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Osmolarity and Growth Phase Overlap in Regulation of *Salmonella typhi* Adherence to and Invasion of Human Intestinal Cells

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The study of the effects of osmolarity and growth phase on *Salmonella typhi* adherence to and invasion of Henle 407 epithelial cells provides the first evidence of a clear overlap between these two environmental stimuli. High-osmolarity conditions are required in the late-log phase for optimum induction of the adherent and invasive phenotypes.

Typhoid fever is a communicable human disease caused by *Salmonella typhi*, which remains a major public health problem in the world (8). In the host, ingestion of *S. typhi*-contaminated food and/or water is followed by the passage of organisms across the epithelial cells of the ileal mucosa and subsequent multiplication and dissemination throughout the host tissues. A variety of experimental tissue culture models have been developed to study steps in invasion and to identify both the genes involved in the invasion process and the factors which regulate those genes (9, 15, 17, 20, 24, 30). The elegant electron micrographs obtained by Yokoyama et al. (31) were the first to show the events involved in internalization of *S. typhi* by HeLa cells. However, little is currently known about the virulence mechanisms involved in the adherence to and invasion of such eukaryotic cells by *S. typhi*, and only two studies to date have addressed the genetic regulation of cell entry by *S. typhi*. Elsinghorst et al. (9) cloned a chromosomal region of *S. typhi* that conferred the ability of a noninvasive *Escherichia coli* strain, HB101, to penetrate Henle 407 cells. However, the same chromosomal region from *Salmonella typhimurium* did not confer the invasive phenotype when cloned into *E. coli*. In addition, Galán and Curtiss (12) characterized a group of genes from *S. typhimurium* that conferred invasive properties on a noninvasive *S. typhimurium* strain. Subsequent studies by those authors demonstrated that functional *inv*-homologous sequences are also present in *S. typhi* (14). Taken together, those studies suggest the existence of at least two distinct chromosomal regions in *S. typhi* which are involved in the invasion of epithelial cells.

Recent studies have suggested that in addition to the presence of a specific gene(s) that regulates entry of bacteria into eukaryotic cells, environmental factors may also play a role in regulating bacterial cell entry (for reviews, see references 5 and 22). Enteric pathogens experience severe environmental changes when they enter their host by the oral route, e.g., low pH, increased temperature, low O_2 tension, high osmolarity, and nutrient deprivation. Studies have shown (i) that bacteria respond to these environmental changes by modulating the expression of different pools of genes and (ii) that many of these genes are coordinately regulated (for reviews, see references 16 and 23). Although several laboratories have demonstrated that osmolarity and growth phase independently affect the virulence of different pathogens (1, 2, 10, 13, 21, 25), in the present report, we provide the first evidence that osmolarity and growth phase together regulate the adherence to, and invasion of, intestinal epithelial cells by *S. typhi*.

S. typhi Ty2 was a gift of E. A. Elsinghorst (Walter Reed Army Institute of Research, Washington, D.C.). *E. coli* HB101 and *Shigella flexneri* 2a 2457T (20) were kindly provided by A. T. Maurelli (Uniformed Services University of the Health Sciences, Bethesda, Md.). In all experiments, the bacteria were grown at 37°C with agitation (200 rpm) in glass tubes (18 by 150 mm) containing 10 ml of Luria-Bertani (LB) medium (28), and the concentration of NaCl was varied to obtain different osmolarities. The overnight cultures were subcultured into 10 ml of fresh medium or grown in 500-ml Erlenmeyer flasks containing 100 ml of medium for the growth phase experiments. Three different osmolarity media which contained different molar concentrations of NaCl were routinely used: LB 0.06 M NaCl (3.5 g of NaCl per liter), LB 0.17 M NaCl (10 g of NaCl per liter), and LB 0.3 M NaCl (17.5 g of NaCl per liter). In other experiments, sucrose was added to increase the osmolarity of the media. Four-tenths molar sucrose was added to LB 0.06 M NaCl to reach the osmolarity of LB 0.3 M NaCl. Osmolarity was measured with a pressure vapor osmometer (Wescor, Inc.) with NaCl solutions of known osmolarity as standards, and the values are given in milliosmoles per kilogram of water. The final pH of the culture media was adjusted to 7.0 in all cases. The growth phase of the bacterial cultures was monitored over time by measuring the absorbance A_{600} in a Pharmacia LKB Ultrospec III spectophotometer and by measuring CFU on LB agar.

