Changes in the virulence of chlorine-injured Yersinia enterocolitica by Mark William LeChevallier
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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
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Abstract:
Experiments were designed to assess the effect of chlorine injury on virulence of waterborne enteropathogens. Higher chlorine doses (1.0-1.5 mg/l) were necessary to produce injured Yersinia enterocolitica, Salmonella typhimurium and Shigella spp. than enterotoxigenic Escherichia coli (ETEC) or coliform bacteria (0.5 mg/l) in the test system used. LD50 experiments using mice showed that injured enterocolitica were 20 times less virulent than uninjured control cells (3300 and 160 cfu, respectively). This decrease in virulence was not related to reduced attachment to Henle 407 intestinal epithelial cells, but could be related to a loss of epithelial cell invasiveness. Loss of epithelial cell invasiveness was measured by four techniques: (i) the HeLa cell roller tube method, (ii) light microscopy on stained Henle cell monolayers, (iii) transmission electron microscopy of HeLa cells infected with Y. enterocolitica, and (iv) enumeration of intracellular bacteria in mouse intestines using fluorescent-antibody methods. In contrast, injured S. typhimurium and ETEC lost the ability to attach to Henle cells. Injured Y. enterocolitica showed reduced oxygen utilization, ATP synthesis and assimilation of radiolabeled thymidine, Uridine and methionine. Inhibition of RNA and protein synthesis by certain antimicrobics inhibited the invasiveness of Y. enterocolitica. Chlorine did not change the hydrophobicity or surface charge of injured pathogens. Preparations of membranes from untreated and antimicrobic treated Y. enterocolitica blocked the invasiveness of virulent Yersinia, while membranes from chlorinated cells were unable to block invasiveness. Electrophoresis of outer membrane proteins from chlorinated cells showed numerous changes as compared to untreated preparations. A theoretical model of how chlorine affected the invasiveness of injured Y. enterocolitica is presented.

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YERSINIA ENTEROCOLITICA

by

Mark William LeChevallier

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ABSTRACT

Experiments were designed to assess the effect of chlorine injury on virulence of waterborne enteropathogens. Higher chlorine doses (1.0-1.5 mg/l) were necessary to produce injured Yersinia enterocolitica, Salmonella typhimurium and Shigella spp. than enterotoxigenic Escherichia coli (ETEC) or coliform bacteria (0.5 mg/l) in the test system used. LD_{50} experiments using mice showed that injured Y. enterocolitica were 20 times less virulent than uninjured control cells (3300 and 160 cfu, respectively). This decrease in virulence was not related to reduced attachment to Henle 407 intestinal epithelial cells, but could be related to a loss of epithelial cell invasiveness. Loss of epithelial cell invasiveness was measured by four techniques: (i) the HeLa cell roller tube method, (ii) light microscopy on stained Henle cell monolayers, (iii) transmission electron microscopy of HeLa cells infected with Y. enterocolitica, and (iv) enumeration of intracellular bacteria in mouse intestines using fluorescent-antibody methods. In contrast, injured S. typhimurium and ETEC lost the ability to attach to Henle cells. Injured Y. enterocolitica showed reduced oxygen utilization, ATP synthesis and assimilation of radiolabeled thymidine, uridine and methionine. Inhibition of RNA and protein synthesis by certain antimicrobics inhibited the invasiveness of Y. enterocolitica. Chlorine did not change the hydrophobicity or surface charge of injured pathogens. Preparations of membranes from untreated and antimicrobic treated Y. enterocolitica blocked the invasiveness of virulent Yersinia, while membranes from chlorinated cells were unable to block invasiveness. Electrophoresis of outer membrane proteins from chlorinated cells showed numerous changes as compared to untreated preparations. A theoretical model of how chlorine affected the invasiveness of injured Y. enterocolitica is presented.

INTRODUCTION

Much progress has been made during the past ten years towards understanding injury of bacteria in drinking water. Bissonnette et al. (17) in 1975 first described injury of bacteria in water while subsequent reports on the physiology of injury have been made by Camper and McFeters (42) Zaske et al. (251, 252) and Domek et al. (54). In addition, the influence of laboratory and environmental factors on the recovery of injured coliforms have been evaluated (16, 54, 133, 153). Recently, new methods for enumerating injured total and fecal coliforms in drinking water have been proposed (134). Despite this work, questions remain about the health significance of injured bacteria. Do indicator bacteria and pathogens injure in the same manner? Are injured pathogens still virulent? What is the physiological mechanism of reduced virulence?

The introduction to this study is divided into three parts. The first section reviews injury including sources of injury in water and factors that affect injury. Most of this work has been in relationship to coliform bacteria. The next part reviews the limited data regarding injury of pathogens. The last section covers Yersinia enterocolitica with special emphasis on the invasiveness of this pathogen.

Injury of Microorganisms in Water

Injury has been defined as the sublethal physiological consequence of exposure to stresses, which cause a loss in the ability of microorganisms to grow normally under conditions that are satisfactory for uninjured cells (15, 17, 42, 153). Injury has also been described as reversible; i.e., under proper conditions of temperature and nutrients, injured organisms can repair the cellular lesion and become indistinguishable from unstressed cells (15, 17, 42, 153). Injury has been observed in all genera of coliform bacteria. This sublethal damage has been observed as an inability of bacteria to proliferate on media containing selective agents that have no apparent inhibitory action on undamaged cells (15, 17, 97, 153, 183, 187). Therefore, most sublethal stresses will result in damaged cells incapable of growing on the selective media commonly used in aquatic microbiology.

Sources of Injury

Although the understanding of all potential sources of injury in water is incomplete, several parameters have been studied extensively.

Disinfectants

Chlorine has been the most widely recognized cause of injury to bacteria in drinking water (6, 16, 24, 42, 81, 83, 138, 140, 154, 157, 175, 177, 232, 249). Both the chlorine dose and the time of exposure have been shown to influence the degree of injury (42, 154). Most bacteria exposed to sodium hypochlorite are rapidly killed once the chlorine demand of the system is satisfied (134). However, a sizable portion of the survivors are injured in such a way that they fail to grow under the selective conditions commonly used to detect bacteria in drinking water. Because of a lack of adequate techniques to measure injury, the extent of bacterial stress in drinking water is largely unknown, although some reports estimate that coliforms are recovered with an efficiency of ≤ 10 percent in chlorinated aquatic environments (15, 16, 24, 42, 58, 152, 155).

Exactly how chlorine injures a microorganism has never been demonstrated experimentally (59). Baker in 1926 (8) theorized that chlorine destroys microorganisms by combining with proteins forming N-chloro compounds. This was extended by Green et al. (82) and others (122, 232) who showed that chlorine had powerful effects on sulfhydral groups of proteins. Other evidence showed that hypochlorous acid (HOCl) converts several a-amino acids by

oxidation into a mixture of corresponding nitriles and aldehydes (177). The exact product of the reaction is dependent on chlorine concentration and pH (50, 51, 247). The action of chlorine on polypeptides can be understood in terms of electrophile-nucleophile interactions where electropositive chlorine reacts with electron-rich centers on the substrate (4). Functional groups in proteins that are susceptible to chlorine include: amino, imidazolium, guanidinium, sulfhydryl and indole residues; phenolic rings and the amide nitrogen of peptide bonds (38, 92, 220, 223). Stelmaszynska and Zgliczynski (220) showed with bovine albumin that ^{36}Cl was incorporated into N-terminal, e-lysine groups and the amide nitrogen of peptide bonds. Other experiments have shown that cytochromes, iron-sulfur proteins and nucleotides are highly vulnerable to oxidative degradation by HOCl suggesting that chlorine causes physiological damage primarily to the bacterial cell membranes (4, 42, 83, 154, 232, 251, 252). Investigators have shown that respiration, glucose transport, and ATP levels all decrease in chlorine-injured populations (42, 83, 154, 232). Electron microscopy of injured cells has demonstrated morphological changes in the cell membrane (252). In addition, chlorination has been reported to cause death as a result of unbalanced metabolism (249), affect protein synthesis, (12, 177), and cause genetic

defects by modifying purine and pyrimidine bases (83, 99, 102, 175, 207).

