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Temperature-Dependent Expression of Virulence Genes in *Shigella* Species

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The pathogenicity of *Shigella* spp. involves the ability of the bacteria to penetrate and replicate within the epithelial cells of the large intestine. Model systems for examining the virulence of shigellae employ Henle intestinal epithelial cells in tissue culture and an in vivo assay for virulence in guinea pig eyes (Sereny test). Using these systems, we studied the genetic and physiological bases for the ability of shigellae to invade epithelial cells. We found that expression of virulence in *Shigella* spp. is dependent on the temperature at which the bacteria are grown. When grown at 37°C, strains of *Shigella flexneri* 2a, *Shigella sonnei*, and *Shigella dysenteriae* 1 were fully virulent and invaded Henle cells. They also produced keratoconjunctivitis in guinea pigs. When grown at 30°C, the bacteria neither penetrated Henle cells nor produced conjunctivitis in the Sereny test and were phenotypically avirulent. Strains grown at 33°C were only partially invasive in the Henle assay, whereas strains grown at 35°C were as invasive as strains grown at 37°C. Using the Henle cell assay, we determined that the loss of ability to penetrate epithelial cells was completely reversed by shifting the growth temperature from 30 to 37°C. The percentage of Henle cells invaded by bacteria increased with increasing time of growth at 37°C. Restoration of invasiveness after growth at 30°C required protein synthesis. When shigellae were grown at 30°C and shifted to 37°C for 2 h in the presence of chloramphenicol, the bacteria remained noninvasive. Similarly treated bacteria grown at 37°C were still invasive. These results suggested that expression of one or more genes required for virulence of *Shigella* spp. are subject to regulation by growth temperature.

Bacteria of the genus *Shigella* are capable of penetrating and multiplying within the epithelial cells of the colon (15). The infection produces ulcerative lesions in the colonic mucosa and results in the bloody mucous diarrhea characteristic of bacillary dysentery (7). Reliable model systems that measure the pathogenic potential of *Shigella* spp. have been developed, and these include infection of guinea pig conjunctiva (Sereny test [25]) and in vitro infection of epithelial cells in culture (9). In both assay systems, virulent strains of *Shigella* spp. which are capable of producing dysentery in humans can invade the epithelial cells and multiply within them, whereas avirulent strains do not invade these cells. These assay systems have been useful in defining genetic determinants required for virulence. For example, Sansonetti et al. have demonstrated that virulence is associated with the presence of a 120-megadalton plasmid in *Shigella sonnei* and a 140-megadalton plasmid (pSf2a140) in *Shigella flexneri* 2a (23, 24).

We are interested in examining the genetics of pathogenesis of *S. flexneri* 2a and in determining how expression of virulence genes may be regulated. In this study we report that the temperature at which the organisms are grown affects the ability of the bacteria to invade epithelial cells in both in vitro and in vivo assays for virulence. Bacteria grown at 37°C are virulent, invade tissue culture cells, and produce a positive Sereny reaction, whereas bacteria grown at 30°C are avirulent and fail to invade epithelial cells in both assays. The temperature-induced loss of virulence is reversed by subsequent growth of the bacteria at 37°C, but protein synthesis is required for virulence to be reexpressed. We also extended these observations to the other common *Shigella* species *Shigella dysenteriae* 1 and *S. sonnei* and conclude that expression of at least some of the genes required for virulence of *Shigella* spp. are subject to regulation by growth temperature.

(A preliminary report of this work was presented in part previously [A. T. Maurelli, B. Blackmon, and R. Curtiss III, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, B124, p. 44].)

MATERIALS AND METHODS

Bacterial strains. All strains used in this paper, except BS98, were kindly provided by T. L. Hale and S. B. Formal (Walter Reed Army Institute of Research, Washington, D.C.). BS98 is a spontaneous avirulent derivative of *S. flexneri* 2a strain 2457T (6) derived in this laboratory. *S. dysenteriae* 1 strain 3818T and its avirulent colonial variant strain 38180 have been previously described (8), as has *S. sonnei* 482-79I (22).

Media. L broth (16) was used as the complete medium for growth of the bacterial strains used. Pigmentation was determined by plating cultures on tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing 0.003% Congo red dye (Sigma Chemical Co., St. Louis, Mo.).