Two human intestinal cell lines were used in this study, Henle 407 (ATCC CCL 6) and Caco-2 (27), which was kindly provided by A. D. O'Brien (Uniformed Services University of the Health Sciences). Cell lines were grown and maintained in minimal essential medium (MEM) (GIBCO, Life Technologies, Inc., Grand Island, N.Y.) to which 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah) and 2 mM glutamine were added. The epithelial cells were incubated at 37°C in a 6% CO_2 atmosphere. Where indicated, 100 μg of gentamicin (Quality Biological, Inc., Gaithersburg, Md.) per ml was added to

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TABLE 1. Effect of osmolarity on the capacity of *S. typhi* to adhere to and penetrate Henle 407 and Caco-2 cells[a]

Cell line	Medium	No. of bacteria added per well	No. of cell-associated bacteria	% Adherence[b]	No. of bacteria surviving gentamicin addition	% Invasion[c]
Henle 407	LB0.06M	$(7.4 \pm 0.9) \times 10^6$	$(4.7 \pm 0.8) \times 10^5$	6.4	$(1.9 \pm 0.7) \times 10^6$	26.3
	LB0.17M	$(8.4 \pm 1.0) \times 10^6$	$(1.7 \pm 0.5) \times 10^6$	20.7	$(5.9 \pm 1.6) \times 10^6$	71.0
	LB0.30M	$(4.5 \pm 1.5) \times 10^6$	$(1.4 \pm 0.1) \times 10^6$	30.4	$(6.8 \pm 1.9) \times 10^6$	149.1
Caco-2	LB0.06M	$(8.9 \pm 1.3) \times 10^6$	$(6.3 \pm 1.3) \times 10^5$	7.1	$(2.0 \pm 0.4) \times 10^6$	22.4
	LB0.17M	$(6.6 \pm 1.5) \times 10^6$	$(1.6 \pm 0.5) \times 10^6$	23.8	$(4.4 \pm 1.1) \times 10^6$	67.7
	LB0.30M	$(3.9 \pm 1.1) \times 10^6$	$(1.5 \pm 0.7) \times 10^6$	39.5	$(5.7 \pm 0.9) \times 10^6$	145.4

[a] The percents adherence of *E. coli* HB101 when grown under the three different osmolarity conditions were the same: 0.73% with LB 0.06 M NaCl, 0.58% with LB 0.17 M NaCl, and 0.45% with LB 0.3 M NaCl. Values shown here represent the averages of duplicate samples of three separate assays.
[b] Percentage of bacteria associated to the monolayer after 1 h of incubation with respect to the initial inoculum.
[c] Percentage of bacteria surviving treatment with gentamicin, with respect to the initial inoculum.

MEM. Monolayers for adherence and invasion assays were prepared by seeding 2.5×10^5 cells into each well of 24-well tissue culture plates (Costar Corp., Cambridge, Mass.) and incubating them overnight at 37°C in 6% CO_2. Duplicate wells were prepared for each sample to be tested.

