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Association with MDCK Epithelial Cells by *Salmonella typhimurium* Is Reduced during Utilization of Carbohydrates

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Association of *Salmonella typhimurium* with MDCK epithelial cells in monolayers, represented primarily by intracellular bacteria after 30 min of contact, with centrifugation followed by vigorous washing, was measured during aerobic and anaerobic growth of the bacteria in brain heart infusion broth. Cell association was greatest during a short period in the late log phase of growth under aerobic conditions. At this time, the pH of the growth medium was changing from acid to alkaline and glucose (0.2% initially) was exhausted. Addition of excess glucose (0.5%) to brain heart infusion broth, which was not exhausted before the bacteria entered the stationary phase of growth, in which cell association dropped sharply, resulted in repression of cell association by the bacteria. The repressive effect of glucose on cell association could not be reversed by exogenous cyclic AMP in the bacterial growth medium. Under anaerobic conditions, the effect of glucose on cell association by the bacteria was not as great and the glucose was not exhausted before the bacteria entered the stationary phase. When *S. typhimurium* was grown in a rich but carbohydrate-free medium, cell association by the bacteria increased earlier in the growth cycle under both aerobic and anaerobic conditions. The addition of glucose and certain other utilizable carbohydrates to this medium caused a repression of cell association by *S. typhimurium* that was greater under aerobic growth conditions. These results show that cell association by *S. typhimurium*, which is accompanied by rapid internalization (cell invasion), is the same under aerobic and anaerobic conditions if the bacteria are grown to the log phase in a carbohydrate-free medium. This suggests that prior reports of greater cell invasion by *S. typhimurium* during anaerobic growth may have arisen from the use of media containing carbohydrates which were found to be more repressive during aerobic growth of the bacteria.

Salmonella typhimurium, like other invasive enteropathogenic bacteria, initiates infection of a host after oral entry by passing through epithelial cells of the intestine as a mechanism for traversing the mucosal barrier. Animal cells in cultures have become popular models for studying attachment (adhesion) to and penetration (invasion) into and through epithelial cells by *S. typhimurium* (7–9, 14, 15, 21, 23, 26, 33, 34). Studies completed with adhesion-invasion-deficient mutants of *S. typhimurium* have indicated that the genetics controlling these processes are complex and involve multiple chromosomal loci (1, 3, 4, 12, 16, 17). Several membrane structures and properties have been examined as potential mediators of cell adhesion-invasion by *S. typhimurium* (2, 6, 18, 20, 22, 24, 25, 27, 39), but none has been singly or definitively identified with what appears to be a complex process having multiple pathways. The close chromosomal association of many of the invasion genes has suggested that the mediating structure on the cell surface of salmonellae may be an "invasion complex" (31) rather than a single protein as found in *Yersinia enterocolitica* (19, 36, 40).

Expression of cell adhesion-invasion by *S. typhimurium* is influenced by various environmental stimuli such as oxygen (11), osmolarity (13, 38), and growth phase (6, 30, 38). Kusters et al. (29) recently challenged previous reports that stationary-phase bacteria were less adherent and invasive than logarithmic-phase bacteria, suggesting that these differences may be due to variations in methods for growing stationary-phase cultures or to the greater proportion of dead bacteria in these cultures. Several studies have found that *S. typhimurium* bacteria in the log phase of growth are more invasive when grown anaerobically than aerobically (6, 30, 37). The study reported herein found that association of *S. typhimurium* with Madin-Darby canine kidney (MDCK) cells, represented primarily as intracellular bacteria, was reduced during utilization of carbohydrates and that the repression of cell association by certain carbohydrates was greater during aerobic growth of the bacteria.

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MATERIALS AND METHODS

Bacteria. This study was completed with *S. typhimurium* SL3201 biotype FIRN, kindly provided by B. A. D. Stocker, Stanford University. This strain of *S. typhimurium* lacks type 1 fimbriae and is highly virulent for BALB/c mice. A stock culture was maintained at −20°C in 1% peptone–40% glycerol. Subcultures were grown in brain heart infusion (BHI) broth overnight (~18 h) at 37°C on a shaker (240 rpm) and used to inoculate experimental media at a dilution of 10^{-3}. Aerobic conditions for experimental cultures (10 ml) were provided by shaking baffle flasks at 240 rpm. Anaerobic conditions were provided by placing broth cultures (5 ml in a baffle flask with a shallow depth; medium conditioned overnight before inoculation in an anaerobe jar) in a sealed bag designed to provide an anaerobic atmosphere (BioBag Environmental Chamber Type A; Becton Dickinson Microbiology Systems, Cockeysville, Md.).

