Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4304–4308, June 1990
Microbiology

The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state

(bacterial invasion/Madin–Darby canine kidney cells/oxygen/stationary phase/*oxrA*)

CATHERINE A. LEE AND STANLEY FALKOW

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305-5402

Contributed by Stanley Falkow, March 29, 1990

ABSTRACT **We have examined the effect of different growth conditions on the ability of *Salmonella* to interact with Madin–Darby canine kidney cells. Two growth conditions that affect the expression of *Salmonella* adherence and invasiveness have been identified. First, bacteria lose their invasiveness in the stationary phase of growth. Second, bacteria growing in oxygen-limited growth conditions are induced for adherence and invasiveness, whereas those growing aerobically are relatively nonadherent and noninvasive. *Salmonella* from cultures aerated with gas mixtures containing 0% or 1% oxygen were 6- to 70-fold more adherent and invasive than those from cultures aerated with a gas mixture containing 20% oxygen. The *Salmonella typhimurium oxrA* gene that is required for the anaerobic induction of many proteins is not involved in the regulation of *Salmonella* invasiveness. We speculate that oxygen limitation might be an environmental cue that triggers the expression of *Salmonella* invasiveness within the intestinal lumen and other tissues.**

Bacterial pathogens are dependent on their ability to overcome host barriers and infect their hosts. For example, *Salmonella*, which normally enters its animal hosts via ingestion, can pass from the intestinal lumen to reach underlying sites (1). The initial site of *Salmonella* infection is the distal ileum where the bacteria associate with the epithelial lining and adjacent Peyer's patch (2, 3). Closer examination of this initial interaction reveals that the salmonellae cause degeneration of the epithelial brush border and then enter the apical surface of enterocytes (4–6). This entry process resembles phagocytosis and results in intracellular bacteria enclosed within membrane-bound vacuoles. To study the molecular details of the initial entry process, *in vitro* assays have been developed for quantitating the ability of *Salmonella* to adhere to and enter cultured mammalian cells (7–13). Recent studies (14–16) have examined the interaction of *Salmonella* with epithelial cells that have formed polarized monolayers *in vitro* (17). In this *in vitro* system, the salmonellae interact with and enter the apical surface of cells in a process morphologically indistinguishable from that observed *in vivo* (14). The ability of *Salmonella* strains to enter mammalian cells *in vitro* also appears to correlate with their ability to invade the ileal mucosa *in vivo* (8).

Previous results from this laboratory (18) showed that bacteria grown overnight in bacterial culture medium were not inherently able to enter cultured mammalian cells. However, after further incubation with the mammalian cells, bacterial adherence and invasion of cells were observed. It appeared that new bacterial proteins were expressed during the incubation period since this process required bacterial RNA and protein synthesis. It was proposed that an epithelial cell component might provide the environmental cue that induced bacterial attachment and entry into the host cell (18).

We now report that the induction of bacterial adherence and invasiveness does not require incubation with mammalian cells. Rather, our analysis shows that growth of *Salmonella* under the appropriate *in vitro* conditions results in expression of bacterial factors that permit attachment and entry into mammalian cells.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *Salmonella choleraesuis* strain SL2824 (19) and *Salmonella typhi* strain 404Ty were obtained from B. A. D. Stocker (Stanford University School of Medicine). 404Ty is a *ΔaroA148* derivative of an Indonesian strain, 3083/30, originally characterized by L. LeMinor (Institut Pasteur, Paris), which has two flagellar antigen phases, d and *z66* (20). *Salmonella typhimurium* strains TN1909 and TN1910, obtained from Charles Miller (Case Western Reserve University, Cleveland), are isogenic strains containing the *oxrA*$^+$ and *oxrA*1 loci, respectively. *S. typhimurium* strain LT2 (DB21) was obtained from David Botstein (Genentech, South San Francisco, CA). *Salmonella* were grown in LB broth and on LB agar medium (GIBCO), alone or supplemented with 2,3-dihydroxybenzoic acid (10 μg/ml) (Sigma), which is required for optimal growth of the *ΔaroA* mutant strain.

Cultures used in some experiments (Table 1 and Table 2, experiment 3) were grown in LB broth containing antifoam (Sigma). The presence of antifoam does not affect the induction or assay of bacterial invasiveness (unpublished observations). Initially, a culture was grown to logarithmic phase in LB broth containing antifoam while bubbled with air from the laboratory air lines for 2½ hr. This culture was diluted to prepare three cultures that were gently bubbled with different gas mixtures, 5% CO_2/95% N_2, 1% O_2/5% CO_2/94% N_2, or 20% O_2/5% CO_2/75% N_2 (Liquid Carbonic Specialty Gas, San Carlos, CA). After 3 hr of additional growth, the final culture density of each culture was $\approx 4 \times 10^8$ colony-forming units (cfu)/ml (Table 1, experiment 1) and $\approx 2 \times 10^8$ cfu/ml (Table 1, experiment 2).

