INFECTION AND IMMUNITY, June 1990, p. 2014–2016
0019-9567/90/062014-03$02.00/0

Vol. 58, No. 6

Anaerobiosis, Type 1 Fimbriae, and Growth Phase Are Factors That Affect Invasion of HEp-2 Cells by *Salmonella typhimurium*

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Received 18 December 1989/Accepted 20 March 1990

The invasion of HEp-2 cells by *Salmonella typhimurium* was studied under various conditions. Anaerobiosis was shown to markedly affect the internalization of bacterial cells by HEp-2 cells. Anaerobically grown bacteria incubated with HEp-2 cells under anaerobic conditions markedly stimulated the rate of invasion. Anaerobiosis may therefore be a controlling factor in the invasion process. Cells obtained during the logarithmic phase of growth invaded at much higher rates than cells obtained during the stationary phase of growth. The presence of mannose-sensitive type 1 fimbriae on the bacterial surface also promoted invasion, and these fimbriae appear to play a role as an accessory virulence factor.

Members of the genus *Salmonella* are characterized by an ability to invade epithelial cells as an early step in pathogenesis. These organisms are able to penetrate the epithelial barrier and gain access to the underlying tissue as well as the blood stream (7). The molecular mechanisms involved in this process are not yet understood but are currently under study in several laboratories (3, 8). We recently reported that type 1 fimbriated strains of *Salmonella typhimurium* adhered in significantly higher numbers to isolated rat enterocytes than did nonfimbriated strains (12). Since adherence is generally presumed to be a prerequisite for invasion, these studies suggested that mannose-sensitive adhesins may play a role in the pathogenic process. Further, it was demonstrated that ongoing metabolic processes were required to maintain stable binding and that bacterium-enterocyte interaction induced the synthesis of new *S. typhimurium* proteins. Finlay et al. (6) subsequently showed de novo synthesis of several new bacterial proteins by *Salmonella choleraesuis* as a result of bacterial interaction with epithelial cells. In the present study, we examined the role of anaerobiosis as a possible controlling factor in bacterial invasion of HEp-2 epithelial cells. The effect of fimbriae and the bacterial growth phase on invasion of HEp-2 cells is also reported in this study.

S. typhimurium SR-11 χ3306 (obtained from R. Curtiss III, Department of Biology, Washington University, St. Louis, Mo.) was utilized for these studies (9) and was maintained as frozen stock cultures at −70°C in the fimbriated (Fim^+) or the nonfimbriated (Fim^-) state (12). Bacteria were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C. Anaerobic growth was achieved by growing bacteria in an anaerobic hood in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2, while aerobic growth was carried out in a gyratory shaker. Bacterial cells were harvested during the logarithmic growth phase, washed in phosphate-buffered saline and suspended in Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.). HEp-2 cells (10) were grown in a 5% CO_2 atmosphere in Eagle minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum (GIBCO), penicillin (50 U/ml), and streptomycin (50 μg/ml). Confluent monolayers (approximately 5×10^5 cells per well) were prepared in a 24-well tissue culture plate. The monolayer was washed with phosphate-buffered saline, and 5×10^7 bacterial cells suspended in 1 ml of Eagle minimal essential medium were introduced. The invasion assay was carried out essentially as described by Finlay and Falkow (4). After infection of the cells, the tissue culture plates were centrifuged at $162 \times g$ for 10 min and then incubated at 37°C in a modular incubator chamber (Billups-Rothenberg, Del Mar, Calif.) with an atmosphere of 5% CO_2 in air or 5% CO_2–95% N_2. The chambers were flushed for a minimum of 5 min before they were clamped to maintain the indicated atmosphere. No further attempt to achieve complete anaerobiosis was made. After 2 h, the monolayers were washed with phosphate-buffered saline three times. Eagle minimal essential medium (1 ml) containing gentamicin (100 μg) was added to each well to kill any extracellular bacteria that remained after the washing, and the incubation continued for an additional hour. The monolayers were rinsed three times with phosphate-buffered saline to remove residual gentamicin. Intracellular bacteria were released by the addition of 1% Triton X-100 and quantitated by plating on Luria Bertani agar (14) plates.