Adherence and invasion assays were a modification of the procedure developed by Elsinghorst et al. (9). Briefly, bacteria were grown overnight at 37°C in a shaking water bath and subcultured by diluting 1:100 in fresh LB medium. Bacteria were grown to an A_{600} of 0.5 unless otherwise indicated. In the growth phase experiments, bacterial samples were concentrated or diluted in LB medium to an A_{600} of 0.5 to obtain the desired number of bacteria. Twenty-five microliters of bacterial suspension per well was added to the intestinal cell monolayer, representing an initial inoculum of 4×10^6 to 9×10^6 CFU per well. Bacteria were allowed to adhere for 60 min at 37°C in a CO_2 incubator, and each well was rinsed six times with Earle's basal salt solution (GIBCO). Monolayer-associated bacteria were released by incubating the infected monolayer with 0.2 ml of a 1% solution of Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) in saline for 10 min, accompanied by vigorous agitation. Saline solution (0.8 ml) was then added to each well and the bacteria were quantified by plating the appropriate dilutions on LB agar. The percent adherence was calculated as follows: (number of bacteria associated with the monolayer/total number of bacteria added) × 100 = percent adherence. For the invasion assays, bacteria were added to the cell monolayers as described above, allowed to adhere for 2 h at 37°C in 6% CO_2, and then washed three times with Earle's balanced salt solution. One milliliter of prewarmed MEM containing 100 μg of gentamicin per ml was added per well and incubated for an additional 2 h to kill extracellular bacteria. Subsequently, the wells were washed three times with Earle's balanced salt solution and internalized bacteria were released by treatment with 0.2 ml of a 1% solution of Triton X-100 in saline and vigorously mixed for 10 min. Saline was added at 0.8 ml per well, and the suspension was diluted in phosphate-buffered saline and plated on LB agar to determine viable counts. The percent invasion was calculated as follows: (number of bacteria surviving treatment with gentamicin/total number of bacteria added) × 100 = percent invasion.

Previous studies with other enteric pathogens suggested that osmolarity is one environmental factor which regulates the interaction of the organisms with eukaryotic cells (1, 2, 13, 25). To determine whether invasiveness of *S. typhi* was regulated by osmolarity, bacteria were grown in LB medium containing increasing concentrations of NaCl or sucrose and the capacity of the organisms to penetrate Henle 407 cells was determined. The results show that the percentage of *S. typhi* able to penetrate Henle 407 cells increased as the osmolarity of the growth medium was increased (Table 1). *S. typhi* was significantly more invasive when grown in LB 0.3 M NaCl (high-osmolarity medium) than when grown in LB 0.06 M NaCl (low-osmolarity medium). To ensure that these results were not restricted to Henle 407 cells, the effect of osmolarity on the invasion of a second intestinal cell line, Caco-2, by *S. typhi* was also evaluated. Table 1 shows that the percent invasion of Caco-2 cells by *S. typhi* was similar to that obtained with Henle 407 monolayers.

Several factors could contribute to the osmoregulated enhancement of invasion, one of which is an increase in adherence. If a higher number of bacteria adhered to the monolayer under high-osmolarity conditions, a higher number of bacteria could potentially penetrate the intestinal cells. To test this hypothesis, the capacity of *S. typhi* to adhere to Henle 407 and Caco-2 cells was analyzed (Table 1). The results demonstrated that the percentage of bacteria associated with the monolayers after 1 h of incubation increased with increments in the osmolarity of the bacterial medium with both cell lines. This increase was approximately fivefold higher with bacteria grown in high-osmolarity medium than with bacteria grown in low-osmolarity medium. The difference between the values of adherence and invasion in Table 1 corresponds to the length of the incubation time of the bacteria with the intestinal cells in the two assays, i.e., 1 h for adherence and 2 h for invasion. Moreover, the bacteria multiply in the tissue culture medium during the incubation period with the monolayer. Taken together, these variables account for the finding that the percent invasion was higher than 100% in some cases. To confirm that osmolarity was the reason for the increase, sucrose was added to the bacterial medium in order to increase the osmolarity to the value of the high-osmolarity medium LB 0.3 M NaCl (676 mosmol). The results indicate that increases in adherence and invasion with sucrose paralleled those observed with NaCl (data not shown). Thus, the increases in *S. typhi* adherence to and invasion of intestinal epithelial cells appear to be osmoregulated.

To determine whether the osmoregulated effect of adherence was a consequence of an increased nonspecific bacterial stickiness, the adherence of noninvasive *E. coli* HB101 to Henle 407 cells, after growth in high- or low-osmolarity medium, was compared with that of *S. typhi*. No differences in adherence to the monolayer were observed between *E. coli* organisms grown in high- and low-osmolarity LB media (Table 1). To demonstrate that increased adherence, as a