Nonagitated cultures were prepared by inoculation of 2 ml of LB broth in 16 × 150 mm borosilicate tubes with 0.04 ml of a stationary-phase culture. For the experiment depicted in Fig. 1, separate cultures were prepared to allow uninterrupted incubation of each sample. It was characteristic of such cultures that, during the initial 3-hr incubation at 37°C, the culture density increased from 10^8 cfu/ml to 4×10^8 cfu/ml. Bacterial growth was much slower during the next 4 hr; the culture density after 7 hr of growth was only 7×10^8 cfu/ml. We considered that bacteria from such cultures were in the late logarithmic phase of growth.

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Abbreviations: MDCK, Madin–Darby canine kidney; cfu, colony-forming unit(s).

Agitated cultures were prepared by inoculation of 1 or 2 ml of LB broth in 16 × 150 mm borosilicate tubes with a stationary-phase culture (a 1:1000 or 1:50 dilution factor) and placing the tubes on a roller drum (New Brunswick Scientific; 80 rpm) at 37°C. Under these growth conditions, logarithmically growing cultures had $\leq 10^8$ cfu/ml, late logarithmic cultures had $\approx 10^9$ cfu/ml, and stationary-phase cultures had $\approx 5 \times 10^9$ cfu/ml. For the experiment depicted in Fig. 2, separate cultures were prepared to allow uninterrupted incubation of each sample.

Overnight cultures were prepared as a source of bacteria in stationary phase by inoculation of 2 ml of LB broth with $<10^6$ cfu and growth with agitation, except for that used for the experiment described in Fig. 3. In this case, a culture was grown overnight without agitation from a heavy inoculum as described (21). Results similar to those shown in Fig. 3 were obtained when stationary-phase bacteria from an agitated culture were used instead (data not shown).

Strains were grown at 37°C with the exception of strains TN1909 and TN1910, which were grown at 30°C. cfu were determined by plating serial dilutions onto LB agar medium.

Madin–Darby Canine Kidney (MDCK) Cells and Growth Conditions. Strain 1 MDCK cells (22) were grown in minimal essential–Eagle medium with Earle's salt (Cellgro/Mediatech, Washington, DC) and 5% fetal bovine serum (GIBCO) without antibiotics. Monolayers for adherence and invasion assays were prepared by seeding $\approx 10^5$ cells into each well of a 24-multiwell tissue culture plate and incubating overnight. MDCK cells between passage 40 and 60 were used for all assays except for those in Table 1 and Table 2 (experiment 3), in which cells between passage 8 and 10 were used. The absolute values for bacterial adherence and invasion were 10-fold lower when MDCK cells between passage 8 and 10 were used vs. when those between passage 40 and 60 were used. It is possible that a property of the MDCK cells changed after many serial subcultures resulting in an increased efficiency of bacterial adherence and invasion. The conclusions of this study appear to be unaffected by this change.

Adherence Assay. The medium overlying the MDCK monolayers was replaced with 1 ml of ice-cold tissue culture medium and the multiwell dish was placed in an ice-water bath for 30 min prior to the assay. Bacterial samples were diluted if necessary in LB broth to approximately 10^8 to 4×10^8 cfu/ml so that addition of 40 μl of each bacterial sample to the MDCK monolayers represented an initial inoculum of 5×10^6 to 2×10^7 cfu. Control wells without mammalian cells were similarly prepared to quantitate nonspecific bacterial adherence to plastic. The bacteria were allowed to adhere on ice for 60 min and then each well was rinsed five times with 1–2 ml of ice-cold phosphate-buffered saline at pH 7.4 (PBS; Sigma). Adherent bacteria were released by incubation with 0.2 ml of 0.1% Triton X-100 (Sigma) for 5–10 min. LB broth (0.8 ml) was then added and each sample was vigorously mixed using a Pasteur pipet. Adherent bacteria were quantitated by plating for cfu on LB agar medium.

To measure internalization of adherent bacteria, bacteria were allowed to adhere as described above. After removal of nonadherent bacteria with the PBS washes, 1 ml of prewarmed tissue culture medium was added to each assay well, and internalization of bacteria was allowed to proceed for 60 min at 37°C in a 50% CO_2 incubator. Intracellular bacteria were quantitated after incubation with gentamicin as described below.

