugation negating the effect, or whether motility really is not a factor, could not be determined with the attachment assay used in this study.

Consequently, most putative mediators of attachment were ruled out during this study, including defective LPS, hydrophobicity, MSHA, and MRHA. However, the role of flagella in the observed attachment remains unclear. Strain St39 is non-flagellated and it does not attach, suggesting that the flagellar structure is required for attachment.

However, strain St39 carries more mutations than just flagellar ones (5), therefore, it cannot be said with complete certainty that flagella are necessary for attachment.

Hohmann *et al.* (39) reported that anti-flagellar antibodies can inhibit attachment. Those results conflict with results from this study in that anti-flagellar antibodies did not inhibit attachment in the experimental assay (Figure 19). It may have been that the anti-flagellar antibody used in this study was not reactive with that part of the flagella active in attachment.

Physical removal of the flagella, or shearing the bacteria, did not decrease attachment. These results are inconclusive since shearing is known to remove the filament portion, but the flagella in its entirety was probably not removed. The hook-filament portion of the flagellum of *S. typhimurium* consists of 5 protein species; flagellin (filament protein), the hook protein, and three hook associated proteins (40). The basal body complex consists of at least eight protein species. Flagellar formation and function involve more than 40 genes clustered in three regions of the chromosome (40). Until mutants are available bearing various flagellar components, it will not be possible to say what the exact role of the flagella is in cell attachment by *S. typhimurium*.

Worton *et al.* (79) presented scanning electron micrographs of *Salmonella* associated with the mucosa. These pictures showed what appeared to be fibrillar structures stretching between the bacterium and the cell and also from bacterium to bacterium. Finlay and Falkow (23) presented pictures of *S. typhimurium* associated with cultured epithelial cells, and the fibrillar structures were apparent in those pictures as well. Giron *et al.* (33) described an inducible bundle-forming pilus on enteropathogenic

E. coli associated with the cells. They went on to say that these pili created networks of fibers that bind together individual organisms. Indirect fluorescent antibody staining performed during the chamber slide assay in this study revealed what appeared to be similar fibrillar structures (Figure 4C). When antiflagellar antibody was used as the first antibody in an indirect fluorescent assay, these structures could be visualized. This suggested that the fibrillar structures were flagella intertwined between the bacteria and the cell. The flagella might be used as a way of physically binding bacteria to each other and to the cells.

Conclusions

The processes of attachment, and the more extensively studied invasion, appear to be multifactorial. It is not clear that the attachment observed in these studies is even related to invasion. This attachment may be just a way of getting the bacteria next to the cell long enough for the invasion mechanisms to engage, or it may precede and be part of the invasion process. These puzzles remain unsolved, yet it is clear, as this study indicates, that environmental signals play an important role in attachment.

This work shows that it is possible to devise methodology that will permit the study of attachment as a separate process from invasion, which has not been the emphasis of most reported studies. Further investigation of attachment as a separate event in infection may eventually lead to the control or treatment of salmonellosis, as it has been suggested that prevention of adherence may result in elimination of invading organisms before establishment of an infection (17).

APPENDIX

Table 8. Growth and attachment after incubation in DME at 37°C (raw data to accompany Figure 2)

Time[a] (h)	Experiment Number 1	2	3
0			9.27×10^{5}[b]
1	2.47×10^{6} 1.77%[c]	1.98×10^{6} 1.14%	
2	1.05×10^{7} 2.31%	8.97×10^{6} 1.43%	
3	2.83×10^{7} 4.56%	1.79×10^{7} 4.36%	
4	4.23×10^{7} 2.88%	3.47×10^{7} 3.42%	
5			7.6×10^{7} 4.25%
6	1.15×10^{8} 2.01%		
7			3.17×10^{8} 2.16%
9			6.47×10^{8} 1.62%
12			1.23×10^{9} 0.20%

[a] The time that the bacteria were incubated anaerobically in DME at 37°C prior to enumerating and adding to the cell monolayers.

[b] The enumerated bacterial density.

[c] The percent of cells attached to cell monolayers.

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