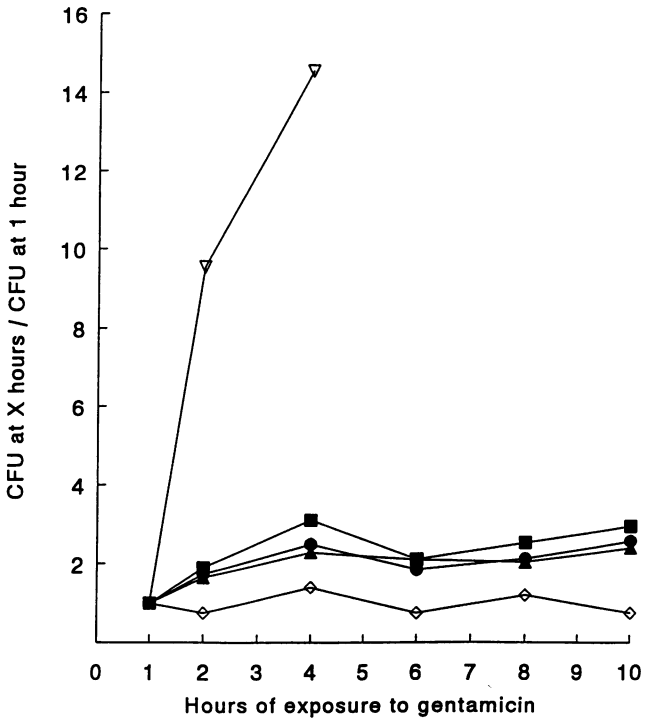

FIG. 1. Intracellular multiplication of *S. typhi* Ty2 in Henle 407 cells. Each point represents the mean of duplicate samples in two separate assays. Symbols: ▽, *S. flexneri* 2457T; ◇, *E. coli* HB101; ●, *S. typhi* Ty2 grown in LB 0.06 M NaCl; ■, *S. typhi* Ty2 grown in LB 0.17 M NaCl; ▲, *S. typhi* Ty2 grown in LB 0.3 M NaCl.

consequence of osmolarity, was the primary reason for the observed increase in invasion, a series of experiments was conducted to eliminate the role of other factors. Elsinghorst et al. (9) had previously shown that *S. typhi* did not significantly multiply within Henle 407 cells. To determine whether the increase in invasion observed in this study was due to an osmoregulated enhancement in intracellular multiplication, the number of intracellular *S. typhi* organisms at various times after the addition of gentamicin was determined. *S. typhi* organisms grown in LB media with increasing concentrations of NaCl were added to Henle 407 monolayers. *S. typhi* organisms were incubated with the monolayers for 2 h, after which gentamicin was added to kill extracellular bacteria per the standard protocol; CFU were determined at 1, 2, 4, 6, 8, and 10 h thereafter. The CFU per milliliter recovered at each time point was compared to the recovery at 1 h (9). An increase in the number of bacteria with time would reflect intracellular multiplication. The results of this experiment are shown in Fig. 1. *S. flexneri* was used as a positive control because it is known to multiply within Henle 407 cells (17). The multiplication kinetics of *S. typhi* grown under different osmolarities were similar and are consistent with those reported by Elsinghorst et al. (9). Thus, these data suggest that osmoregulated intracellular multiplication was not the reason for the observed increase in invasion. Changes in the sensitivity of *S. typhi* to gentamicin were also investigated. Bacteria grown in LB 0.06 M NaCl, LB 0.17 M NaCl, and LB 0.3 M NaCl were incubated in the presence of gentamicin at 37°C in 6% CO_2 for 2 h, the same incubation time used in our standard invasion assay, and the number of viable bacteria was determined. The results indicate that growth of *S. typhi* in high-osmolarity medium did not alter the sensitivity of the organism to gentamicin (data not shown). Thus, the osmoregulated increase in the number of bacteria able to penetrate the monolayer was not due to a higher resistance of *S. typhi* to gentamicin treatment after growth in LB 0.3 M NaCl.