Although few data exist that describe injury from other water disinfection agents such as ozone, chlorine dioxide, and ultraviolet irradiation, it has been suggested that these treatments also cause injury (15, 39, 188). Chlorine dioxide has been reported to inhibit membrane-bound enzymes and protein synthesis (12, 188). Ultraviolet irradiation is known to cause damage to nucleic acids as well as alter membrane functions (15).

Environmental Factors

Environmental factors such as heat, freezing, and sunlight are also known to cause injury (15, 39, 68, 100). Exposure to sunlight can inactivate 90 percent of the indicator bacteria in stream water within 13 to 32 minutes (68). Although these parameters are probably of little importance in water contained in distribution systems, they may affect bacteria in source waters and reservoirs. Changes in pH resulting from acid rain or acid mine drainage (15, 55) or from lime or soda additions in the treatment plant may also cause injury to indicator bacteria in surface water. pH is known to affect substrate transport across membranes (15) and the soluability of heavy metals.

The role of transition metals in the injury process in drinking water was recently described (54). A combination of laboratory studies and field data indicated that copper, at concentrations normally found in drinking water, could cause significant injury to <u>Escherichia coli</u>. Although other metals such as cadmium, lead, and zinc were also detected in drinking water samples, these metals or combinations of the metals were not as important in the injury process as copper alone. Copper has been detected in 65.2 percent of 380 finished drinking water supplies in the United States, with a mean concentration of 0.043 mg/L and a range of 0.001-1.060 mg/L (222). Experiments have indicated that 90 percent injury occurs in <u>E. coli</u> populations within two days at copper concentrations near the national average (54).

Statistical analysis of data from 30 drinking water samples suggested that pH, alkalinity, and temperature influenced the extent of injury by copper, presumably by altering the chemical species and activity of the dissolved copper. Other factors, such as total organic carbon levels and the species of bacteria present, are thought to influence injury by copper. Copper-injured cells show impaired respiratory activity and are unable to utilize oxygen during the repair process (54).

Sources of copper in drinking water include copper pipe material, brass, faucets, and other attachments

(222). Copper may also be introduced as copper sulfate, which is used to treat algal problems in reservoirs (76), and as trace contaminants in flocculation material. In addition, copper may enter water from geological sources. This process may be especially important in some areas, such as Montana, where copper is naturally abundant.

Biological Interactions

Another source of injury to indicator bacteria in drinking water is biological interactions (133). Some strains of heterotrophic or standard plate count (SPC) bacteria in drinking water have been shown to suppress coliform detection (74, 75, 101, 185, 243). It has been demonstrated that coliform suppression in municipal and rural water supplies can at least partly be attributed to elevated SPC numbers (3, 22, 75). For example, increased SPC densities have been noted prior to, or associated with, waterborne disease outbreaks (3, 75, 150, 162). The presence of pathogens has also been documented in the absence of detectable coliforms in samples with high SPC numbers (3). Data from the National Community Water Supply Study (150) showed that the frequency of coliform detection decreased when SPC levels exceeded 500-1000 cfu/ml.

Interactions of SPC bacteria with coliforms may also cause injury. For example, experiments have shown that interactions between Pseudomonas maltophilia and E. coli

can produce 55 percent injury over a 7-day period (133).
The decline in viable bacteria for the control and mixed
coliform cultures was identical, but injury was observed
only in the mixed culture. Injury did not occur until P.
maltophilia levels reached 10^4-10^5 cfu/ml. The ratio of
coliforms to P. maltophilia (1:10^4-10^5) in those
experiments is similar to the ratios for coliforms and SPC
bacteria in drinking water in which coliform suppression
has been reported (coliforms--1-10 cfu/100ml; SPC-->500
cfu/ml) (48, 75, 163). Such interactions are important,
particularly when samples are transported or stored.
Previous studies have shown that coliform levels may vary
significantly during sample transport (163, 164), but
injury in these experiments was not controlled or examined.

Finally, the role in the injury process of the many
toxic organic substances that may be dissolved in surface
waters is completely unknown. As awareness of injured
bacteria in drinking water increases, other causes of
injury may become apparent.

Factors Affecting Injury

In addition to the primary causes of injury to
bacteria in drinking water, a number of factors can
influence the extent or severity of stress.

Environmental Factors

Environmental factors that influence the extent of injury include time and temperature of exposure of the bacteria in water. As mentioned previously, >90 percent injury can occur in an E. coli population within two days when exposed to 0.05 mg copper/L (54). Similarly, Bissonnette et al (17) showed that >90 percent of E. coli became injured when exposed for 2 days in mountain streams. Other investigators have reported 97 percent injury in E. coli populations exposed to reagent-grade water for 3 days (252). Results of experiments in which 230 membrane diffusion chambers containing bacteria were immersed in Bozeman, Montana drinking water indicate that 90 percent injury occurred in E. coli populations within 2-4 days (134). In general, as the temperature of the water increased, the time necessary to produce significant levels of injury decreased (7, 52, 187). Temperature was also shown to be an important variable in a computer model developed to predict injury in drinking water (54). These findings might be explained by the observation that higher water temperatures increase the activity of injurious agents such as chlorine, metals, and biological antagonists (7, 52, 54, 187). Other environmental factors that probably influence extent of injury include the bacterial strain, the physiological state of the organism, and the

concentration of dissolved nutrients, although little
information regarding these factors is available.

Sample Handling

The way a water sample is collected and handled prior
to examination greatly influences the viability of injured
bacteria in that sample. Sodium thiosulfate is usually
added to drinking water samples to neutralize any chlorine
residual (6, 20, 72). A chelator such as EDTA is sometimes
added to remove heavy metals, particularly if there is an
extended period between sample collection and enumeration
(20, 54, 72).

Laboratory manipulations involving exposure to
diluents may cause underestimations of bacterial densities
in drinking water (153). If diluents are maintained at
approximately refrigerator temperature (around 4°C), their
composition and exposure time are of minimal impact on
enumeration efficiency. However, substantially lower
recoveries of injured bacteria have been associated with
use of diluents maintained at room temperature and with
extended exposure times (153). The enrichment of diluents
with low concentrations (0.05-0.1 percent) of organic
material such as peptone, gelatin, tryptone, or milk has
been demonstrated to be of value in the enumeration of
stressed aquatic bacteria (15, 24, 40, 104, 120, 183, 221,
145, 246, 248). In addition, diluents (such as standard

methods buffer) that contain magnesium ($MgSO_4$ or $MgCl_2$) and phosphate (K_2HPO_4 or KH_2PO_4) (15) are known to help recover metabolically injured cells (72).

Method of Enumeration

The choice of enumeration methodology probably has the greatest impact on the recovery efficiency of injured bacteria. Important factors to be considered when choosing a method to enumerate injured cells include the surface pore morphology of the membrane filter, the enumeration technique and the composition of the selective medium (21, 47, 73, 88, 96, 98, 132, 134, 139, 182, 208, 214, 225, 226). The influence of these factors on coliform enumeration has been reviewed (134). Recently, new membrane filter techniques have been proposed for enumerating stressed total and fecal coliforms (134). A new medium (m-T7 agar) remains selective and at the same time minimizes the inhibition of stressed coliform bacteria (134).