Cell cultures. MDCK cells (ATCC CCL34) were grown in Dulbecco's modified Eagle's medium (DME) with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 10% fetal bovine serum (FBS), penicillin (50 U/ml), streptomycin (50 U/ml), and amphotericin B (2.5 μg/ml). Cell cultures were replaced after 20 passages. Confluent monolayers for infection with bacteria were prepared in 24-well tissue culture plates. Each well was seeded with 2×10^5 cells suspended in DME–10% FBS without antibiotics and incubated for 24 ± 2 h at 37°C in an atmosphere containing 5% CO_2. The monolayers were washed once with Dulbecco's phosphate-buffered saline (PBS) before the bacteria were added.

Cell association. Bacteria were diluted directly from broth cultures in DME (NaH_2PO_4 added in place of $NaHCO_3$, pH adjusted to 6.6) at an average density, determined by plate counting with Trypticase soy–0.6% yeast extract (TSY) agar, of 5.3×10^6 CFU/ml (standard deviation = 2.0×10^6 for $n = 79$). The bacteria (0.2 ml) were added to triplicate cell monolayers in all experiments at a multiplicity (bacterium/cell ratio) of about 2.5 (±1.0), based on the average plate count of inocula and allowing for one doubling of MDCK cells during the 24-h incubation of seeded wells. This multiplicity is well below cell saturation, so that small variations in numbers of added bacteria among experiments did not influence the results.

In order to maintain a constant multiplicity, the amount of carryover BHI in

DME varied, being the highest at 1 h of incubation (20% for aerobic and 50% for anaerobic cultures). However, at 2.5 h of incubation the carryover BHI was <10%, and after 4 h and thereafter was <1%. Cell association by *S. typhimurium* at 1 h, when the carryover BHI concentration in DME was greatest, was the same as for time zero, when the BHI concentration was ≤0.1%. Consequently, there was no evidence that the carryover BHI in DME had any influence on cell association.

The tissue culture plate containing inoculated cell monolayers was immediately centrifuged at 500 × *g* for 30 min at room temperature. These conditions for centrifugation, using forces slightly higher than those used by most other investigators, were chosen in order to maximize the number of bacteria coming into physical contact with the cells and thereby remove any influence of bacterial motility and diffusion on bacterium-cell interactions. No artifact was created by centrifugation; that is, when the bacteria were unable to establish irreversible cell association, for example those from stationary-phase cultures, the number recovered after centrifugation was usually <1% (see Fig. 1). After centrifugation, the cell monolayers were promptly washed five times with PBS (0.5 ml per well), each wash including 2 min on a rotary shaker, to remove unassociated bacteria. Cells were lysed, and the bacteria were suspended by adding *N*-lauroylsarcosine (0.5% in saline) (1.0 ml per well) and shaking the plate vigorously for 5 min. Aliquots (0.5 ml per well) from triplicate wells were composited, and dilutions were prepared in saline for counting recovered bacteria with TSY agar. The total number of bacteria recovered from each cell monolayer, representing adherent plus intracellular bacteria, was referred to as "association." This number was converted for relating cell association to percentage of the inoculum. Each experiment was repeated three times, and the final results are reported as the arithmetic mean ± the standard error of the mean.

Intracellular bacteria (internal) were measured by treating infected monolayers after the centrifugation and washing with gentamicin (100 μg/ml in DME–10% FBS) (0.5 ml per well) for 1 h at 37°C. The antibiotic solution was removed by aspiration, the monolayers were washed twice with PBS, the cells were lysed, and bacteria were suspended for counting as described above. The activity of gentamicin in situ was confirmed by treating cell monolayers with cytochalasin D (1 μg/ml) for 1 h before adding the bacterial suspension, which also contained cytochalasin D, an inhibitor of phagocytosis and, consequently, bacterial invasion. Bacterial association with cytochalasin D-treated cells was nearly equivalent to that with untreated cells (39% ± 1.5% versus 48% ± 2.2%). Exposure to gentamicin for 1 h resulted in an average kill of 99.8%; consequently, more than 99% of the survivors of gentamicin treatment could be considered in all experiments as representative of internalized (intracellular) bacteria.