Assays for virulence. (i) Sereny test. Bacterial cultures were grown overnight with aeration to a density of about 2×10^9 cells per ml. The cultures were washed once and suspended in 50 μl of phosphate-buffered saline. A 25-μl portion of a washed culture was carefully dripped onto the eye of an adult albino guinea pig. Strains which produced keratoconjunctivitis within 96 h were considered virulent (25).

(ii) Tissue culture infection. Bacterial strains were assayed for virulence in tissue culture cells with the Henle 407 cell line (12). The cells were maintained in basal Eagle medium (Flow Laboratories, Inc., McLean, Va.) supplemented with

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15% newborn calf serum (Flow) and 2 mM glutamine (Flow). Cells were plated at 1 to 2 × 10^5 cells per tissue culture dish (35 by 10 mm; Lux Scientific Corp., Newbury Park, Calif.) and grown at 37°C in 5% CO_2 for 48 h before infection with bacteria. Cell monolayers were infected with bacteria by a modified version of the procedure of Hale and Formal (10). Bacterial cultures were harvested in exponential phase, washed once with phosphate-buffered saline, and suspended in basal Eagle medium to a concentration of 1 to 2 × 10^8 cells per ml. Samples of bacteria (1 ml) were added to Henle monolayers in tissue culture dishes. The dishes were immediately centrifuged at room temperature at 3,000 rpm for 10 min in a Sorvall GLC-2B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The dishes were then incubated for 3 h at 37°C in 5% CO_2 to allow for invasion of the Henle cells by the bacteria and for growth of the bacteria within the cells. After 3 h, the monolayers were washed with phosphate-buffered saline, fixed with methanol, and stained with Giemsa solution. Stained monolayers were examined by phase-contrast microscopy for bacterial invasion of Henle cells.

Isolation of plasmid DNA and agarose gel electrophoresis. The procedure of Kado and Liu (14) was used to isolate plasmid DNA from all of the strains used. A 10-μl sample of the phenol-chloroform-extracted aqueous phase was mixed with 3 μl of tracking dye (25% sucrose, 0.05% bromophenol blue, 0.1% sodium dodecyl sulfate, 5 mM sodium acetate) and loaded onto a 0.7% horizontal agarose gel. Electrophoresis with a Tris acetate buffer system (11) was carried out at 2.5 V/cm until the dye entered the gel. Voltage was then increased to 7.5 V/cm for the rest of the run. Gels were stained in a solution of 2 μg of ethidium bromide per ml (Sigma), and a short-wavelength UV light source (Transilluminator model C-61, Ultra-Violet Products, Inc., San Gabriel, Calif.) was used to visualize the DNA in the gel.

RESULTS

Effect of growth temperature on virulence of *Shigella* spp. The determination of virulence for *Shigella* spp. was done in both a tissue culture invasion model and an animal model for invasiveness. With Henle 407 human intestinal epithelial cells, we measured the ability of invasive strains of three different *Shigella* species to invade the cells after growth at different temperatures. When virulent strains were grown at 37°C before adding them to Henle monolayers, the bacteria were invasive, and usually greater than 90% of the cells in a monolayer could be seen to have bacteria in the cytoplasm (Fig. 1a). However, when virulent bacteria were grown at 30°C and then added to monolayers, the bacteria failed to invade the cells. Less than 10% of the cells in a challenged monolayer were seen to have bacteria in the cytoplasm (Fig.