Injury of Pathogens

The question concerning the significance of injured bacteria in water has been a problem not adequately answered. Presumably, conditions that stress coliforms would also injure waterborne pathogens, although there are no data to support this hypothesis. Some investigators

have examined the virulence of injured foodborne pathogens (49, 71, 209, 218). Sorrells et al. (218) found no difference in the virulence of Salmonella gallinarum for 6 week old chicks after injury by freezing. Similarly, Simon et al. (209) reported that the virulence for mice of three strains of Salmonella typhimurium did not change appreciably when samples were freeze-dried and stored for 1 or 2 years at 5°C. Fung and Vanden Bosch (71) and Collins-Thompson et al. (49) reported that Staphylococcus aureus regained the ability to produce enterotoxin B after resuscitation in a non-selective medium. Other pathogens including Yersinia enterocolitica, Campylobacter jejuni, and Pseudomonas aeruginosa have been injured by a variety of stresses (heat, freezing, chlorine and ionizing irradiation), but the virulence of the injured cells was not assessed (61, 62, 80, 142, 174, 199). E. coli injured by heating, freezing, drying or gamma irradiation became more sensitive to hydrophobic antibiotics (142). While these data indicate that pathogens are injured in foods and by food processing, the results may not be directly applicable to the aquatic environment.

Walsh and Bissonnette (237) studied the attachment of chlorine injured enterotoxigenic E. coli (ETEC) to human peripheral leukocytes. They concluded that reduced adhesive ability was due to the loss of surface structures

resulting from sublethal chlorination. While they did not test the in vivo virulence of chlorine-injured ETEC, Walsh and Bissonnette (237) did imply that a reduced ability to colonize the small intestine would result in an inability of this organism to initiate disease.

Recently, a "viable-but-nonculturable" stage of Salmonella enteritidis (189) and Vibrio cholerae (250) has been described. In this condition, cells exposed to the aquatic environment for prolonged periods of time become nonculturable by conventional techniques but could be resuscitated by additions of nutrients (189). Pathogens in this "viable-but-nonculturable" stage have been reported to remain virulent (Roszak, D.B., D.J. Grimes and R.R. Colwell, 1984, Abst. Annu. Mtg. Am. Soc. Microbiol.,I 21, p. 125).

Yersinia enterocolitica

The genus Yersinia is currently comprised of seven Gram-negative coccobacillary species, Y. enterocolitica, Y. pseudotuberculosis, Y. pestis, Y. intermedia, Y. frederiksenii, Y. kristensenii and Y. ruckeri (14, 26, 27, 31, 230). Formerly classified as Pasteurella species, the genus is now named for the French bacteriologist, A. J. E. Yersin, who first isolated the causative agent of plague in 1894. The most notorious of the species, Y. pestis, has a "well defined heritage of human morbidity and mortality"

(29), while <u>Y. enterocolitica</u> has, until the late 1960´s,
remained virtually unknown. First described in 1939
(198), <u>Y. enterocolitica</u> has been etiologically
incriminated in a broad range of clinical entities, the
more frequent manifestations of which include: acute
mesenteric lymphadenitis, terminal ileitis,
gastroenteritis, septicemia, nonsuppurative arthritis,
erythema nodosum and localized abscesses in liver and
spleen (23, 93, 217).

Biochemical Characteristics

<u>Y. enterocolitica</u> has been characterized as
"biochemically polydiverse and thermally perceptive" (23,
93, 119). The organism usually ferments glucose, sucrose,
arabinose, galactose, sorbitol, trehalose, xylose and
cellobiose at both 22° and 35^OC within 48 h. Rhamnose,
raffinose, dulcitol and adonitol are not utilized; acid
from salicin and esculin hydrolysis is strain variable; and
maltose is utilized at 22^OC. <u>Y. enterocolitica</u> lacks
enzymatic activity for gelatinase, oxidase, phenylalanine
deaminase, citratase, lysine decarboxylase and arginine
dihydrolase, but possesses catalase, B-galactosidase
(except some strains), ornithine decarboxylase, nitrate
reductase type B and urease activity. Although the
rapidity of utilization of the aforementioned substrates
may vary slightly with temperature, the tests for

acetylmethyl carbinol (Voges-Proskauer), growth in
potassium cyanide and motility are temperature dependent
and are expressed at 22°C but lacking at 35°C.

Growth temperature affects expression of genes on the
40- to 48-megadalton plasmid associated with virulence of
Y. enterocolitica. Temperature affects calcium dependent
growth, serum resistance, autoagglutination, synthesis of V
and W antigens, and other polypeptides (18, 56, 90, 148,
178, 180, 211, 213). Growth temperature is known to affect
the structure of Y. enterocolitica membranes including the
O-specific sugars and fatty acid composition (11, 114,
239), surface properties of the bacterium (64, 124, 125),
attachment to epithelial cells (148, 168, 172), production
of fimbriae (64, 113, 168, 172) and sensitivity to certain
bacteriophage (114, 115). While many genes in the
chromosome and in the plasmid genome appear to be
temperature dependent, their exact role in pathogenesis is
not well understood. Some investigators (116) have
presented evidence that lipopolysaccharide produced during
growth in vivo resembles, antigenically, that produced
during growth in vitro at 25°C. Clearly, Y. enterocolitica
is an organism that can tolerate temperatures
characteristic of both the aquatic environment and a warm
blooded host.

Environmental Sources

It is well established that the animal kingdom serves as the major reservoir for Yersinia species (23, 45, 93). Y. enterocolitica of serotype and phage type identical to those recovered from human hosts have been isolated from animals, particularly the pig (23, 45, 93). Water, however, can serve as a vehicle for transmission of Y. enterocolitica to humans. There have been a number of reports of waterborne yersiniosis (60, 118, 131, 147) and many investigators have isolated Yersinia spp. from water (70, 94, 110, 111, 130, 156, 190, 197, 244), although usually these bacteria are avirulent. Keet (118) showed that Y. enterocolitica O:8 isolated from a mountain stream had the same phage and serotype as a strain recovered from a man suffering from septicemia after a hunting trip in the Adirondack Mountains of New York. He postulated that the water may have been contaminated by deer or other animal feces. Martin et al. (147) reported a family outbreak of yersiniosis in northern Saskatchewan, Canada, where the source of the etiologic strains could have been river water contaminated by a cow or her calf. Saari and Jansen (190) state that "an increasing number of atypical North American human Y. enterocolitica isolates suggest that water contamination may play an important role in the transmission of some forms of yersiniosis to man".

Virulence Determinants

While there are at least 50 serogroups of Y. enterocolitica (242), only a few serotypes are commonly associated with disease in man (45, 112, 117, 160, 195, 229). Human infections are most often associated with serotypes O:3, O:8, O:9, O:5,27, and O:13 (5, 45, 117, 160, 194, 195, 229). Virulence determinants associated with these serotypes include: autoagglutination, calcium dependency at 37°C, serum resistance, heat-stable enterotoxin production, tissue culture invasiveness, and the presence of V and W antigens (77, 103, 117, 136, 160, 170, 195, 203, 229).