A third factor considered to explain the apparent osmoregulated increase in invasion was the multiplication of bacteria in tissue culture medium during the 2 h of incubation prior to the addition of gentamicin. Since the tissue culture medium (MEM) offers a rich environment for bacterial growth, the possibility that bacteria which adapted to grow in high-osmolarity medium would multiply faster in this rich tissue culture medium was explored. *S. typhi* was grown overnight in LB 0.06 M NaCl, LB 0.17 M NaCl, and LB 0.3 M NaCl and subcultured (1:100) into fresh media of the same osmolarities. These subcultures were incubated at 37°C in a shaker water bath until an A_{600} of 0.5 was reached. Twenty-five microliters of the bacterial suspension was inoculated into 1 ml of MEM and incubated for 2 h at 37°C in 6% CO_2, after which samples were taken and the appropriate dilutions were plated on LB agar. No significant difference was observed in the multiplication rates of the bacteria grown in high- and low-osmolarity media (data not shown). Taken together, the results of these experiments indicate that the osmoregulated increase in invasion observed in our experiments appears to be a consequence of the increase in the number of bacteria able to specifically adhere to the intestinal cell monolayer.

Previous studies have suggested that *Salmonella* species and other pathogens would most likely be found in the stationary growth phase when they are in the environment (19, 21). If so, it could be surmised that *Salmonella* species and other organisms are likely to be in the stationary phase when they enter the host through contaminated food and water. Thus, we considered the possibility that the growth phase of *S. typhi* may influence the capacity of the organism to interact with eukaryotic cells. This possibility is supported by findings which show that the growth phases of *S. typhimurium* and *Salmonella choleraesuis* influence the ability of the organisms to penetrate cultured mammalian cells (10, 21). To determine whether the growth phase affected the capability of *S. typhi* to adhere to epithelial cells, *S. typhi* was grown overnight at 37°C in a shaker water bath in LB media containing three different concentrations of NaCl. The cultures were subcultured (1:100) into fresh media and the growth phase was monitored as described in Materials and Methods. Because the adherence and invasion assays were standardized with cultures with an A_{600} of 0.5, and, in order to inoculate the same quantity of bacteria to the monolayers from the different time points, we concentrated or diluted the samples in different states of growth to an A_{600} of 0.5. Twenty-five microliters of each culture was added to Henle 407 monolayers, and the percent adherence was quantitated as described in Materials and Methods. The results are presented in Fig. 2 and show that the growth phase dramatically affects the adherence of *S. typhi* to Henle 407 cells but only under certain osmolarity conditions (Fig. 2). Thus, the growth phase did not affect the adherence of *S. typhi* organisms to Henle 407 cells when bacteria were grown in low-osmolarity medium, LB 0.06 M NaCl. However, when the bacteria were grown in LB 0.17 M NaCl, the adherence increased during the logarithmic phase of growth, reaching an optimal value at the late-log phase. This increase in adherence was even more dramatic when the bacteria were grown in high-osmolarity LB 0.3 M NaCl. Interestingly, *S.*

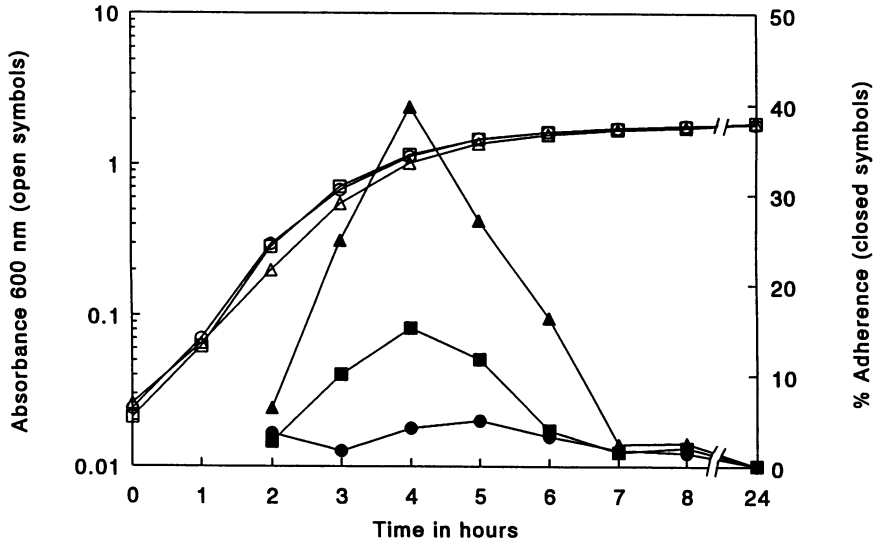

FIG. 2. Effects of osmolarity and growth phase on the invasion of Henle 407 cells by *S. typhi*. Samples were taken at different time points during growth, and the optical density at A_{600} and bacterial adherence were measured. The data presented are from a single representative experiment which was repeated four times. Symbols: ○ and ●, *S. typhi* Ty2 grown in LB 0.06 NaCl; □ and ■, *S. typhi* Ty2 grown in LB 0.17 M NaCl; △ and ▲, *S. typhi* Ty2 grown in LB 0.3 M NaCl.