Invasion Assay. Prior to all invasion assays, the medium overlying each MDCK monolayer was replaced with 1 ml of prewarmed tissue culture medium. Bacterial samples were then added as described above. Invasion assays were incubated at 37°C in a 5% CO_2 incubator. Assays were terminated by replacing the overlying medium with 1 ml of tissue culture medium supplemented with 100 μg of gentamicin per ml. Gentamicin, an aminoglycoside antibiotic, kills extracellular bacteria while intracellular bacteria remain viable (23). After incubation with the gentamicin for 120 min at 37°C in a 5% CO_2 incubator, the cell monolayers were washed once with PBS and the viable intracellular bacteria were released by incubation with 0.2 ml of 1% Triton X-100 for 5–10 min. Samples were vigorously mixed with 0.8 ml of LB broth using a Pasteur pipet. Viable bacteria were quantitated by plating for cfu on LB agar medium.

For the experiment depicted in Fig. 3, initially, 1-ml aliquots of tissue culture medium inoculated with 1.4×10^7 cfu of *S. choleraesuis* from a stationary-phase culture was placed either on MDCK cell monolayers or in empty wells. In the first case, the entry of *Salmonella* into MDCK cells was measured hourly by replacing the overlying medium with tissue culture medium supplemented with 100 μg of gentamicin per ml, as described above, at t_x. In this case, calculations were performed to determine the incremental increase in the number of bacteria that entered the MDCK cells during each 1-hr period. The invasiveness of the bacteria incubated in tissue culture medium alone was determined by transferring the infected medium onto MDCK cell monolayers at different times. In this case, the number of bacteria that were able to enter the cells during a 1-hr assay was determined, as described above.

Biosynthetic Radiolabeling of *Salmonella* Proteins. Bacterial cultures were grown at 37°C for 5 hr to a final culture density of $\approx 10^7$ cfu/ml. Cultures containing tissue culture medium were grown in a 5% CO_2 incubator. Bacteria from 0.4 ml of each culture were washed with 1 ml of PBS and then resuspended in 80 μl of M9 medium (GIBCO) containing 0.1% glucose and 15 μCi of [^{35}S]methionine (Amersham; 1 Ci = 37 GBq). Samples were incubated at 37°C for 1 hr and then centrifuged to harvest the bacteria. The samples were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (24).

RESULTS

Effect of Growth Conditions on *Salmonella* Invasiveness. We have been studying the ability of *Salmonella* to adhere to and enter cultured mammalian cells. Previous studies from our laboratory examined bacteria that were grown overnight in LB broth to stationary phase (18, 21). It was considered that *Salmonella* in the environment were likely in stationary phase prior to ingestion by a susceptible host (25). It was found that such bacteria were not inherently able to enter mammalian cells (18). *Salmonella* that were grown to logarithmic phase in LB broth were also found to be inherently nonadherent (15, 18). In the course of our experiments, we measured the invasiveness of *Salmonella* that were grown to logarithmic and late logarithmic phase and found that the invasiveness of these bacteria varied dramatically. To understand this variation, we decided to more carefully measure the invasiveness of bacteria during the course of growth of a culture. We examined nonagitated and agitated LB broth cultures.

Nonagitated cultures of *S. choleraesuis* were prepared by inoculation of 2 ml of LB broth with 0.04 ml of a stationary-phase culture (a 1:50 dilution factor) at t_0. As previously reported, we found that bacteria grown overnight to stationary phase were not able to enter MDCK cells during a 1-hr assay (Fig. 1). However, after growth of bacteria in nonagitated LB broth for 3–7 hr, we observed a 1000-fold increase in bacterial invasiveness, 0.02% to 20% (Fig. 1).

Agitated cultures of *S. choleraesuis* were prepared by inoculating 2 ml of LB broth with a stationary-phase culture (a 1:1000 or 1:50 dilution factor) at t_0. When we measured the ability of bacteria from agitated culture to enter MDCK cells,

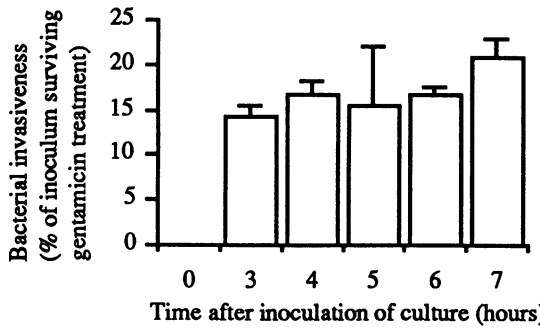

FIG. 1. *S. choleraesuis* invasiveness measured during the course of growth of a nonagitated culture. Bacterial invasiveness was quantitated as the percent of the inoculum that could enter MDCK cells during a 1-hr assay and thus survive subsequent treatment with gentamicin. Values represent the average of two separate assays.