Measurement of glucose. Glucose concentrations in bacterial growth media containing this sugar were determined with a commercial kit (Sigma no. 115-A) which incorporates the hexokinase method described by Carroll et al. (5). The sensitivity of this method with serum is 0.04%. This procedure will also detect other hexoses. Analysis of BHI broth found the glucose concentration to be 0.2% (Fig. 2C), which is the labelled concentration of glucose, indicating that this medium contained no other hexoses.

Experimental media. Experimental media were as follows: BHI broth (contains 0.2% glucose) (Difco Laboratories); BHI supplemented with additional glucose (0.5% = 0.0277 M) (BHG), lactose (0.0277 M) (BHL), or xylose (0.5%) (BHX); heart infusion-phosphate-peptone (HPP) broth prepared by adding to heart infusion broth (Difco Laboratories) Proteose Peptone (1.5%) (Difco Laboratories)–Na_2HPO_4 (0.25%) and adjusting the pH before autoclaving to 7.4 to 7.6; and HPP with 0.2% glucose (HPG), sorbitol (HPS), xylose (HPX), mannitol (HPM), or glycerol (HPL).

RESULTS

Cell association during aerobic and anaerobic growth. Cell association by aerobically and anaerobically grown *S. typhimurium*, representing the total number of bacteria remaining with each cell monolayer after a 30-min centrifugation and vigorous washing, was measured at various times during growth of the bacteria in BHI broth (Fig. 1). Time zero represents bacteria from the overnight (~18 h) aerobic BHI culture diluted in DME before addition to the cell monolayers. The first measurable increase in cell association occurred after 2.5 h of incubation when both aerobic and anaerobic cultures had entered the early logarithmic phase of growth. The greatest level of association by anaerobically grown bacteria occurred during the late logarithmic phase of growth, just before the growth rate began to slow preceding onset of the stationary phase (Fig. 2A). This level of cell association showed little change between 4 and 9 h of anaerobic growth and then began to gradually decline. Bacteria from overnight anaerobic cultures showed greater cell association (~5%) (no data presented) than did bacteria from overnight aerobic cultures

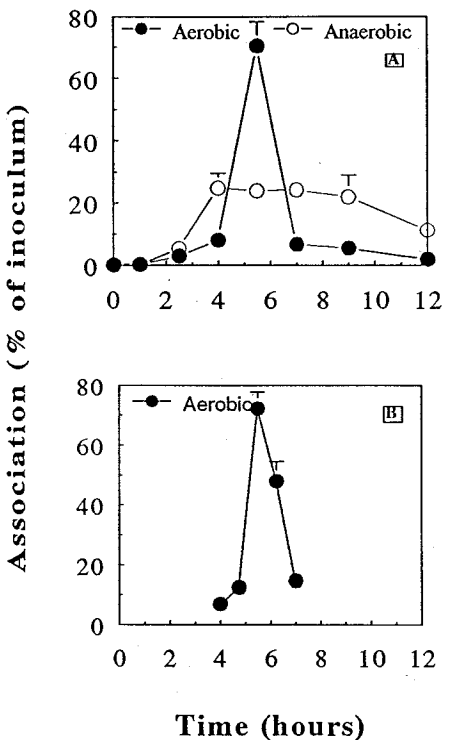

FIG. 1. (A) Association (total) of *S. typhimurium* with MDCK cells after aerobic and anaerobic growth of the bacteria in BHI broth. (B) Verification of transient peak of cell association occurring between 4 and 7 h of aerobic growth of the bacteria. Each point represents the arithmetic mean for three experiments using composites of triplicate cell monolayers. Error bars represent standard errors of the means.

(<1%) (time zero in Fig. 1), indicating the greater lability of the mediator(s) of this process under aerobic conditions.