The microflora in the gut reflects the essentially anaerobic atmospheric environment in the gastrointestinal tract and consists mainly of anaerobic or facultative anaerobic microorganisms (15). Thus, when *S. typhimurium* reaches the distal ileum, it must adapt to an anaerobic environment in order to carry out essential metabolic activities. It is under these conditions that penetration of intestinal epithelial cells likely occurs. However, the role of anaerobiosis in *S. typhimurium* invasion of epithelial cells has, to the best of our knowledge, not been addressed. The invasion of HEp-2 cells was carried out under aerobic (CO_2-air) or anaerobic (CO_2-N_2) conditions with aerobically or anaerobically grown bacterial cells. Growth of *S. typhimurium* was initiated from a glycerol stock that was stored in the fimbriated state. The results were determined as mean percentage of invaded bacteria ± standard deviation in at least three experiments; each experiment was run in triplicate. Growth of bacteria under aerobic and anaerobic conditions, followed by incubation with HEp-2 cells under aerobic conditions, resulted in similar rates of invasion (aerobically grown bacteria, 3.4% ± 1.1%; anaerobically grown bacteria, 3.0% ± 1.2%). However, incubation of anaerobically grown bacterial cells with HEp-2 cells under anaerobic conditions showed marked stimulation of invasion, giving rise to at least three times (9.2% ± 2.3%) the aerobic rates of invasion. The level of

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TABLE 1. Effect of fimbriation on invasion[a]

Carbohydrate addition	% Invasion[b] of phenotype:	
	Fim$^+$	Fim$^-$
None	7.2 ± 0.9	1.8 ± 0.8
0.1% Galactose	7.5 ± 0.0	1.7 ± 0.5
0.1% Mannose	1.6 ± 0.3	1.9 ± 0.4

[a] Bacteria were grown under anaerobic conditions, and the invasion assay was carried out in an atmosphere of 5% CO_2–95% N_2. The tested carbohydrates were added at the beginning of the invasion assay.

[b] Mean percentage of invaded bacteria ± standard deviation of at least three experiments; each experiment was run in triplicate. Growth of fimbriated (Fim$^+$) cells was initiated from a glycerol stock that was stored in the fimbriated state; growth of nonfimbriated (Fim$^-$) cells was initiated from a glycerol stock that was stored in the nonfimbriated state.

invasion of aerobically grown cells incubated with HEp-2 cells under anaerobic conditions was 4.0% ± 1.9%. We did not detect any reduction in the ability of gentamicin to kill *S. typhimurium* under anaerobic conditions, indicating that the results shown above were not due to an artifact of the killing action of the antibiotic under the different conditions. We also assessed the viability of the HEp-2 cells under the anaerobic and aerobic conditions of the invasion assay. No detectable differences in trypan blue exclusion were detected. Thymidine and leucine uptakes by HEp-2 cells under anaerobic conditions were found to be 85 and 76%, respectively, of the values observed under aerobic conditions. We therefore conclude that stimulation of *S. typhimurium* invasion of HEp-2 cells is due to bacterial factors that are induced under anaerobic conditions.

It is becoming increasingly clear that environmental signals may coordinately regulate virulence factors involved in bacterial pathogenesis (5, 13). Environmental signals such as plant exudates, changes in osmolarity, nutrient deprivation, repellants, and attractants act as sensory stimuli that regulate the expression of genes that are components of a global regulatory network. Perhaps in the case of *S. typhimurium*, anaerobiosis is an environmental signal which regulates the synthesis of virulence factors. To explore this possibility, we recently employed Mu d-*lac* operon fusion technology (11) to identify anaerobically inducible loci. Anaerobically inducible insertion mutants with greatly reduced abilities to invade HEp-2 cells have been isolated, and we are currently in the process of characterizing these mutants in order to define the molecular components involved in invasion.