dramatic differences were seen depending on the stage of growth of the bacteria sample (Fig. 2). We found that *S. choleraesuis* from cultures in logarithmic phase were not maximally invasive. However, when the culture was in late logarithmic phase, bacterial invasiveness increased at least 10-fold. The bacterial culture then lost invasiveness in stationary phase, which is consistent with previous observations that bacteria grown overnight in LB broth to stationary phase are noninvasive. Similar results were also found for nonagitated and agitated cultures of *S. typhi* (data not shown).

The dramatic differences in the invasiveness of *Salmonella* from different cultures were not affected by inhibition of bacterial growth and *de novo* protein synthesis during the assay period (data not shown). Thus, in our assay, the difference in the ability of *Salmonella* to enter MDCK cells reflects an inherent property of the bacterial inoculum.

Effect of Oxygen Availability on *Salmonella* Invasiveness. The above experiments show that *S. choleraesuis* and *S. typhi* from nonagitated cultures and agitated cultures in late logarithmic phase are at least 10-fold more invasive than those from agitated cultures in logarithmic phase. We considered that one commonly cited difference between these growth conditions might account for the differences in *Salmonella* invasiveness—that is, the availability of oxygen (26, 27).

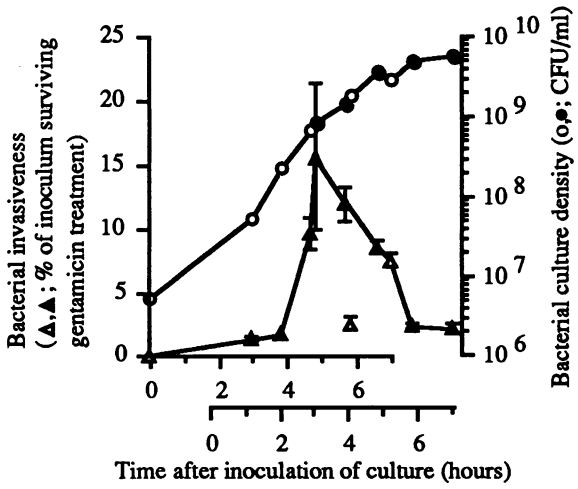

FIG. 2. *S. choleraesuis* invasiveness measured during the course of growth of an agitated culture. During the course of growth of agitated cultures (○, △ and ●, ▲ represent a 1:1000 and 1:50 t_0 culture, respectively), samples were analyzed to determine culture density (○, ●) and bacterial invasiveness (△, ▲); see legend to Fig. 1 for details. The results from the two cultures were superimposed such that their growth curves overlapped. The upper time scale corresponds to the course of growth of the 1:1000 culture, whereas the lower corresponds to that of the 1:50 culture.

We attempted to vary the amount of oxygen available during bacterial growth by bubbling gas mixtures containing different amounts of oxygen into the culture medium. Unlike the nonagitated and agitated cultures, these bubbled cultures are more comparable since they can be grown with similar mixing and to similar culture densities. We found that *S. choleraesuis* and *S. typhimurium* grown with gases containing 0% or 1% oxygen were 6- to 70-fold more invasive than those grown with gas containing 20% oxygen (Table 1). These results confirm that *Salmonella* invasiveness is induced by oxygen-limited growth conditions.

Invasiveness of a *S. typhimurium* Mutant Defective in Anaerobic Regulation. Thirty-three proteins of *S. typhimurium* detected by two-dimensional gel electrophoresis have been identified whose synthesis is increased in response to anaerobiosis (28). The *oxrA* locus of *S. typhimurium* is required for the regulation of 14 of these proteins (26, 28). The OxrA protein is homologous to the Fnr protein of *Escherichia coli*, which functions as an oxygen-sensitive transcriptional activator during anaerobiosis (26, 29, 30). Since the expression of *Salmonella* invasiveness is induced in oxygen-limited growth conditions, we examined the invasiveness of isogenic *oxrA*$^+$/*oxrA*$^-$ *S. typhimurium* strains grown in aerobic and oxygen-limited conditions. We found that the *oxrA*$^+$ and *oxrA*$^-$ strains did not differ in their ability to enter MDCK cells. Both strains exhibited a 100-fold increase in invasiveness when grown in oxygen-limited vs. aerobic conditions (data not shown). Thus, the *oxrA* locus does not regulate the expression of *Salmonella* invasiveness.