What was most surprising was the high level of cell association (~70% of inocula) that occurred with aerobically grown *S. typhimurium* at 5.5 h of incubation (Fig. 1). This transient peak of cell association occurred during the late logarithmic phase of growth, just before the growth rate began to slow preceding the stationary phase (Fig. 2A). The results were confirmed with a second set of three experiments in which cell association was measured every 0.75 h between 4 and 7 h of incubation (Fig. 1B). The transient peak of cell association was clearly reproducible, rising abruptly between 5.0 and 5.5 h and then decreasing rapidly during the next 1.5 h of growth. Again, this peak occurred during the late logarithmic phase of growth (data not shown).

The location of cell-associated bacteria was determined in separate experiments using a duplicate set of triplicate cell monolayers treated after infection with bacteria and washing with gentamicin. The number of associated and internalized (i.e., gentamicin survivors) bacteria were essentially equal, the internalized bacteria representing 109 and 96% of the associated bacteria for aerobic and anaerobic cultures, respectively. These results confirmed the reports of other investigators that the entry of salmonellae into cultured epithelial cells is very rapid. The longest bacterium-cell contact time obtained with the procedure used in this study was about 45 min (from start of centrifugation to end of last wash).

pH and glucose concentration during growth in BHI broth. The peak in cell association by *S. typhimurium* from an aerobic BHI culture, which occurred in the late logarithmic phase of

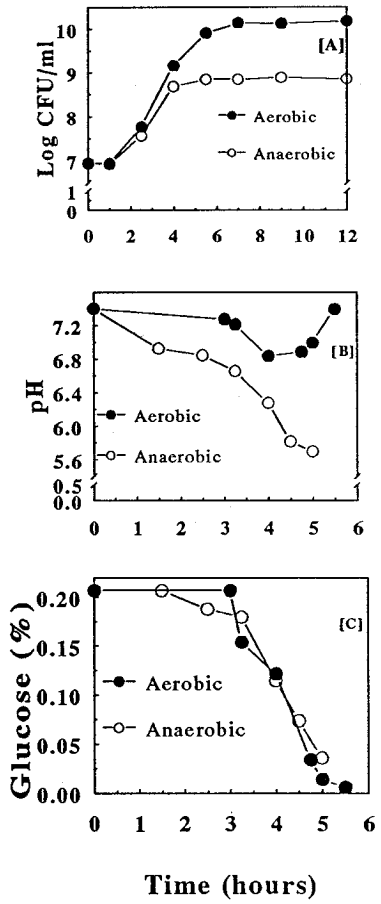

FIG. 2. Changes in cell density (A), pH (B), and glucose concentration (C) during aerobic (●) and anaerobic (○) growth of *S. typhimurium* in BHI broth.

growth, coincided with two significant changes. First, this peak occurred after the pH had passed through a low, representing the production of acids from utilization of carbohydrates, to an alkaline pH, representing utilization of amino acids, which begins with deamination, which increases the pH (Fig. 2B). Secondly, this rise in pH occurred after exhaustion of glucose (Fig. 2C), which agrees with the interpretation regarding substrate utilization based on changes in pH. A rapid decrease in cell association occurred as the bacteria entered the stationary phase of growth. This demonstrates the lability of structures mediating cell association and the necessity of continuous active metabolism for maintaining cell adhesion-invasion activity.

Maximum cell association by *S. typhimurium* grown anaerobically in BHI broth, which was considerably less than observed with aerobic cultures, also occurred in the late logarithmic phase of growth (Fig. 1A). However, the pH remained acid under anaerobic conditions, suggesting that utilization of amino acids as secondary carbon-energy sources did not occur. The repression of cell association by glucose was less under anaerobic conditions; however, it was not possible to judge whether this repression would eventually have been relaxed since considerable glucose (>0.1%) remained as the bacteria entered the stationary phase, in which other signals and/or reduced metabolism also resulted in repression of cell association.

Influence of glucose supplementation of BHI broth. If BHI broth, which contains 0.2% glucose as received, was supplemented with an additional 1.0% glucose, cell association was repressed up to 6.5 h, when the pH was still declining (data not shown). This experiment was repeated with addition of 0.5% (0.0277 M) glucose; it was found that cell association after 5.5 h of incubation, when the glucose concentration was still 0.26%, was severely depressed (Fig. 3). When an equal concentration (0.0277 M) of lactose was added to BHI broth, cell association after 5.5 h of incubation was nearly the same as that observed with bacteria grown in BHI broth alone. Lactose is not utilized by *S. typhimurium*, indicating that the repression of cell association that occurred with addition of glucose resulted from carbohydrate utilization and not merely a change in osmotic conditions. Nearly all of the bacteria, under all conditions, were internal (intracellular).