typhi lost its ability to adhere to eukaryotic cells during the stationary phase. Invasion kinetics of *S. typhi* paralleled adherence kinetics (data not shown).

Our findings that *S. typhi* became less invasive as the cultures entered the stationary phase of growth are consistent with those reported by other investigators (10, 11, 21). In a study by Lee and Falkow (21), it was reported that *S. choleraesuis* cultures in the stationary phase appeared to adhere to MDCK cells, but the organisms invaded less. In contrast, our results showed that the decrease in invasion observed in stationary cultures of *S. typhi* was due to a parallel decrease in the number of bacteria able to adhere to the epithelial monolayer (Fig. 2). The differences between our results and those of Lee and Falkow (21) could correspond to differences in the control of the growth phase on the synthesis of adherence determinants in different *Salmonella* species or to differences in the interaction of bacteria with cell lines of different origins. In summary, our findings show a clear overlap between growth phase and osmolarity in the regulation of *S. typhi* adherence to and invasion of intestinal cells. Thus, a high-osmolarity medium is required during the late-log phase for optimum induction of the invasive phenotype.

Other investigators have shown that two-component regulatory systems might be involved in osmoregulation of virulence of different pathogens (1, 3, 4). Moreover, DNA supercoiling changes in response to growth conditions such as osmolarity, growth phase, and anaerobiosis have been reported (6, 7, 13, 18, 26). In addition, an overlap between osmolarity and anaerobiosis has been shown to affect the expression of the *proU*, *ompC*, and *tppB* genes in *E. coli* (26). Galán and Curtiss have shown that osmoinducible changes in DNA supercoiling regulated the expression of the *invA* invasion gene, which permits *S. typhimurium* to penetrate tissue culture cells (13). Anaerobiosis has also been shown to affect the ability of different *Salmonella* species to invade eukaryotic cells (10, 11, 21, 29). These studies showed that bacteria grown under low oxygen tension are more invasive than aerobically grown cultures. Although it is intriguing to speculate on the effects of the two-component regulatory systems and DNA supercoiling on the regulation of *S. typhi* adherence and invasion by osmolarity and growth phase, the studies have yet to be completed.

When *S. typhi* enters its host, the organism faces a very special environment. Under these conditions, the bacterium is exposed to a variety of stimuli that will most likely affect the expression of genes required for its survival and initiate the development of pathogenic events. In the intestinal lumen, osmolarity may be one of the signals which allow the bacterium to adhere more efficiently to certain mucosal surfaces. However, the osmolarity values of the small intestine, in which *S. typhi* enters its human host, are not well documented. We were interested in determining the optimal osmolarity values for *S. typhi* adherence to and invasion of intestinal cells as well as the highest NaCl concentrations that would still allow synthesis of the factors required by the organism to attach to and penetrate eukaryotic cells. To address these issues, LB media with increasing concentrations of NaCl were prepared to give osmolarities from 283 to 1,467 mosmol/kg of water. *S. typhi* Ty2 was grown in media of different osmolarities overnight at 37°C and subcultured into fresh media of the same osmolarities until an A_{600} of 0.5 was reached. Cultures were inoculated onto the Henle 407 monolayers, per the standard adherence assay, and adherence was quantified. As shown in Fig. 3, the results indicate that the adherence of *S. typhi* to Henle 407 cells increased with the osmolarity of the medium until a maximal value at 676 mosmol, which was equivalent to that of LB 0.3 M NaCl, was reached, after which the adherence suddenly dropped. The growth kinetics for the bacteria in the different media were similar (data not shown). Only bacteria in the highest-osmolarity medium, LB 0.6 M NaCl and LB 0.7 M NaCl, showed slightly slower overall growth, but, as mentioned above, all samples were taken in the same phase of growth. These results suggest that the synthesis of factors involved in the adherent phenotype may decrease significantly at high