Adherence and Entry of *S. choleraesuis* to MDCK Cells. It has been proposed that *Salmonella* initially bind to the mammalian cell surface and are subsequently internalized by a host-directed endocytic/phagocytic process (21, 31). Thus, alteration of either the binding properties or the efficiency of the internalization process would affect bacterial invasiveness. We examined whether *S. choleraesuis* from different cultures differed in their ability to bind or subsequently enter MDCK cells. A similar assay for adherence and internalization has been used to examine the interaction between the *Yersinia pseudotuberculosis* invasin protein and its mammalian receptor (32, 33).

Although *S. choleraesuis* does not bind efficiently to MDCK cells in this assay, we found that bacteria from

Table 1. Effect of oxygen availability on *S. choleraesuis* and *S. typhimurium* invasiveness

Exp.	Bacterial species	Bacterial culture	Bacterial invasiveness,* %
1	*S. choleraesuis*	Nonagitated, late log	100 ± 5
		Agitated, midlog	8 ± 0.4
		0% O_2	75 ± 0
		1% O_2	62 ± 4
		20% O_2	13 ± 2
2	*S. typhimurium*	Nonagitated, late log	100 ± 0
		Agitated, midlog	0.1 ± 0
		0% O_2	41 ± 1
		1% O_2	218 ± 18
		20% O_2	3 ± 0.3

log, Logarithmic. Bacterial cultures were grown as described in the text.

*The ability of bacteria to enter into MDCK cells (passage 8–10) during 1 hr was measured as described in the text. Assay values represent the average of two separate assays and were normalized such that the invasiveness of bacteria from the nonagitated cultures equals 100%. The actual percentage of the inoculum from the nonagitated cultures that entered the MDCK cells was 1.6% and 1.7% for experiments 1 and 2, respectively.

oxygen-limited cultures were able to adhere to MDCK cells 20- to 100-fold better than bacteria from aerobic cultures (Table 2). Bacteria from stationary phase also appeared to adhere to MDCK cells. When we measured the number of adherent bacteria that were able to subsequently enter the MDCK cells, we found that a relatively high percentage were internalized in all cases, except for the bacteria from stationary phase. Adherent stationary-phase bacteria were internalized 100-fold less efficiently, which is consistent with previous observations that such bacteria are not invasive.

Induction of *S. choleraesuis* Invasiveness in the Presence and Absence of MDCK Cells. Our laboratory reported that *S. choleraesuis* grown overnight in LB broth were not inherently able to enter MDCK cells. However, after further incubation with the mammalian cells in tissue culture medium, bacteria adherence and invasiveness were induced. It was proposed that induction might occur in response to exposure to the epithelial cell surface (18). Considering our results, we wondered if the induction seen in the previous study was actually due to the presence of the MDCK cells or to a change in bacterial growth state during the incubation period. We decided to compare the invasiveness of *S. choleraesuis* incubated with MDCK cells vs. *S. choleraesuis* incubated identically in tissue culture medium but in the absence of MDCK cells. We found that regardless of the presence of MDCK cells, bacterial invasiveness was induced 40-fold after 4 hr of incubation (Fig. 3). Thus, our results show that, in this *in vitro* system, induction of *Salmonella* invasiveness can be accounted for by growth in tissue culture medium alone.

Analysis of Proteins Synthesized by Invasive *Salmonella*. To identify the major proteins expressed by invasive *Salmonella*, we radiolabeled *S. choleraesuis* and *S. typhi* from different cultures. We compared invasive bacteria from nonagitated cultures with bacteria from agitated culture, which are at least 10-fold less invasive. We observed proteins that were preferentially produced by invasive *Salmonella* (Fig. 4). By comparison to previously published protein patterns, it appears that both of these proteins have been identified as expressed by *Salmonella* adherent to MDCK cells (18).

Table 2. Adherence and subsequent internalization of *S. choleraesuis* into MDCK cells

Exp.*	Bacterial culture	Bacterial adherence at 0°C,† %	Internalization of adherent bacteria at 37°C,‡ %
1	Nonagitated, late log	100 ± 15	59 ± 7
	Agitated, late log	26 ± 1	35 ± 2
	Agitated, midlog	0.8 ± 0.5	54 ± 11
	Stationary phase	22 ± 2	0.3 ± 0.1
2	Nonagitated, late log	100 ± 8	40 ± 5
	Agitated, late log	40 ± 0.5	16 ± 9
	Agitated, midlog	6 ± 0.4	15 ± 6
	Stationary phase	83 ± 16	0.4 ± 0.1
3	Nonagitated, late log	100 ± 16	7 ± 0.1
	0% O_2	83 ± 8	17 ± 2
	1% O_2	58 ± 7	9 ± 0.3
	20% O_2	2 ± 1	—§

log, Logarithmic. Bacterial cultures were prepared as described in the text.