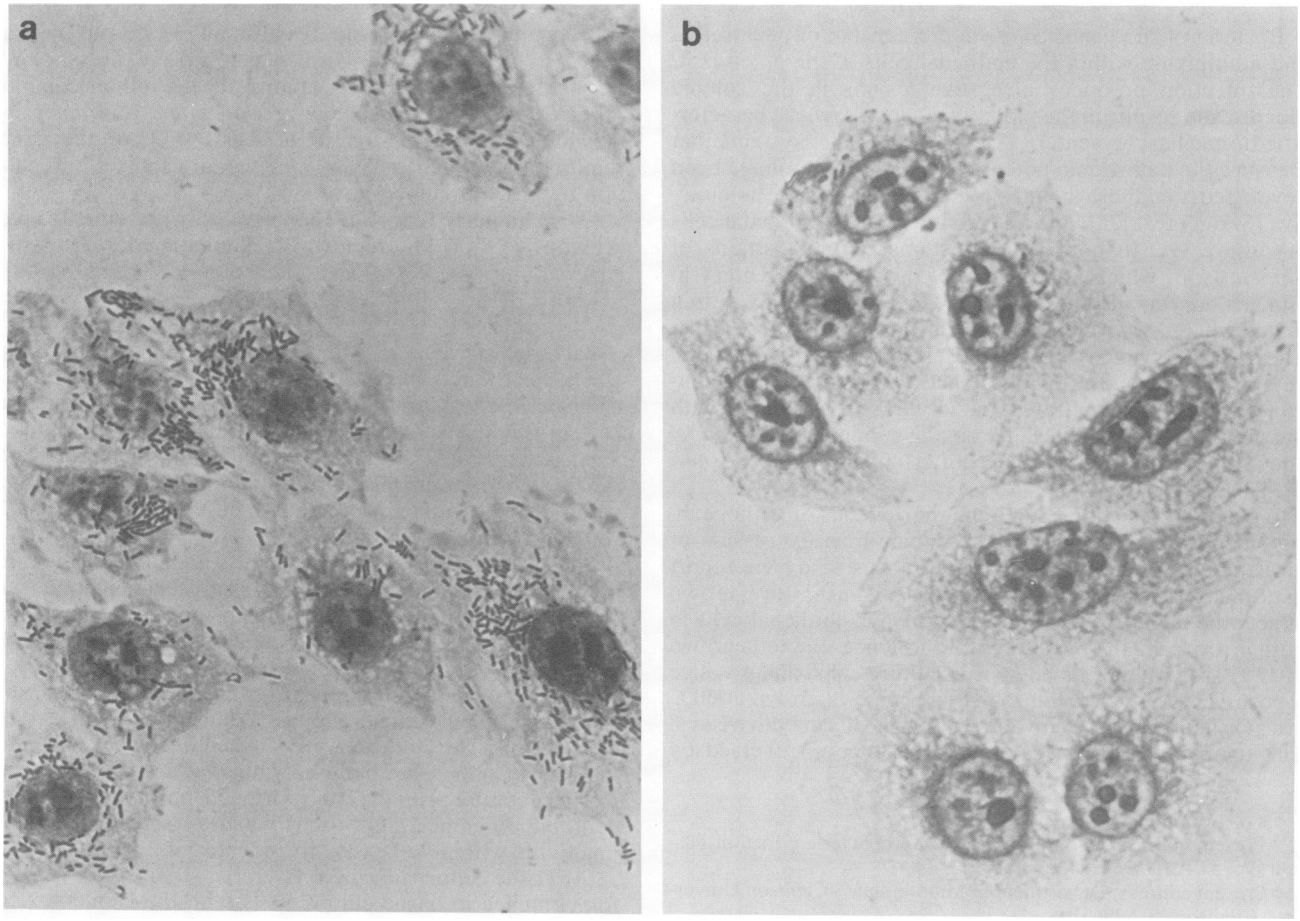

FIG. 1. Henle epithelial cell monolayers challenged with *S. flexneri* 2a. Phase-contrast photomicrograph of monolayers after 3 h of incubation with bacteria grown at 37°C (a) and 30°C (b).

1b). Cells in these monolayers were also free of adherent bacteria and, except for the occasional infected cell, looked like monolayers which had been challenged with an avirulent strain. *S. flexneri* 2a strain 2457T was also grown at 35 and 39°C, and the bacteria still retained their ability to invade over 90% of the Henle cells. When grown at 33°C, strain 2457T penetrated less than 50% of the cells in the challenged monolayer. We found that growth of virulent strains of *S. flexneri* 2a, *S. sonnei*, and *S. dysenteriae* 1 at 30°C inhibited expression of invasiveness of these strains for Henle cells.

The Sereny test (25) is frequently used as a reference assay for virulence of invasive pathogens, and positive Sereny tests have been shown to correlate well with demonstrated virulence in experimental infections involving *Shigella* spp. We, therefore, sought to determine whether the temperature at which a virulent culture was grown would affect its ability to produce a positive Sereny reaction. Strains grown at 30 and 37°C were inoculated onto the eyes of guinea pigs to assay for virulence. *Shigella* cultures which were grown at 37°C produced keratoconjunctivitis within 3 days after inoculation, thus demonstrating ability to invade the corneal epithelium. These same strains which were virulent when grown at 37°C failed to produce conjunctivitis when the bacteria were grown at 30°C before infection of the eye. Therefore, the temperature at which the bacteria were grown affected the expression of invasiveness in both tissue culture cells and the Sereny test. Analysis of 23 biochemical properties of *S. flexneri* 2a was performed using a microtube system (API 20E; Analytab Products, Plainview, N.Y.). No differences were found between the biochemical reactions of *S. flexneri* 2a grown and tested at 30 and 37°C.