A number of virulence determinants are associated with the 40- 48-megadalton plasmid. The plasmid appears to be essential for resistance of Y. enterocolitica against host defenses (148, 253). Strains that have lost the plasmid are avirulent. The virulence plasmids have been shown to code for at least 20 different polypeptides in minicells (181) of which one to four are associated with the outer membrane of the bacterium (19, 180, 181, 211). The plasmids of serotypes O:3 and O:9 share 90% homology with each other while the plasmids harbored by serotype O:8 have 75% sequence homology with those of O:3 and O:9 (89). The high degree of molecular relatedness of these plasmids is also reflected by the incompatibility phenomenon (10).

However, recently a mobilizable virulence plasmid from serotype O:8 was constructed and transferred to plasmid negative strains of O:3, O:5 and O:8 (91).

Calcium dependency for growth at 37°C is a plasmid associated trait that has received much attention (13, 28, 78, 109, 169). It has been speculated that calcium dependency reflects the ability of the bacteria to respond to their intracellular environment (28, 29, 169). Growth restriction occurs with the production of the V and W antigens when calcium levels are low, but growth resumes and the antigens are repressed when enough calcium is present to permit cell division (2.5 to 4.0 mM) (28, 29, 169). This calcium restriction reflects a normal metabolic step down characterized by shut off of stable RNA synthesis and decreased adenylate energy charge (28, 29).

Autoagglutination is another plasmid associated virulence determinant of Y. enterocolitica (112, 129). The bacteria produce a fluffy precipitate when grown in a tissue culture medium at 37°C but not at 25°C or when the plasmid has been lost (112, 129). It has been speculated that autoagglutination may be promoted by ancillary outer membrane proteins coded by the virulence plasmid (112).

Recently, Heesemann et al. (90) have presented evidence that the virulence plasmid also promotes attachment in some strains of Y. enterocolitica. Transconjugants that received the plasmid from serotypes

0:3 or 0:9 were tested for their ability to associate with HEp-2 cells. One strain (serotype 0:5) was able to interact with HEp-2 cells only after receipt of the cointegrate. However, strains of serotypes 0:3, 0:8 and 0:9 were able to interact with epithelial cells regardless of whether they contained the plasmid or not (90).

Invasiveness

Studies of the pathogenicity of Y. enterocolitica have revealed that the virulence of this organism is multifactorial consisting of both plasmid and chromosomal determinants (67, 215, 219). The ability of Y. enterocolitica to invade tissue is a key chromosomal virulence trait for this organism (146, 180, 194, 233).

Methods for evaluating bacterial attachment and invasiveness include light microscopy, viable plate counts and radiolabel assays (36, 53, 143, 151, 206). Light microscopy has the advantage of being able to estimate the percentage of cells colonized and/or infected but has the disadvantages of poor precision, is subjective and cannot distinguish live from dead cells (143). Recently, Nomarski differential interference contrast microscopy and UV incident light microscopy applied on the same microscope have been used to evaluate bacterial attachment and invasiveness (35, 36). The authors claim that the method is more accurate, sensitive, and simpler to use than

ordinary light microscopy (35, 36). Viable plate counts have the advantages of being objective, reproducible, and detect only viable bacteria (143). The accuracy of the method is limited by difficulty in obtaining homogeneous suspensions of individual bacteria (143). The roller tube method developed by Devenish and Schiemann (53) allows for the enumeration of intracellular Y. enterocolitica by killing extracellular bacteria with gentamicin. The system is simpler than microscopy, allows stricter control of multiplicity and provides a quantitative index for comparing the relative infection capability of Y. enterocolitica for HeLa cells (53). Other investigators have used different approaches to determine intracellular bacterial densities. Shaw et al. (206) used UV-irradiated bacteriophage to kill extracellular Yersinia and Shigella spp. in tissue culture infectivity assays. McCoubrey et al. (151) used incorporation of [^{14}C]methionine into Y. enterocolitica in the presence of streptomycin and cycloheximide as a measure of intracellular bacterial levels. Since streptomycin does not cross the tissue cell membrane and cycloheximide inhibits eucaryotic protein synthesis, only the intracellular bacteria are active and able to incorporate the label. Radiolabel assays are objective and reproducible but generally are more expensive than the other test procedures (143).

A number of advantages are associated with the use of the _in_ _vitro_ tissue culture system for studying invasiveness. It is possible to choose cell lines from different tissue types and different animal species. The assays are simple, rapid and inexpensive to perform and the use of laboratory animals is avoided. Most importantly, _in_ _vitro_ assays permit attachment or invasiveness to be studied isolated from other factors that influence the result of an infection _in_ _vivo_. Many investigators have noted strong correlations between tissue culture cell infectivity _in_ _vitro_ and the ability to penetrate epithelial cells _in_ _vivo_ (37, 145, 228). Une (227, 228) after studying the process of invasion of _Y_. _enterocolitica_ both _in_ _vivo_ in rabbits and _in_ _vitro_ using HeLa cells, concluded that "the HeLa cell infection system well reflected the interaction between _Yersinia_ and epithelial cells _in_ _vivo_". However, since the precise biochemical nature of the invasion mechanism is not known, it cannot be stated for certain that the processes that occur with the engulfment of bacteria by HeLa cells (derived from a cervical carcinoma) are the same as human intestinal epithelial cells _in_ _vivo_.

Since it is clear that the cell monolayer test for invasiveness does not completely reflect all of the virulence characteristics of enteropathogenic bacteria, use of _in_ _vivo_ tests are also necessary. One model used for

determining the in vivo virulence of enteric organisms is the Serény test (204). This test qualitatively measures the capacity to cause conjunctivitis in the eye of a guinea pig. A positive Serény test correlates well with virulence in Y. enterocolitica O:8 (65, 146, 193). Infection of intact intestinal epithelial linings has been used by some investigators to measure invasiveness (43, 44, 227). This method is more direct since intracellular bacteria can be observed using immunofluorescence techniques (67). Other virulence models include use of rabbits, mice, gerbils, and monkeys (43, 44, 45, 65, 193, 227).

Invasive Process

"Attachment" and "adhesion" are used here to denote the binding of a bacterium to a surface, while "invasion" is used to indicate the process of penetrating tissue. "Association" or "interaction" refers to the combined process of attachment and invasion where the two processes have not been delineated.

Investigators (85, 228, 253) have noted that when pathogenic strains of Yersinia and Shigella attached to HeLa cells in tissue culture a ruffling movement of the cell membrane stimulates a pinocytotic process by which the adherent bacteria become incorporated into the epithelial cell. Bacteria initiate this process by stimulating a phagocytic depression in the tissue culture cell

cytoplasmic membrane (79, 85, 123, 228, 253). The
organisms are then incorporated into the cells by numerous
fine appendages (microvilli). Modification of the
epithelial cell surface by interferon, viruses, and
chemicals can inhibit endocytosis of virulent bacteria (32,
33, 34, 85). Intracellular Y. enterocolitica are enclosed
in phagocytic vesicles which accumulate near the nucleus
(228).

Numerous investigators have studied the role of the
bacterium in the invasive process (2, 30, 84, 86, 158, 171,
176, 191, 192, 216, 231, 234, 241, 242; also reviewed in
67, 87, 137, 215, 219). From these studies two
requirements appear to be necessary for invasiveness; (i)
the organism must be viable and metabolically active, and
(ii) the organism must have certain surface components to
initiate engulfment. Brunius et al. (30) showed that
pretreatment of Y. pseudotuberculosis with formalin,
glutaraldehyde or proteases inhibit its interaction with
HeLa cells. Hale and Bonventre (84) have shown that
virulent Shigella strains participate actively in the
infection of tissue culture cells. They found that heating
the organisms at 50°C for 2 min inhibited the infectivity
of S. flexneri 2a for Henle cells and that a sublethal
pulse of UV irradiation also diminished infectivity. Heat
killed Y. enterocolitica have been reported by many authors
to be noninvasive (168, 176, 216, 234). Okamoto et al.