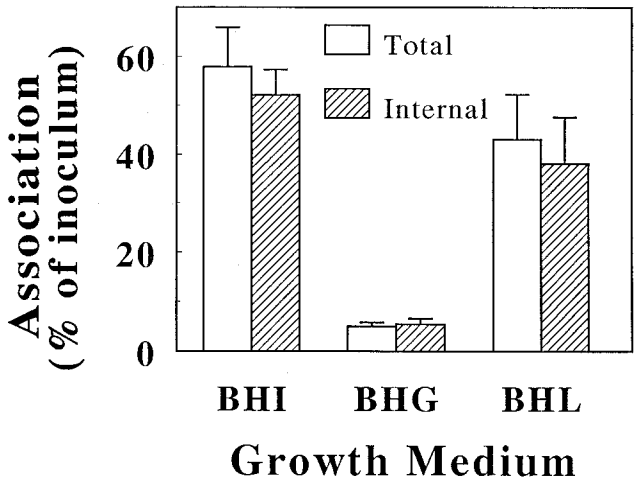

FIG. 3. Effects of added glucose (BHG) or an equal concentration (0.0277 M) of lactose (BHL) in BHI broth on cell association (total) and invasion (internal) by *S. typhimurium* after 5.5 h of aerobic growth.

Effect of cAMP. Glucose, which is utilized in preference to any other available carbon-energy source, including amino acids, is known to repress inducible enzymes through a control mechanism effected by catabolites of glucose utilization. Catabolite repression involves a multigene system regulated by cyclic AMP (cAMP) and can be relieved by adding exogenous cAMP. cAMP was added to BHI broth at 2 and 5 mM, and cell association by *S. typhimurium* was measured after 4 h of aerobic growth, before glucose had been exhausted; cell association remained repressed. Cell association by bacteria grown in the presence of cAMP was equal to that of bacteria from a BHI control, indicating that the repression by glucose was not relieved by exogenous cAMP (data not shown). The effect of cAMP was examined again using three BHI cultures of *S. typhimurium* grown aerobically. At 4 h of incubation, one culture was supplemented with glucose (stock solution prepared in BHI broth) to a final concentration of 0.1% (BHD), the second culture was supplemented with an equal concentration of glucose plus 2 mM cAMP (BHC), and a control culture was supplemented with fresh BHI broth. Incubation was continued to 5.5 h when cell associations by bacteria from the three cultures were compared. Cell association was repressed by the addition of glucose with or without cAMP (Fig. 4), indicating again that the inhibitory effect of glucose on cell association by *S. typhimurium* could not be relieved by exogenous cAMP.

Influence of carbohydrates in HPP broth. HPP broth was formulated to resemble BHI broth but without inclusion of glucose (BHI broth from Difco contains 0.2% glucose) (see "Experimental media" above). Cell association by *S. typhi-*

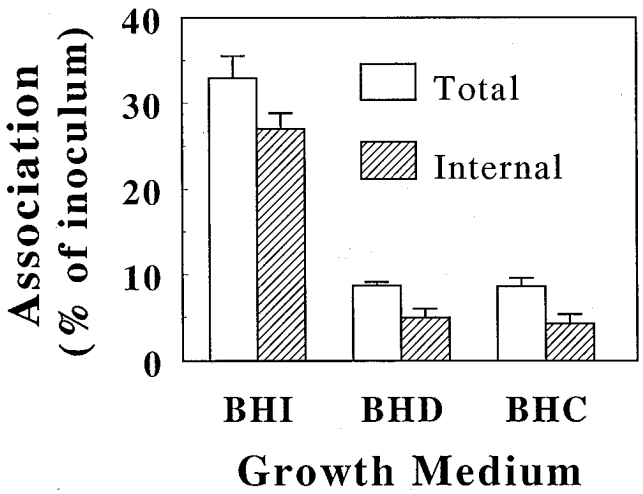

FIG. 4. Cell association (total) and invasion (internal) of MDCK cells by *S. typhimurium* after 5.5 h of aerobic growth of the bacteria in three media: BHI broth (BHI), BHI broth supplemented at 4 h with glucose (0.2%) (BHD), and BHI broth supplemented at 4 h with glucose and cAMP (BHC).