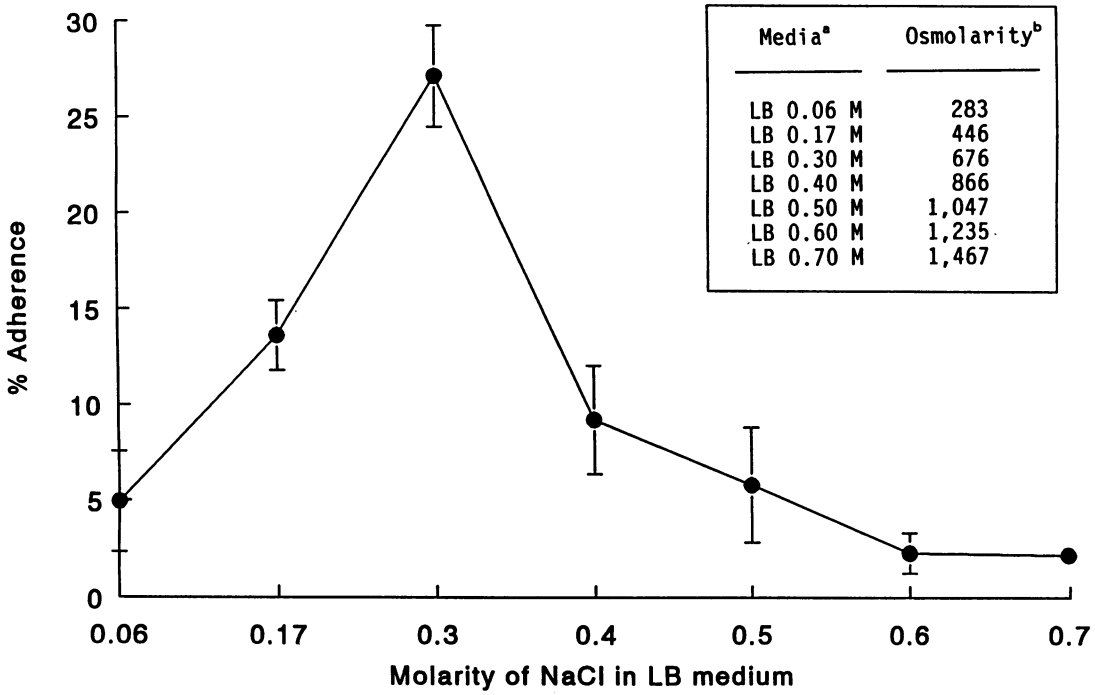

FIG. 3. Effect of increasing osmolarity on the adherence of *S. typhi* to Henle 407 cells. Bacterial cells were grown in media with increasing concentrations of NaCl until an A_{600} of 0.5 was reached, and adherence to Henle 407 cells was measured. Values represent the means of duplicate samples in three independent experiments ± the standard deviations. *a*, molarity of NaCl in LB medium; *b*, osmolarity in milliosmoles per kilogram of water.

concentrations of NaCl or that changes in the outer envelopes of the bacteria impede the transport of products required for adherence which are encoded by these osmoinducible genes.

In summary, our findings that adherence occurs optimally in the late-log portion of the growth phase suggest that *S. typhi* acquired from contaminated food and/or water may undergo several rounds of division while in the intestine before it is physiologically adapted for optimal adherence and invasion. Additionally, the maximal *S. typhi* expression of the factors required for adherence and invasion at 676 mosmol suggests that the osmolarity encountered in the distal ileum, in which *S. typhi* initiates its pathogenic effect, is high. Finally, the observation that growth phase and osmolarity together enhance the expression of virulence determinants involved in the adherence and uptake of *S. typhi* by intestinal cells will increase our capacity to identify and characterize the gene(s) and gene product(s) involved in the initial stages of *S. typhi* virulence. The characterization of this product(s) with the Tn*phoA* mutation is currently underway in our laboratory.

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