(168) found Y. enterocolitica killed by UV irradiation or formalin lost their capacity to attach to HeLa cells. However, other researchers have indicated that formalin or UV killed bacteria still interacted with epithelial cells (176, 216, 234). The reason for this discrepancy is not clear, but it may have to do with the techniques employed to measure attachment and invasion. All of these investigators used microscopic methods which I have previously noted (page 19) as being imprecise.

A requirement for specific surface characteristics for invasiveness has been extensively studied with Shigella (2, 86, 171, 191, 192, 241, 242). Plasmids of Shigella flexneri, Shigella sonnei and enteroinvasive E. coli have been shown to code for specific outer membrane proteins, nine of which were similar in all three strains (86, 192). When the plasmid of S. flexneri was transferred to E. coli K-12 (normally avirulent and noninvasive) the recipient became invasive for HeLa cells (191). E. coli minicells containing the plasmid incorporated [^{35}S]methionine into 14 outer membrane proteins (86). Full pathogenicity for a variety of laboratory models including the Serény test and the rabbit ileal loop required the plasmid and three chromosomal segments (191). Hale et al. (86) have hypothesized that the outer membrane proteins encoded by the virulence plasmid bind to receptors on host enterocytes and stimulate engulfment. Maurelli et al. (149) showed

that restoration of invasiveness after growth at 30°C

required protein synthesis. When shigellae were grown at

30°C and shifted to 37°C for 2 h in the presence of

chloramphenicol, the bacteria remained noninvasive. Adamus

and Romanowska (2) found significant quantitative

differences in the outer membrane protein composition

between virulent and avirulent strains of Shigella sonnei

phase I. Other researchers have indicated that the

composition of lipopolysaccharide in Shigella flexneri is

important for the survival of the organism against host

defenses and that glycolipids and lipoproteins are required

to induce engulfment of the bacilli by epithelial cells

(171, 173, 179). These data have been extended by others

who have indicated that a small plasmid of Shigella

dysenteriae is required to produce specific O-

polysaccharide side chain structures of lipopolysaccharides

which are necessary for invasiveness (241, 242). Similar

reports have been made for Salmonella typhimurium

indicating that the organism requires both plasmid-derived

and lipopolysaccharide determinants to be adhesive and

invasive in the HeLa test (105, 106, 158). The

invasiveness of Y. pestis and Y. pseudotuberculosis has

been related to the presence of chemical structures on the

bacterial surface sensitive to the action of ether.

Certain surface characteristics have also been shown

in Y. enterocolitica to be important for invasiveness (176,

216). It has been suggested that boiling cells destroys some heat-labile surface components essential for penetration of epithelial cells (176, 216). Hydrophobicity has been reported by some investigators to be important in the processes of attachment and invasion of tissues (124, 196). Schiemann and Swanz (196) found that all strains of Y. enterocolitica which were able to attach to Henle cells were also hydrophobic. Lachica and Zink (124, 125) correlated increased surface charge and hydrophobicity to the presence of the 40- to 48- megadalton plasmid. They suggested that hydrophobicity may be associated with the ability of the plasmid positive strains to colonize and survive in the gastrointestinal tract of mice. Hydrophobicity and electric charge have been shown to influence the phagocytosis of type 1 fimbriae-bearing E. coli (167, 205). One strain possessing a hydrophobic surface with weak negative charge was efficiently ingested while another strain which was hydrophilic and had an uncharged smooth lipopolysaccharide, resisted phagocytosis. The authors concluded that whether attached bacteria are ingested or not depends on their underlying physicochemical surface properties (167, 2050. Similar results have been noted for Salmonella (105, 107, 108). Hydrophobic interactions and electric charge were shown to be responsible for irreversible attachment and engulfment by HeLa cells.

Statement of Research Problem

The present study was conducted to investigate the changes in virulence of chlorine-injured Y. enterocolitica. This study had three objectives: (i) to compare the susceptibility of coliforms and pathogens to injury by chlorine; (ii) to evaluate with combinations of in vitro and in vivo tests the step(s) in the pathogenic process affected by chlorine; and (iii) to try to determine the physiological basis for reduced virulence.

MATERIALS AND METHODS

Bacterial Strains

The organism of primary interest in this study was a strain of Yersinia enterocolitica serotype O:8 (E661) donated by D. A. Schiemann, Montana State University. This organism was originally isolated from a human case of yersiniosis and has been previously characterized as autoagglutination positive, calcium dependent for growth at $37^{\circ}C$ on magnesium oxlate agar(95, 180), invasive for HeLa cells, able to produce conjunctivitis in guinea pigs and lethal for mice (194, 195, 196). Biochemically, this serotype belongs to Knapp and Thal (121) biotype 2, Nilehn (165) biotype 2, Wauters (G. Wauters, Ph.D. thesis, Vander, Louvain, Belgium) biotype 1, Brenner et al. (25) biotype 1, and Schiemann and Devenish (194) biotype I (Table 1). The organism was resistant to a sulfamethoxyazole and trimethoprim combination (190 ug/ml:10 ug/ml), but sensitive to various levels of 8 other antimicrobics (Table 2).

In addition, several other Yersinia enterocolitica serotypes (also donated by D. A. Schiemann) were used in some experiments. These included: O:3 (E752), O:13 (E887; isolated from a monkey), O:20 (Em062), O:21 (E750), O:4,32 (E759), and O:5,27 (E771). All of the enteropathogens

(except E887) were originally isolated from humans. The virulence characteristics of most of these isolates have been published previously (194, 195, 196).

Table 1. Biochemical characteristics of <u>Y. enterocolitica</u> O:8 (E661).

Test	Incubation Temperature $^{\circ}$C	Reaction
Kligler iron agar	35	K/A no gas, H_2S^-
Christensen's urea agar	35	+
Ornithine decarboxylase	35	+
Arginine decarboxylase	35	−
Lysine decarboxylase	35	−
Indole	30	+
Xylose	22	+
Salicin	35	−
Esculin	22	−
Sorbitol	35	+
Voges-Proskauer	22	+
ONPG	35 or 22	−
Sucrose	22	+
Sorbose	35	+
Lecithinase	22	+
Rhamnose	22	−
Raffinose	22	−
Mellibiose	22	−
a-Methylglucoside	22	−
Citrate	22	−
Motility	22	+
Motility	35	−

<u>Salmonella</u> <u>typhimurium</u> isolates were obtained from the State Laboratory of Hygiene, Madison, Wisconsin and from H. Lior, Canadian Laboratory Center for Disease Control, Ottawa, Ontario. Two of the enterotoxigenic <u>Escherichia</u> <u>coli</u> (ETEC) cultures were provided by M. Levine, The Center

for Vaccine Development, University of Maryland School of

Medicine, and a third ETEC isolate was obtained from R.A.

Table 2. Minimal inhibitory concentration (MIC) of various
          antimicrobics for Y. enterocolitica O:8.

Antimicrobic	Minimal Inhibitory Concentration (ug/ml)		
	at 22°C		at 35°C
Nalidixic acid	2	(2)[a]	2
Rifampicin	4	(8)	8
Chloramphenicol	8	(16)	8
Nitrofuratoin	50	(100)	50
Sulfamethoxyazole/ Trimethoprim (19:1)	4	(>200)	>200
Spectinomycin	16	(32)	16
Tetracycline	2	(4)	2
Penicillin G	16	(>32)	32
Gentamicin	1	(6)	1

a) Numbers in parentheses are 72 h MIC values. All other
    values are 24 h standard MIC results (240).