murium grown aerobically in HPP broth was already substantial after just 3.5 h of incubation (Fig. 5), although there was no measurable association (<1%) at 2.5 h (data not shown). This was very unlike the experience with BHI broth (Fig. 1). Cell association increased between 3.5 and 4.5 h of incubation and then decreased between 4.5 and 5.5 h (Fig. 5). The densities of *S. typhimurium* in HPP broth at these times were 7.4×10^7, 6.0×10^8, 1.5×10^9, and 3.0×10^9, respectively. These counts indicated that *S. typhimurium* had passed the mid-log phase of growth before substantial cell association occurred and that the decline between 4.5 and 5.5 h of incubation coincided with slowing of growth upon entering the stationary phase.

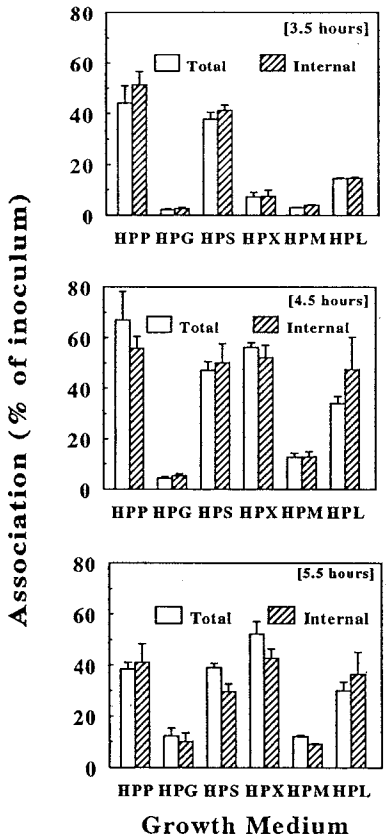

FIG. 5. Cell association (total) and invasion (internal) by *S. typhimurium* during aerobic growth of the bacteria in a carbohydrate-free medium (HPP) and carbohydrate-supplemented (0.2%) HPP: HPG = glucose, HPS = sorbitol, HPX = xylose, HPM = mannitol, HPL = glycerol.

TABLE 1. Changes in pH during aerobic growth of *S. typhimurium* in the presence of different carbohydrates

Growth medium[a]	pH at time (h)			
	0	3.5	4.5	5.5
HPP	7.26	7.23	7.26	7.46
HPG	7.26	6.88	6.59	7.23
HPS	7.28	7.10	7.17	7.39
HPX	7.14	6.92	7.00	7.34
HPM	7.15	7.03	6.75	7.47
HPL	7.57	7.42	7.43	7.55

[a] Carbohydrates added to HPP broth at 0.2% before autoclaving: HPG = glucose, HPS = sorbitol, HPX = xylose, HPM = mannitol, HPL = glycerol.

The addition of 0.2% glucose to HPP (HPG) severely depressed cell association, with little recovery between 3.5 and 5.5 h of growth (Fig. 5). This lack of recovery of cell association was unlike the results obtained with BHI broth. The pH profile in HPG (Table 1) was similar to that observed in BHI broth (Fig. 2); however, at 4.5 h the glucose concentration in HPG was 0.03% and did not change between 4.5 and 5.5 h (data not shown), when the growth was slowing. The repression of cell association due to carbohydrate utilization and repression due to the onset of the stationary phase are both evident in this system.

Four additional carbohydrates utilizable by *S. typhimurium* (confirmed by acid formation from each in purple broth base) were examined individually by addition at 0.2% in HPP broth. Cell association was measured after aerobic incubation for 3.5, 4.5, and 5.5 h (Fig. 5). When repression of cell association was evident, it was greatest at 3.5 h of growth, least after 4.5 h, and then reduced at 5.5 h, when growth was slowing upon entry into the stationary phase. Sorbitol showed the least amount of repression of cell association. Nearly full recovery of cell association occurred with prolonged incubation of all media, with the exception of HPG (containing glucose) and HPM (containing mannitol). This was explainable by the pH profile (Table 1), which showed that in HPM, as in HPG but not the other media, the pH continued to decline between 3.5 and 4.5 h, indicating that repression continued during formation of acids from utilization of the carbohydrate. The lack of recovery of cell association between 4.5 and 5.5 h was due, as shown by other results, to slowing of the bacterial growth rate upon entering the stationary phase.