*MDCK cells between passage 40 and 60 were used for experiments 1 and 2, whereas those between passage 8 and 10 were used for experiment 3.

†Assay values represent the average of two separate assays and were normalized such that the adherence of bacteria from the nonagitated cultures equals 100%. The actual percentage of the inoculum from the nonagitated cultures that were adherent was 1.6%, 1.2%, and 0.12% for experiments 1, 2, and 3, respectively.

‡Values represent the average of two separate assays.

§Values were too low to allow calculation.

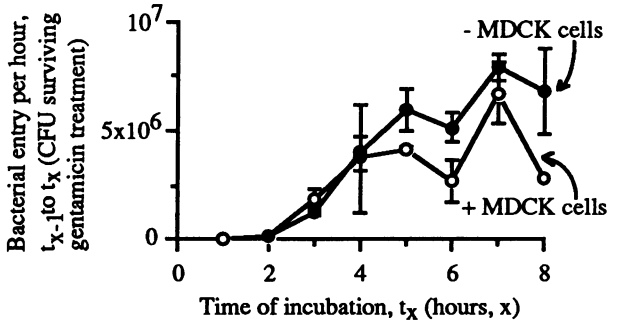

FIG. 3. Induction of *S. choleraesuis* invasiveness in the presence and absence of MDCK cells: Entry of bacteria into MDCK cells during successive 1-hr periods after incubation in the presence of MDCK cells (○) and the absence of MDCK cells (●).

DISCUSSION

This study confirms previous reports that the ability of *Salmonella* to enter mammalian cells is regulated (14, 18). Our work clarifies and extends these previous observations by identifying two distinct conditions that affect the expression of *Salmonella* invasiveness. (*i*) Stationary phase results in loss of bacterial invasiveness. In one experiment, highly invasive bacteria lost 90% of their invasiveness after a 2-hr period in stationary phase. After prolonged time in stationary phase—for example, after overnight culture—*Salmonella* were virtually noninvasive. (*ii*) Oxygen-limited growth conditions were found to induce the expression of bacterial adherence and invasiveness. Bacteria from oxygen-limited cultures were 10- to 100-fold more adherent and invasive than bacteria from aerobically growing cultures.

Thirty-three proteins of *S. typhimurium* have been identified whose synthesis is increased in response to oxygen limitation (28). We have begun to eliminate some of these proteins as candidates for mediating bacterial adherence and invasion. For example, the *oxrA*, *oxrB*, and *oxrC* loci of *S. typhimurium* are required for the induction of 19 of these proteins; *oxrA* alone is required for the induction of 14 (34). We have found that the *oxrA* locus is not essential for the regulation of *Salmonella* invasiveness. Thus, the 14 *oxrA*-regulated proteins are not required for bacterial invasion. Although, many genes have already been identified that are required for adherence and invasion, it is not known whether

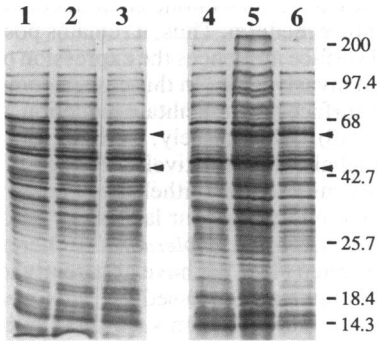

FIG. 4. Proteins synthesized by *Salmonella* grown in agitated vs. static conditions: Autoradiograph of 10% polyacrylamide gel showing [^{35}S]methionine-labeled proteins from *S. choleraesuis* (lanes 1–3) and *S. typhi* (lanes 4–6). Prior to radiolabeling, bacteria were grown in agitated LB broth (lanes 1 and 4), in nonagitated 50% LB broth/50% tissue culture medium (lanes 2 and 5), or in nonagitated tissue culture medium (lanes 3 and 6). Arrowheads indicate major proteins preferentially produced by *Salmonella* grown in tissue culture medium. The mobilities of prestained protein molecular weight standards (BRL) are indicated in kilodaltons.

any are induced by oxygen-limited growth conditions (16, 35).