Wilson, E. coli Reference Center, Pennsylvania State

University. All three isolates were reported by Wilson to

be colonization factor antigen positive. Shigella flexneri

and S. sonnei were obtained from the University of

Washington Clinical Laboratories, Seattle, Washington. The

S. dysenteriae isolate was part of the Montana State

University stock culture collection. The coliforms were

isolated in our laboratory from drinking water using the

membrane filter technique (6), and included five strains of

E. coli, two stains of Klebsiella oxytoca, two strains of

Enterobacter cloacae, and one strain each of Enterobacter aerogenes and Citrobacter freundii.

All cultures were stored at $-70^{\circ}C$ in a solution of 10% peptone and 40% glycerol. For each experiment, cultures were taken from the frozen stocks and subcultured only once. Therefore, changes in the virulence of the enteropathogens would not be expected to occur due to repeated passage of the culture.

Preparation of Injured Cultures

Bacterial cultures were grown 24 h in 100 ml of tryptic soy broth without dextrose supplemented with 1% lactose and 0.3% yeast extract (TLY broth). All cultures were grown at $35^{\circ}C$ except the Y. enterocolitica isolates which were grown at $22^{\circ}C$. Cultures were diluted to 1×10^6 cells/ml in cold sterile reagent grade water and chlorinated with varying levels of free available chlorine (prepared daily from stock solutions of chlorine bleach; sodium hypochlorite). Cells were chlorinated for 10-30 min at $4^{\circ}C$ and pH 6.5-7.0 in the dark. In this test system, carry over of nutrients from the TLY broth (1/1000 dilution) created a chlorine demand of 1.0 ppm in 5 min and 1.7 ppm within 10 min (measured by the N,N diethyl-p-phenylenediamine colorimetric method). Therefore, in most cases injury resulted from a combination of free and combined chlorine. The system was designed to reproducibly

injure bacteria and not intended to simulate the natural environment. Thus, the chlorine levels used in this study may not be directly compared to other reports. Samples were dechlorinated using sodium thiosulfate (final concentration 0.01%) (6) which was added to unchlorinated control cultures as well.

When high densities of injured cells were needed (approximately 5 x 10^8 cells/ml), cultures were washed twice in cold sterile reagent grade water to remove the culture medium and chlorinated as described above.

The level of chlorine injury was measured by the percent difference in plate counts between a non-selective medium (TLY agar) and a selective medium (TLY agar plus 0.1% deoxycholate; TLY-D (153). TLY and TLY-D plates were incubated at $35^{\circ}C$ for all bacteria.

LD_{50} Determinations

Differences in virulence between healthy and injured Y. enterocolitica were determined using LD_{50} assays in CD-1 mice (Charles River Breeding Laboratory Inc., Wilminington, MA). Cultures were injected intraperitoneally (seven mice per dose). Mice were observed for up to 21 days. LD_{50} calculations were made according to Reed and Muench (184). Statistical analyses were made using a generalized linear interactive modeling program (GLIM) on natural logarithmic transformed data (9, 66).

Tissue Culture Attachment Assay

The procedures for measuring association between
enteropathogens and epithelial cells have been described
previously (196). Henle 407 intestinal epithelial cells
(ATCC CCL-6) were maintained in Eagles's Basal Medium with
Hanks' Balanced Salts (BME) containing 15% fetal bovine
serum, penicillin G (50 IU/ml) and streptomycin (50 ug/ml).
The epithelial cells were grown in tissue culture flasks
until the monolayer was confluent. On the day prior to
use, fresh medium without antimicrobial agents was
provided. Before use, the cells were washed twice with
Dulbecco's phosphate buffered saline without calcium (PBS)
and harvested by scraping the monolayer off the flask wall.
The cells were suspended in BME and the number of viable
cells was determined microscopically by trypan blue
exclusion. The cell density of the suspension was adjusted
to 5.6×10^3/ml by dilution with BME.

Bacteria were grown at room temperature (22°C) for 48
h (Y. enterocolitica) or at 35°C for 24 h (E. coli and S.
typhimurium) in 0.13 M phosphate buffered salts medium
(BSYE) containing 20 uCi/ml [^{35}S] methionine (196).
Bacteria were washed in cold sterile reagent grade water
and adjusted to 5×10^8/ml. Cells grown in this medium
were injured by chlorine as described above. Aliquots of
the bacterial suspension (0.2 ml) were added to 1.8 ml of

the Henle cells to give a multiplicity of $10^4:1$ bacteria to epithelial cells.

The mixture was incubated at $35^{\circ}C$ for 5 min. This short incubation time was chosen so that only the process of bacterial attachment would be examined assuming that 5 min was too brief for engulfment to occur. The contents of the tube were transferred to 100 ml of PBS and filtered through a 5 um pore size Nuclepore membrane (Nuclepore Corp., Pleasanton, CA) that had been pretreated with 50 ml of 1 mM disodium (ethylenedinitrilo)tetraacetate (EDTA) in 2% peptone (pH 8.2). After filtration under gravity, an additional 100 ml of PBS was added to rinse the membrane. Each test was performed in triplicate. To test for non-specific adhesion of bacteria to the 5.0 um Nuclepore filters, control suspensions containing bacteria alone were prepared and filtered in a similar manner.

The specific activity of the radio-labeled bacteria was determined by filtering a known amount of bacteria through a 0.4 um Nuclepore filter and determining the radioactivity. Membranes were placed in scintillation vials containing 10 ml of Aquasol (New England Nuclear, Boston, MA) and radioactivity was determined by scintillation counting in a Tri-Carb 460 Liquid Scintillation System (Packard Instrument Co. Inc, Downers Grove, IL). Counts were converted to disintegrations/min by reference to an efficiency curve based on carbon-14.

The radioactivity per bacterial cell was calculated and used to determine the average number of bacteria attached to each Henle cell.

Microscopic Evaluation of Attachment

Attachment of enteropathogens was also observed by direct microscopic observations. Henle cell monolayers grown on cover slips were infected with healthy and injured bacteria (5 x 10^8 cells/ml) for 30 min at 35°C. Infected monolayers were fixed in methanol, stained with May-Grunwald and Giemsa stains and examined using a light microscope. At least 50 Henle cells from each preparation were observed.

Tissue Culture Invasiveness

Procedures for the HeLa cell infection technique have been described elsewhere (53, 194), HeLa cells (ATCC CCL-2) were maintained in Minimal Essential Medium (Eagle) (MEM) with Earle Salts plus 10% fetal bovine serum and 50 IU of penicillin G and 50 ug of streptomycin per ml. Washed cells were recovered in antibiotic-free MEM and the density of recovered HeLa cells was determined by microscopic counting in a Fuchs-Rosenthal chamber. The concentration was adjusted to 2 x 10^5 cells/ml and one milliliter of this suspension was added to plastic 16 x 125

mm tissue culture tubes.   Each experiment was performed in triplicate.