Cell association during anaerobic growth on carbohydrates. Cell association by *S. typhimurium* after anaerobic growth in HPP broth (Fig. 6) was similar to that observed with aerobic growth in this medium (Fig. 4), increasing between 3.5 and 4.5 h. This association was repressed by the presence of glucose, although to a slightly lesser degree under anaerobic conditions (64 versus 95% inhibition at 3.5 h, and 85 versus 95% inhibition at 4.5 h). The glucose concentration after 4.5 h of anaerobic growth, when repression of cell association was still very evident (85% inhibition), was 0.05%. Repression of cell association by addition of mannitol to HPP broth was much less under anaerobic than aerobic growth conditions (29 versus 93% inhibition at 3.5 h, and 53 versus 79% inhibition at 4.5 h).

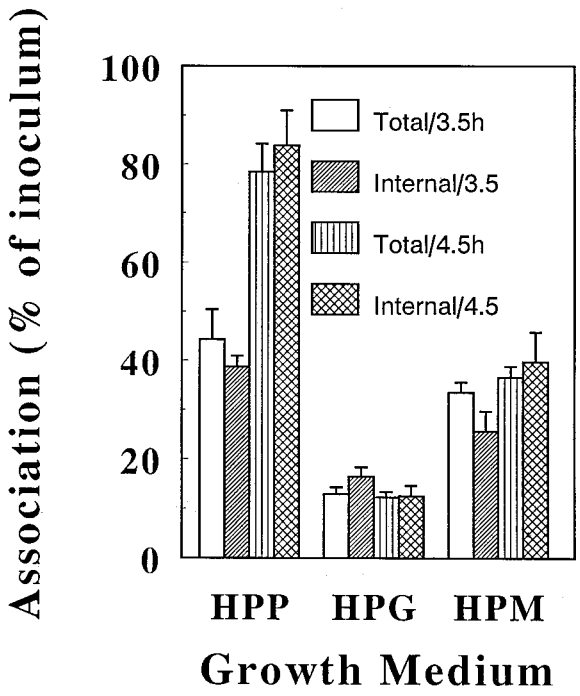

FIG. 6. Cell association (total) and invasion (internal) by *S. typhimurium* during anaerobic growth of the bacteria in a carbohydrate-free medium (HPP) and carbohydrate-supplemented (0.2%) HPP: HPG = glucose, HPM = mannitol.

DISCUSSION

In an early report, Finlay et al. (10) showed that *Salmonella choleraesuis* grown overnight to stationary phase was unable to enter cultured mammalian cells. Both adherence and invasion by the bacteria increased during incubation with cells, which were thought to induce new bacterial proteins necessary for mediating these processes by both *S. choleraesuis* and *S. typhimurium*. The loss of invasiveness by *S. choleraesuis* after growth overnight to stationary phase was confirmed in a subsequent study by Lee and Falkow (30), who reported that induction of invasiveness did not require the presence of MDCK cells but followed growth of the bacteria in medium alone. Adherence and invasiveness were greatest during the late logarithmic phase, and the investigators concluded, were induced by oxygen limitation. Ernst et al. (6) subsequently reported that invasion of HEp-2 cells by *S. typhimurium* was greatest for bacteria from the logarithmic phase of growth and when the bacteria were grown anaerobically or incubated with the cells under anaerobic conditions. Francis et al. (11) used an assay with a short bacterium-cell interaction period to confirm again that *S. typhimurium* bacteria from the stationary phase of growth were somewhat adherent but less so than bacteria grown under low-oxygen conditions, which were also invasive for both HEp-2 and MDCK cells. Furthermore, only bacteria grown under low-oxygen conditions, but not bacteria from stationary-phase cultures, elicited rapid changes in cell morphology, internal actin filament rearrangement, and cell entry. Schiemann and Shope (37) suggested that the increased uptake of anaerobically grown *S. typhimurium* by Henle 407 epithelial cells, which was also observed with mouse peritoneal cells, may have resulted from repression rather than synthesis of new proteins under anaerobic conditions. The work reported herein indicates that oxygen repression of cell association by *S. typhimurium* occurs only when the bacteria are utilizing carbohydrates and that greater association, represented primarily by rapid invasion, by anaerobically grown bacteria is observed when the comparison is made with an aerobic culture in which carbohydrate repression is occurring. In the absence of a utilizable carbohydrate, cell association is essentially equal for logarithmic-phase bacteria grown aerobically or anaerobically. Furthermore, the bacteria have only to enter the logarithmic phase and not wait until late in this phase of growth to exhibit maximal cell association when they have been grown in the absence of a utilizable carbohydrate. Behlau and Miller (3) found that a PhoP-repressed gene in *S. typhimurium* that promotes cell invasion was most highly expressed at neutral pH when the bacteria were grown aerobically to late log phase. These were the same physiological conditions under which maximum cell association by *S. typhimurium* was observed in this study.