In a previous study, we demonstrated that mannose-sensitive type 1 fimbriae plays a role in the attachment of *S. typhimurium* to rat enterocytes (12). However, because of the fragility of the enterocytes, we could not determine whether fimbriated bacterial cells also promote invasion. In this study, we compared the abilities of fimbriated and nonfimbriated cells to invade HEp-2 cells. As can be seen in Table 1, fimbriated bacterial cells invaded much more effectively than nonfimbriated bacterial cells. The cultures used in these studies were initiated from glycerol stocks in which the bacteria were stored either in the fimbriated (Fim$^+$) or nonfimbriated (Fim$^-$) state. During the culture period prior to the invasion assay, fimbriated bacteria remained Fim$^+$ and nonfimbriated bacteria remained Fim$^-$, as determined by guinea pig erythrocyte hemagglutination. Invasion was inhibited by mannose but not by galactose, suggesting that mannose-sensitive type 1 fimbriae promoted invasion. Presumably, contact between bacteria and epithelial cells through fimbrial attachment to mannose-containing glycoproteins initiates the endocytic process. Similar results were

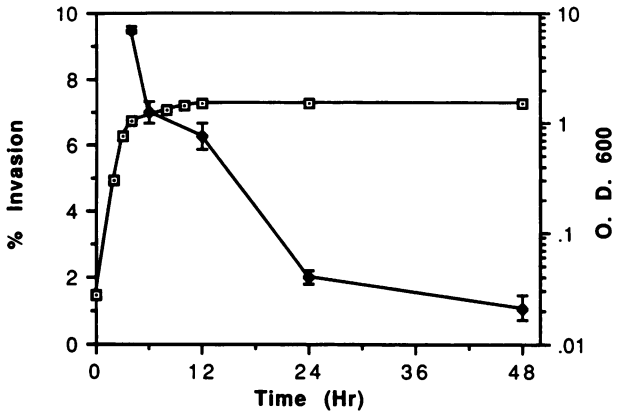

FIG. 1. Effect of growth phase on invasion. Bacteria were grown under anaerobic conditions, and the invasion assay was carried out in an atmosphere of 5% CO_2–95% N_2. Each result shown for percent invasion represents the mean percentage of invaded bacteria ± the standard deviation of two experiments; each experiment was run in triplicate. Bacterial cell growth was initiated from a glycerol stock of fimbriated cells. Symbols: ⊡, optical density (O.D.) at 600 nm; ◆, percent invasion.

obtained with isogenic *fim*$^+$ and *fim* mutant strains of *S. typhimurium* (data not shown). Nonfimbriated bacterial cells do invade, but at considerably lower levels. Thus, fimbriae are not an absolute requirement for invasion but likely play a role as an accessory virulence factor. These results are in accord with observations which have indicated that nonfimbriated as well as fimbriated cells can cause disease in orally infected mice (2). However, fimbriated cells have some advantage, since these strains cause significantly more infections and death (16). In the case of nonfimbriated bacterial cells, stable association with epithelial cells may be carried out by bacterial surface components that have not yet been defined. To obtain controls for this study, we determined the levels of invasion of HEp-2 cells by two noninvasive *Escherichia coli* strains, HB101 (1) and K-12(W3110). The percent invasions for HB101 and K-12 were less than 0.05 and 0.1, respectively.

The penetration of bacterial cells into HEp-2 cells was found to be markedly dependent on growth phase (Fig. 1). The highest rate of invasion was obtained with cells from the logarithmic phase of growth. Bacterial cells became less invasive as the culture progressed into the stationary phase of growth. The higher rates of invasion observed for cells in logarithmic growth may reflect the need to carry out protein synthesis at sufficiently high rates so that new proteins necessary for the invasion process are induced. This conclusion is supported by the effect of choramphenicol on the invasion of HEp-2 cells by *S. typhimurium*. For example, the percent invasions (± standard deviations) observed in the presence of 15 and 30 μg/ml of chloramphenicol were 3.3 ± 0.3 and 1.4 ± 0.1, respectively, compared with 7.2 ± 1.7 observed in the absence of chloramphenicol. These data do not rule out the possibility that invasion proteins have a relatively high turnover rate and must be continually synthesized in order to maintain maximum invasion rates.

This work was supported by Public Health Service grant DE8240 from the National Institute of Dental Research; a grant-in-aid from the New York State Science and Technology Foundation, Center for Advanced Technology Program; and funds made available to the School of Medicine, State University of New York at Buffalo.

We express our appreciation for the helpful advice given by David Dyer throughout the course of this work.

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