Our results lead to interesting speculation about the normal events in *Salmonella* pathogenesis. Logically, the invasive property of *Salmonella* would be induced when the bacteria are within a susceptible host. Although host tissues require oxygen, the concentration of free oxygen is relatively low (pO_2 of 5–40 mmHg) since most is bound by hemoglobin (36). Thus, oxygen limitation might be one environmental cue that triggers *Salmonella* invasiveness in its host—for example, in the intestinal lumen or other tissues. The actual regulatory signal might be the concentration of molecular oxygen or another physiological property that correlates with the growth states that we have identified. For example, the anaerobic induction of cobinamide biosynthesis in *S. typhimurium* appears to be a result of a partial block in electron transport rather than the lack of oxygen *per se* (37). Once *Salmonella* invasion factors are identified, it would be instructive to examine their expression in bacteria located in different sites of infected animals. Such studies might lead to a greater understanding of the role of invasion in bacterial pathogenesis.

Bacterial growth phase may also be an important factor during pathogenesis since our studies showed that *Salmonella* lose invasiveness in stationary phase. Interestingly, such bacteria apparently remain adherent, although electron microscopy and other types of adherence assays need to be conducted to establish whether stationary-phase bacteria actually adhere to the epithelial cells. If they do, analysis of the mechanism of their adherence should be interesting since it is not understood why adherence of certain bacteria to mammalian cells leads to their internalization and adherence of others does not (38).

It was previously observed that *Salmonella* invasiveness was induced after incubation in the presence of mammalian cells. Consequently, it was proposed that invasiveness might be expressed in response to exposure to epithelial cell surfaces (18). Our study shows that induction of bacterial invasiveness does not require direct contact with epithelial cells. We have found that growth in tissue culture medium alone results in induction of bacterial invasiveness. In view of our results, the simplest explanation for the previous observation is that the bacterial inoculum was from stationary phase and thus was noninvasive, but, after a lag phase, the bacteria grew in the tissue culture medium overlying the epithelial cell monolayer and thus became invasive. Unfortunately, we could not determine the effect of exposure of bacteria to epithelial cells without simultaneous growth of the bacteria in culture medium. Thus, it remains possible that the epithelial cell surface influences the expression of *Salmonella* adherence and invasiveness. In this case, a component of the epithelial cell surface might facilitate induction, as previously hypothesized (18). Alternatively, perhaps the environment near the epithelial cell is relatively depleted of oxygen and thus close proximity to the epithelial cell leads to induction.

In a previous report from our laboratory, proteins preferentially synthesized by *S. choleraesuis* adherent to MDCK cells were identified (18). We have apparently identified two of the same proteins as expressed by *Salmonella* that were grown in tissue culture medium such that the bacteria were highly invasive. We speculate that in the previous study the adherent bacteria represent a particular population of the inoculum that changed growth state and, as a result, became able to adhere to the MDCK cells. Thus, these results together suggest that the expression of the identified proteins is indicative of a change in growth state and the ability to adhere to and enter MDCK cells, further supporting our conclusion that *Salmonella* adherence and invasiveness is modulated by changes in growth state. Although the *Salmonella* adherence and invasion factor(s) can be expressed in the absence of mammalian cells, our results do not preclude the possibility that other *Salmonella* proteins are specifically synthesized in response to exposure to mammalian cells.

We hope our results will aid in the biochemical and genetic analysis of the *Salmonella* adherence and invasion factor(s) since we have found that simply by changing *in vitro* growth conditions their expression in bacteria can be controlled.

We thank D. Pierson for advice in analyzing the data in Fig. 3. We also thank R. Isberg, D. Pierson, J. Bliska, J. St. Geme, C. Francis, and M. Starnbach for critical reading of the manuscript. This work was supported by Public Health Service Grant AI26195 (to S.F.) and a Stanford University Digestive Disease Center (Public Health Service Grant DK38707) Pilot/Feasibility Study Award (to C.A.L.). C.A.L. was a Fellow of the Arthritis Foundation.