Healthy and injured Y. enterocolitica O:8 (E661) were prepared as described above.   After chlorine injury, bacterial cells were centrifuged and resuspended in BME to a density of 2 x 10^6/ml.   One ml of the bacterial suspension was added to the tissue culture tube containing the HeLa cells giving an approximate multiplicity of 10 bacteria per epithelial cell.   The mixture was placed on a roller apparatus (Labline Instruments Inc., Melrose Park, N.J.) and incubated at 35°C for 2 h.   Resuscitation of injured cells did not occur to any appreciable extent during this time period.   After 2 h infection time, 0.1 ml of gentamicin solution (2.5 mg/ml) was added to each tube and tubes were reincubated under the same conditions. Since gentamicin does not cross the epithelial cell membrane (53, 194), it killed only the extracellular bacteria and prevented further infection.   After this period, the tube was centrifuged (5500 g, 10 min, 20°C) and the supernatant removed.   One ml of a 1.0% solution of N-lauroylsarcosine in physiological saline was added to lyse the epithelial cells. The contents of the three tubes were combined and plated out in triplicate on TLY agar.   The average colony count was used to calculate the invasiveness index (intracellular bacteria per epithelial cell).   Since it was a concern that sarcosine may have a deleterious

effect on intracellular injured Y. enterocolitica, HeLa cells were also lysed by homogenization (Virtis Company, Gardiner, New York) at 25,000 rpm and plated out as described above.

The effectiveness of gentamicin on extracellular bacteria in the presence of tissue culture cells was also examined. HeLa cells were prepared as described above and chilled to 4°C in an ice bath. Since HeLa cells are incapable of engulfing bacteria at 4°C (30), all the Y. enterocolitica remained extracellular. After 2 h, gentamicin (2.5 mg/ml) was added to each tube and the suspensions were reincubated at 35°C for 1 h. The tubes were centrifuged, the supernatant withdrawn, the epithelial cells lysed using sarcosine, and the contents plated out as described above.

Microscopic Evaluation of Invasiveness

The invasion of epithelial cells by Y. enterocolitica was also assessed by direct microscopic observations. Cover slips containing HeLa cell monolayers (ca. 1.7×10^5 cells/cover slip) were infected with healthy and injured bacteria (ca. 3.5×10^6 cells) for 2 h at 35°C. Infected monolayers were vigorously washed with PBS, fixed in methanol, stained with May-Grunwald and Giemsa stains (for 10 and 20 min, respectively) and examined using a light microscope at 1250 x magnification. The experiment was

performed in triplicate and the number of intracellular bacteria in 100 HeLa cells from each preparation was recorded.

Infected HeLa cells for transmission electron microscopy (TEM) were prepared according to the HeLa cell invasiveness procedure described above. Cells were fixed using 3% glutaraldehyde in 0.1 M potassium-sodium phosphate buffer (PSPB) and post-fixed using 2% osmium tetroxide in PSPB. Samples were dehydrated with ethanol (50%, 70%, 95%, 100%; three changes, 10 min each) and embedded in Spurr's resin. Embedded samples were thin sectioned using an ultramicrotome and stained with uranyl acetate and Reynold's lead citrate. Samples were examined using a JEOL model JEM-100 CX scanning-transmission electron microscope.

In Vivo Model of Invasiveness

The invasiveness of healthy and injured Y. enterocolitica was also evaluated using an in vivo model. Healthy and injured Y. enterocolitica (ca. 1 x 10^9 cells) were injected into intestinal loops of CD-1 mice. The animals were fasted 24 h before the experiment. Methoxyflurane (Metofane; Northwest Veterinary Supply, Great Falls, MT) was used to anesthetize the mice. Intestinal loops were made by ligating the intestine 3 cm below the pylorus, washing down the intestinal contents with 0.5 ml saline, and then adding another tie 5 cm below

the first. Each ligation was made in duplicate using cotton thread. Controls included a noninvasive strain of Y. enterocolitica O:5. After an appropriate incubation period, the mouse was sacrificed, the intestinal loops excised, fixed in buffered formalin and prepared for histological examination using the "Swiss roll" technique (159). Tissues were stained with a Giemsa stain and by direct and indirect immunofluoresence (224). Antiserum used in the indirect procedure to serotype O:8 was provided by D. A. Schiemann. Fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG was purchased from Sigma Chemical Co. (St. Louis, Mo). FITC conjugated antiserum to serotype O:5 was provided by L. Mann. The number of intracellular bacteria in 100 villi of each preparation was recorded.

Assimilation of Radio-labeled Compounds

Y. enterocolitica was grown and injured as described above. Cells were pelleted by centrifugation and resuspended in 4 ml of Scheusner's minerals salts medium (per liter: 7.0 g K_2HPO_4, 3.0 g KH_2PO_4, 0.1 g sodium citrate, 1.0 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, and 10.0 g glucose). One ml of the cell suspension was added to 1 ml of mineral salts medium containing 100 uCi per ml of either [35S]methionine, [3H]uridine or [3H]thymidine. Samples were incubated at room temperature (22°C) for 2 h. Samples (0.2 ml) were withdrawn and filtered through a 0.4 um

Nuclepore filter. Filters were washed with 100 ml of PBS
and placed in a scintillation vial containing 1 ml lysis
buffer (50 mM Trizma, pH 12; 4% SDS, 10 mM EDTA). After at
least 10 min incubation in the lysis buffer, 10 ml of
Aquasol was added and the vials were counted using a Tri-
Carb 460 Liquid Scintillation System. The assimilation rate
of the compounds were calculated as cpm/5 x 10^6
bacteria/min.

ATP and Oxygen Measurements

Adenosine triphosphate (ATP) levels in healthy and
injured bacteria were determined by the luciferin-
luciferase method using a Turner Designs model 20e
Luminometer and Turner Designs reagents (Mountain View,
CA). Healthy and injured cells were suspended in
Scheusners mineral salts medium. The amount of ATP per
cell was calculated from triplicate experiments according
to the manufacturer's instructions.

Oxygen uptake measurements were performed using a
Gilson model KM oxygraph equipped with a YSI model 4004
Clark-type electrode. The chamber of the instrument was
maintained at $32^{\circ}C$. Tryptic Soy Broth containing 1.0%
glucose (1.8 ml) was added to the chamber and allowed to
equilibrate for 2 min, followed by 0.2 ml of a 2 x 10^9 cell
suspension. The oxygen uptake rates were determined

graphically from the slope of the lines from triplicate experiments (54).

Effect of Metabolic Inhibitors on Invasiveness

The invasiveness of Y. enterocolitica treated with various metabolic inhibitors was examined using the HeLa cell roller tube technique described above. The inhibitors (chloramphenicol, tetracycline, spectinomycin, nalidixic acid, nitrofuratoin or rifampicin; for concentrations used see Table 5) were added to the bacterial suspension just before the bacteria were added to the HeLa cells. No significant loss of bacterial or epithelial cell viability occurred, due to the inhibitors, during the 2 h incubation period. Changes in the invasiveness of Y. enterocolitica treated with the inhibitors was compared to an untreated control.

Measurement of Hydrophobicity and Electric Charge

Changes in surface hydrophobicity and electric charge of chlorine-treated Y. enterocolitica were determined by the methods used by Schiemann and Swanz (196). Cultures of Y. enterocolitica were grown in BSYE broth 48 h at 22°C. Bacteria were centrifuged and resuspended in 4 M NaCl in 10 mM phosphate buffer (pH 7.2) at concentrations about 10^9 cells/ml. Salmonella and ETEC isolates were grown on CFA agar (per liter: 1.5 g yeast extract, 0.05 g MgSO$_4$, 0.005 g

$MnCl_2$, 10.0 g casamino acids and 20.0 g agar; pH 7.0) at

$35^{\circ}C$ for 24 h. Bacteria were washed off the plate using 4

M NaCl and adjusted to a concentration of about 10^6

cells/ml. Sepharose CL-4B and phenyl-Sepharose CL-4B

(Pharmacia, Inc. Piscataway, NJ) were washed five times

with equal volumes of 4 M NaCl and added to Pasteur

pipettes plugged with glass wool to achieve a column of 25-

30 mm after settling. The bacterial suspension (0.1 ml)

was layered on top of the column and 5 ml of 4 M NaCl was

added for elution. The number of bacteria added to the

column and the number of bacteria recovered in the eluate

were determined by plate counts on TLY agar and the percent

adherent to the column was calculated. Since it was a

concern that the high salt buffer might stress the

bacteria, the same experiments were repeated using 10 mM

Tris (Trizma) buffer.