Temperature-dependent pigmentation on Congo red medium. The absorption of the dye Congo red by virulent *Shigella* strains is a useful way of distinguishing these strains from avirulent *Shigella* strains (20). On Congo red agar, colonies formed by virulent shigellae appear pigmented due to binding of the dye, whereas avirulent shigellae are nonpigmented. When Congo red plates spread with virulent shigellae were incubated at 30°C, the colonies appeared nonpigmented and were indistinguishable from colonies of avirulent shigellae. This temperature-dependent capacity to bind Congo red reflects the actual invasive ability of these strains when grown at 30 or 37°C. Table 1 summarizes the results of the virulence assays and the effects of growth temperature.

Stability of the invasive phenotype. We measured the rate of loss of invasive ability of strain 2457T when the culture was shifted from growth at 37 to 30°C. When shifted to the lower temperature, the ability of 2457T to invade Henle cells steadily decreased with increasing time of growth at 30°C (Fig. 2). After about two to three generations (90 min) at 30°C, the strain was able to invade only 60% as many cells as could a control culture grown at 37°C.

TABLE 1. Summary of virulence properties of shigellae grown at 30 and 37°C

Strain	% of infected Henle cells at:		Sereny assay at:		Pigmentation on Congo red medium at:	
	30°C	37°C	30°C	37°C	30°C	37°C
S. dysenteriae 1						
3818T	<1	90	−	+	−	+
38180	<1	<1	ND[a]	−	−	−
S. flexneri 2a						
2457T	7	90	−	+	−	+
BS98	<1	<1	ND	−	−	−
S. sonnei 482-79I	ND	ND	−	+	−	+

[a] ND, Not determined.

Plasmid profiles of virulent shigellae grown at 30 and 37°C. The above results demonstrated that expression of virulence in shigellae is dependent on the temperature at which the bacteria are grown. We next wished to determine whether the loss of virulence on growth at 30°C was due to a genotypic or phenotypic change in the organism; that is, whether the growth temperature induced a mutation-like event resulting in loss of virulence or whether temperature regulated the expression of virulence.

Sansonetti et al. (23) have demonstrated that virulence in *S. sonnei* is associated with the presence of a 120-megadalton plasmid and that virulence in *S. flexneri* involves genetic determinants on a 140-megadalton plasmid (24). We, therefore, examined our virulent *Shigella* strains to determine whether the plasmids associated with virulence were lost during growth of the strains at 30°C. The plasmid profiles of these strains were identical whether they were grown at 30 or 37°C (Fig. 3). Thus, we can exclude a temperature-induced curing of the virulence plasmid as an explanation for the loss of virulence after growth at 30°C.

Low-temperature-induced avirulence is reversed by subsequent growth at 37°C. If growth at 30°C induced a mutational event (genotypic change) which abolished avirulence, we would expect that the loss of virulence would be irreversible, in the case of a deletion, or reversed only at a low frequency in the case of other types of genetic alterations. The other possibility is that growth at 30°C causes a physiological (phenotypic) change in the organism such that either essential virulence components are not synthesized or they are present but not functional. To distinguish between these possibilities, we asked whether shigellae grown at 30°C could reexpress the virulent phenotype after growth at 37°C and how long it takes to reexpress virulence.

Strain 2457T, a virulent strain of *S. flexneri* 2a, was grown overnight in L broth at 30°C. The overnight culture was diluted 1:100 into tubes containing 1.2 ml of L broth. The tubes were then shaken at 30°C in a gyratory water bath before shifting to 37°C. After incubation with shaking at 37°C for different lengths of time, the cultures were tested for their ability to invade Henle cells. Strain 2457T grown at 30°C was unable to invade Henle cells (Fig. 4). Shifting the growth temperature from 30 to 37°C restored invasiveness to the strain. After growth at 37°C for about 75 min before being added to Henle cells, 2457T expressed 50% of the invasive ability of a control culture grown at 37°C. This period of growth at 37°C represents two and one-half to three doublings of the culture. A 100% restoration of the invasive capability of the culture grown at 30°C resulted after growing the bacteria at 37°C for 150 min (approximately four and one-half to five doublings). In similar experiments with *S. dysenteriae* 1, we found that loss of invasiveness due to growth at 30°C was also completely reversed by subsequent growth of the strain at 37°C (data not shown). These experiments demonstrated that the loss of invasiveness of shigellae grown at 30°C is transient and is reversed by a shift in growth temperature to 37°C.