Kusters et al. (29) recently challenged previous reports by stating that there was no difference in cell adhesion and invasion by *S. typhimurium* during the logarithmic and stationary phases of growth. The basis for their interpretation is not clear from the data presented, which show graphic differences in time sequence curves for mid-logarithmic- and stationary-phase *S. typhimurium* (Fig. 1 in Kusters et al. [29]). The assay used in these studies monitored changes in the number of bacteria over time during incubation on the cells, conditions suitable for growth and under which stationary-phase bacteria would quickly initiate active metabolism. Logarithmic-phase bacteria were recovered from cultures by centrifugation, a procedure, especially if conducted at a low temperature, that can quickly shut off the continual protein synthesis necessary for maintaining cell adhesion and invasion activity by *S. typhimurium* (32). Kusters et al. (29) suggested that the observations of other workers may derive from a greater proportion of dead bacteria in stationary-phase cultures, which can compete for a limited number of adhesion sites on the cells. This appears to be a valid point, especially for older cultures which have entered the decline phase. However, there are few if any dead bacteria in the early stationary phase when cell association, including invasion, declines drastically (Fig. 1) (30), a change that cannot be explained by the presence of dead bacteria.

Glucose repression of inducible enzymes, and repression by catabolic intermediates of glucose utilization, are well-known control mechanisms in bacteria. Catabolite repression involves a multigene system regulated by cAMP and can be relieved by exogenous cAMP. That was not the case, however, in this study, in which the addition of cAMP during aerobic growth of *S. typhimurium* did not reverse the inhibitory effect of glucose on cell association. There are, however, bacteria, especially several gram-positive species, that demonstrate cAMP-independent catabolite repression, and synthesis of some enzymes subject to glucose repression is not relieved for these organisms by exogenous cAMP (28).

This study adds to the list of environmental stimuli that control the capability of salmonellae to associate with and invade epithelial cells in vitro. Oxygen repression described in other studies was found during this work to occur only during utilization of carbohydrates. Exhaustion of a preferred carbohydrate substrate in the presence of other potential carbon-energy sources represents a nutrient limitation that can stimulate a responsive change in network proteins. *Escherichia coli* synthesizes at least 30 proteins when deprived of a carbon source, which has been described as occurring in a "burst." Cell association by *S. typhimurium* also occurred, coincidentally, in a burst at the point of glucose exhaustion during aerobic growth in BHI broth. Carbohydrate utilization during

anaerobic growth also repressed cell association, whereas anaerobic growth in a carbohydrate-free medium (HPP broth) resulted in a level of cell association essentially equal to that demonstrated by aerobically grown *S. typhimurium.*

The onset of the stationary phase of growth resulted in a rapid loss of cell association by *S. typhimurium*. If onset occurred before complete exhaustion of a utilizable carbohydrate substrate, cell association remained depressed, indicating an overlap of environmental stimuli similar to that described by Tartera and Metcalf (38) for osmolarity and growth phase in their study of cell adhesion and invasion by *Salmonella typhi*. The stationary phase is a stimulus for a multigene system that results in an increase in protein turnover and also the synthesis of new proteins (35). Cell invasion by *S. typhimurium* requires continual metabolism, and any slowing of metabolic activity, such as must occur with cessation of cell division upon entry into the stationary phase, results in degeneration of this process (32). The full spectrum and interrelationships of the environmental stimuli that regulate cell adhesion and invasion by *S. typhimurium* have, quite obviously, not yet been fully elucidated.

ACKNOWLEDGMENT

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