Hydrophobicity of healthy, injured and chlorine-killed

cells was also determined by partitioning in an aqueous-

hydrocarbon mixture (196). Cultures were grown as

described above, but were washed twice with 0.85 % NaCl.

The density of each cell suspension was adjusted

photometrically to an absorbance (400 nm; path length 1 cm)

of 1.2 to 1.4 (DMS 80 Spectrophotometer, Varian Associates,

Mulgrave, Australia). Four ml of the bacterial suspension

were added to an acid-washed glass test tube and 0.5 ml of

the hydrocarbon (hexadecane) was added. The tube contents

were mixed on a vortex mixer for 1 min and then allowed to stand for 15 min. The decrease in the absorbance of the aqueous phase at 400 nm was recorded.

Attachment of the bacteria to DEAE-Sepharose was used as a measure of surface charge. Protocols for these experiments were similar to those for the phenyl-Sepharose chromatography except DEAE-Sepharose CL-4B (Pharmacia, Inc.) and 10 mM Tris buffer were used.

Outer Membrane Studies

Isolation of cell membranes was similar to the procedure of Achtman et al. (1). Three 3-liter batches of Y. enterocolitica were grown for 48 h at $22^{O}C$ in TLY broth and concentrated by tangental flow filtration (Pellicon Cassette System, Millipore Corp, Bedford, MA) to approximately 50 ml. The cells were washed twice by centrifugation in 0.85 % NaCl. The first batch was set aside as an untreated control. The second batch was treated with 1 mg chlorine per ml for 30 min at $22^{O}C$, while the last batch was treated with 32 ug tetracycline and 32 ug rifampicin for 2 h at $22^{O}C$. The cells were then centrifuged and resuspended in 25 ml of 20 mM Trizma (pH 7.2), 1 mM EDTA, 0.5 mM $MgCl_2$, 1 ug/ml DNase-I, 1 ug/ml RNase-A (bovine pancreas) and 0.02 mM phenyl methyl sulfonyl fluoride (a protease inhibitor). The cells were passed three times through a Sorvall model RM cell

fractionator and centrifuged at 3,000 x g for 10 min to remove whole cells. The membrane fractions were collected by centrifugation at 50,000 x g for 1 h and the pellet resuspended in 20 ml sterile reagent grade water. Fractions were examined under 1250 x phase contrast microscopy to confirm presence of the membranes and absence of whole cells. Membranes were stored at $-20^{\circ}C$ until use.

The effect of the membrane preparations on the ability of Y. enterocolitica to attach to epithelial cells was examined using the Henle cell attachment assay. Approximately 0.05 ml of each membrane preparation (75 ug/ml dry weight of membrane material, final concentration) was added to a 2 ml suspension of Henle cells (1×10^5 cells/ml) and bacteria (5×10^8 cells/ml). Differences in the levels of bacterial attachment in samples treated with various membrane preparations were compared to an unamended control.

Changes in the biological activity of outer membranes of Y. enterocolitica after chlorination were measured by the ability of the membranes to block invasiveness of Y. enterocolitica in the tissue culture system. Approximately 0.05 ml of each membrane preparation was added to a 2 ml suspension of HeLa cells and bacteria (final concentration: 75 ug/ml dry weight of membrane material). The effect of the membrane preparation on the viability of HeLa cells was

examined microscopically by trypan blue exclusion after the
2 h incubation time.

Electrophoresis of Outer Membrane Proteins

Equal amounts (4.5 mg dry weight) of the three
membrane preparations were sedimented for 30 min in a
microcentrifuge, and the supernatant fluid discarded. The
sediment was suspended in 1.8 ml of Triton X-100 buffer (10
mM Trizma (pH 8.0), 5 mM $MgCl_2$, and 2% Triton X-100) and
allowed to incubate 20 min at room temperature. The outer
membranes were washed two additional times in Triton X-100.
buffer with the last time being resuspended in 500 ul
electrophoresis buffer (127, 128). Samples (50 ul) were
subjected to SDS polyacrylamide gel electrophoresis in a
10% gel (a 5% stacking gel) using a modified Laemmli
discontinuous buffer system (127, 128). The following
purified proteins were used as molecular weight standards:
thyroglobulin (200,000), B-galactosidase (107,000),
phosphorylase B (97,400), bovine serum albumin (66,000),
ovalbumin (45,0000) and carbonic anhydrase (29,000). Gels
were stained with Coomassie brilliant blue and analyzed
using a Gilford Multi Media Densitometer interfaced to a
Gilford System 2600 for data manipulation and storage.
Tracings were drawn using a Hewlett Packard model 7225B
plotter.

Statistical Analysis

Statistical analyses were performed using the MSUSTAT interactive statistical package developed by R. E. Lund, Montana State University. The GLIM program was run with the help of Dr. M. A. Hamilton, Department of Mathematical Sciences, Montana State University.

Materials and Reagents

Tryptic soy broth without dextrose, casamino acids, and yeast extract were obtained from Difco Laboratories, Detroit, MI. Eagle's Basal Medium with Hanks' Balanced Salts was obtained from Irvine Scientific, Santa Ana, CA. Minimal Essential Medium with Earle Salts and Dulbecco's phosphate buffer saline were obtained through Gibco Laboratories, Grand Island, N. Y. [^{35}S]methionine (specific activity, 400 Ci/mM) was obtained from New England Nuclear, Boston, MA. [methyl-^{3}H]thymidine (specific activity, 63 Ci/mM) and [5,6-^{3}H]uridine (specific activity 39 Ci/mM) were purchased from ICN Radiochemicals, Irvine, CA. Fetal bovine serum, chloramphenicol, tetracycline, spectinomycin, nalidixic acid, nitrofuratoin, rifampicin, gentamicin, penicillin G, streptomycin, n-hexadecane, DNase, RNase, phenyl methyl sulfonyl fluoride, Trizma and sarcosine were obtained from Sigma Chemical Co., St. Louis, MO.

RESULTS

Chlorine Injury

Coliforms serve as indicators of potable water quality by indicating the possible presence of enteric pathogens. It has been suggested that conditions which stress coliforms would also injure waterborne pathogens. Because there are no data to support this hypothesis, I investigated the relative susceptibilities of coliforms and pathogens to injury by chlorine. Data presented in Figure 1 show that 10 strains of coliform bacteria became injured (approximately 90% injury) when exposed to 0.25 to 0.50 mg/l chlorine. Decreases in viability for coliforms in this range of chlorine concentrations were usually less than 0.5 log. At higher chlorine concentrations (>0.75 mg/l) the bacteria rapidly died. Figure 2 shows the susceptibility of three strains of ETEC to injury by chlorine. For comparison, the shaded area (in this figure and the three following) indicates the range of chlorine concentrations causing injury to the coliform bacteria. Figure 2 demonstrates that ETEC show the same response to chlorine as the coliform group. The sensitivity of seven strains of S. typhimurium to chlorine-injury is shown in Figure 3. Higher chlorine doses were required to injure