The ability of the bacteria to bind Congo red was also reexpressed after a shift in growth temperature to 37°C. Strains of virulent shigellae were grown at 30°C in L broth and plated on Congo red plates. The plates were incubated at 37 and 30°C. All of the colonies formed on Congo red plates

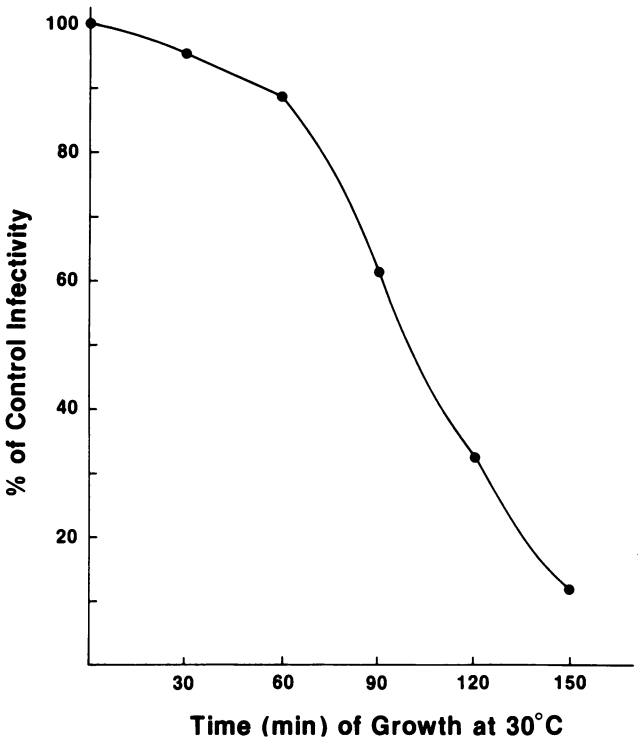

FIG. 2. Loss of invasive ability of *S. flexneri* 2a after downshift from growth at 37°C to growth at 30°C. Strain 2457T was grown at 37°C overnight with aeration, diluted 1:100 into L broth, and grown at 37°C with aeration. Cultures were shifted to 30°C for different lengths of time and then assayed for invasiveness in Henle monolayers. The percentage of infected cells was scored after 3 h of incubation of bacteria with monolayers. A control sample, subcultured from a 37°C overnight culture and grown at 37°C, infected 95% of the Henle cells in the monolayers.

incubated at 37°C were pigmented, whereas colonies on Congo red plates incubated at 30°C were nonpigmented. None of the colonies were sectored. Nonpigmented colonies from the 30°C plate were replica plated to fresh Congo red plates and incubated at 37°C to assess the extent of reexpression of pigmentation. All 350 colonies replica plated from the 30°C-plate became pigmented when incubated at 37°C for 18 h. This suggested that the entire population of virulent shigellae grown at 30°C was capable of reexpressing the ability to bind the dye after a shift in temperature to 37°C.

Induction of virulence by temperature upshift requires protein synthesis. We considered two hypotheses which could explain the physiological effect which growth temperature has on the display of virulence by shigellae. First, temperature-induced conformational changes in proteins essential for virulence could alter the virulence capabilities of a pathogen. Since the ambient temperature of the host (human) is 37°C, it is possible that this is the optimal temperature for function of proteins involved in cell-cell interactions of shigellae with the colonic epithelium. This hypothesis predicts that the macromolecules required for virulence are present at 30°C and can be restored to function by shifting the temperature to 37°C. One may argue that the temperature shift would be followed by an immediate recovery of function of these proteins and, therefore, immediate reexpression of the virulence phenotype. The data in Fig. 3 would, therefore, appear to rule out this hypothesis since there is a delay in reexpression of virulence. A second hypothesis is that growth temperature is the signal which regulates the expression of virulence genes in shigellae at the transcriptional or translational level. This model predicts that de novo protein synthesis at 37°C is required for a strain grown at 30°C to express the virulent phenotype. The following experiment was designed to determine whether protein synthesis at 37°C was required to restore the virulent phenotype to a strain grown at 30°C. Overnight cultures of *S. flexneri* 2a grown at 30 and 37°C were diluted 1:100 into L broth and shaken at 30 and 37°C, respectively, for 2 h. Titers were determined for the cultures, which had reached a density of about 1.5×10^8 cells per ml, and chloramphenicol, a reversible inhibitor of protein synthesis, was added to a final concentration of 1.25 μg/ml. This level of chloramphenicol was sufficient to rapidly shut down protein synthesis as measured by incorporation of [^{3}H]leucine into hot trichloroacetic acid-precipitable activity. Both cultures were incubated at 37°C for another 2 h, washed with phosphate-buffered saline to remove chloramphenicol, and then tested for invasiveness in the Henle tissue culture assay. Control cultures that were shifted from 30 to 37°C without adding chloramphenicol were also assayed for invasiveness. Culture densities were adjusted so that Henle monolayers were challenged

