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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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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 ($p<0.0001$).

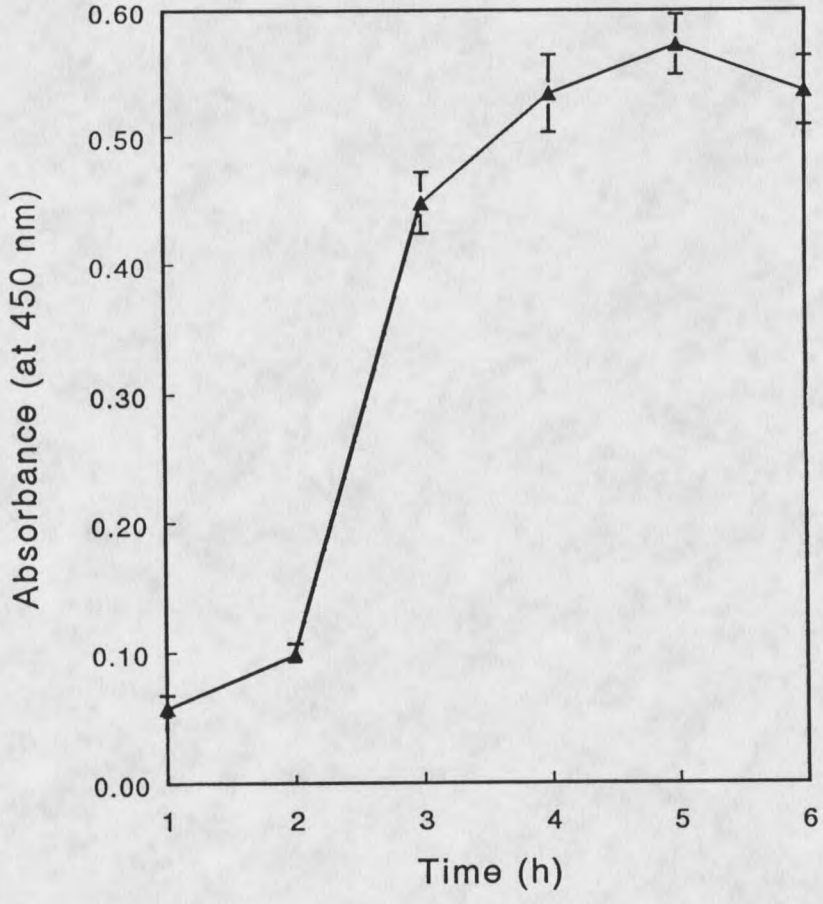

Figure 1. *S. typhimurium* attachment after incubation with monolayers in DME with 0.5% FBS. Results expressed as the mean ± standard error of the mean for three replicate monolayers. One way ANOVA showed a significant difference in attachment over time ($p < 0.0001$).

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.