1. Rubin, R. H. & Weinstein, L. (1977) *Salmonellosis: Microbiologic, Pathologic, and Clinical Features* (Stratton Intercontinental Medical Book, New York), pp. 25–34.
2. Carter, P. B. & Collins, F. M. (1974) *J. Exp. Med.* **139,** 1189–1203.
3. Heffernan, E. J., Fierer, J., Chikami, G. & Guiney, D. (1987) *J. Infect. Dis.* **155,** 1254–1259.
4. Takeuchi, A. (1966) *Am. J. Pathol.* **50,** 109–136.
5. Wallis, T. S., Starkey, W. G., Stephen, J., Haddon, S. J., Osborne, M. P. & Candy, D. C. A. (1986) *J. Med. Microbiol.* **22,** 39–49.
6. Worton, K. J., Candy, D. C. A., Wallis, T. S., Clarke, G. J., Osborne, M. P., Haddon, S. J. & Stephen, J. (1989) *J. Med. Microbiol.* **29,** 283–294.
7. Baloda, S. B., Faris, A. & Krovacek, K. (1988) *Microbiol. Immunol.* **32,** 447–459.
8. Gianella, R. A., Washington, O., Gemski, P. & Formal, S. B. (1973) *J. Infect. Dis.* **128,** 69–75.
9. Jones, G. W. & Richardson, L. A. (1981) *J. Gen. Microbiol.* **127,** 361–370.
10. Jones, G. W., Richardson, L. A. & Uhlman, D. (1981) *J. Gen. Microbiol.* **127,** 351–360.
11. Kihlstrom, E. & Edebo, L. (1976) *Infect. Immun.* **14,** 851–857.
12. Lindquist, B. L., Lebenthal, E., Lee, P.-C., Stinson, M. W. & Merrick, J. M. (1987) *Infect. Immun.* **55,** 3044–3050.
13. Tavendale, A., Jardine, C. K. H., Old, D. C. & Duguid, J. P. (1983) *J. Med. Microbiol.* **16,** 371–380.
14. Finlay, B. B., Fry, J., Rock, E. P. & Falkow, S. (1989) *J. Cell Sci. Suppl.* **11,** 99–107.
15. Finlay, B. B., Gumbiner, B. & Falkow, S. (1988) *J. Cell Biol.* **107,** 221–230.
16. Finlay, B. B., Starnbach, M. N., Francis, C. L., Stocker, B. A., Chatfield, S., Dougan, G. & Falkow, S. (1988) *Mol. Microbiol.* **2,** 757–766.
17. Rodriguez-Boulan, E. & Nelson, W. J. (1989) *Science* **245,** 718–725.
18. Finlay, B. B., Heffron, F. & Falkow, S. (1989) *Science* **243,** 940–943.
19. Nnalue, N. A. & Stocker, B. A. D. (1986) *Infect. Immun.* **54,** 635–640.
20. Guinee, P. A. M., Jansen, W. H., Maas, H. M. E., LeMinor, L. & Beaud, R. (1981) *Ann. Microbiol. (Paris)* **132A,** 331–334.
21. Finlay, B. B. & Falkow, S. (1988) *Biochimie* **70,** 1089–1099.
22. Gumbiner, B. & Simons, K. (1986) *J. Cell Biol.* **102,** 457–468.
23. Vaudaux, P. & Waldvogel, F. A. (1979) *Antimicrob. Agents Chemother.* **16,** 743–749.
24. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
25. Kjelleberg, S., Hermansson, M., Marden, P. & Jones, G. W. (1987) *Annu. Rev. Microbiol.* **41,** 25–49.
26. Strauch, K. L., Lenk, J. B., Gamble, B. L. & Miller, C. G. (1985) *J. Bacteriol.* **161,** 673–680.
27. Khosla, C. & Bailey, J. E. (1988) *Nature (London)* **331,** 633–635.
28. Spector, M. P., Aliabadi, Z., Gonzalez, T. & Foster, J. W. (1986) *J. Bacteriol.* **168,** 420–424.
29. Spiro, S. & Guest, J. R. (1988) *Mol. Microbiol.* **2,** 701–707.
30. Trageser, M. & Unden, G. (1989) *Mol. Microbiol.* **3,** 593–599.
31. Moulder, J. W. (1985) *Microbiol. Rev.* **49,** 298–337.
32. Isberg, R. R. (1989) *Mol. Microbiol.* **3,** 1449–1453.
33. Isberg, R. R., Voorhis, D. L. & Falkow, S. (1987) *Cell* **50,** 769–778.
34. Alibadi, Z., Park, Y. K., Slonczewski, J. L. & Foster, J. W. (1988) *J. Bacteriol.* **170,** 842–851.
35. Galan, J. E. & Curtiss, R., III (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6383–6387.
36. Guyton, A. C. (1977) *Basic Human Physiology: Normal Function and Mechanisms of Disease* (Saunders, Philadelphia), pp. 408–423.
37. Andersson, D. I. & Roth, J. R. (1989) *J. Bacteriol.* **171,** 6734–6739.
38. Finlay, B. B. & Falkow, S. (1989) *Microbiol. Rev.* **53,** 210–230.