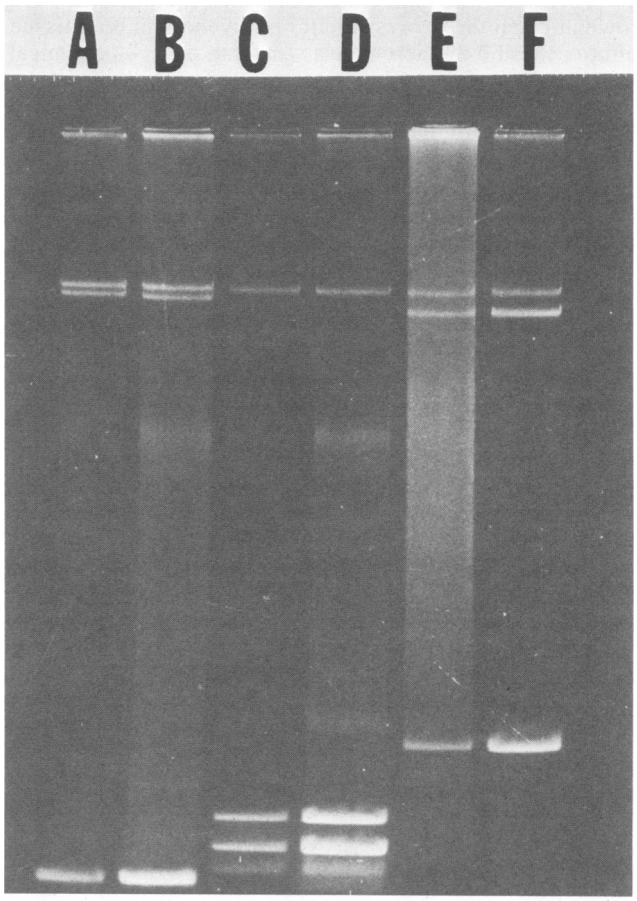

FIG. 3. Agarose gel electrophoresis of plasmid DNA from *Shigella* spp. grown at 30 and 37°C. Plasmid DNA was prepared from *Shigella* strains by the procedure of Kado and Liu (14) and electrophoresed on a 0.7% horizontal agarose gel. Lanes: A, *S. flexneri* 2a strain 2457T grown at 30°C; B, strain 2457T grown at 37°C; C, *S. sonnei* 482-79I grown at 30°C; D, strain 482-79I grown at 37°C; E, *S. dysenteriae* 1 strain 3818T grown at 30°C; F, strain 3818T grown at 37°C.

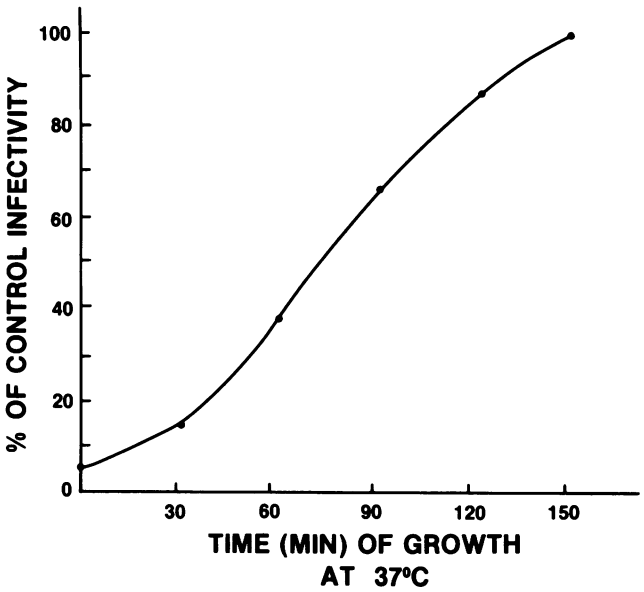

FIG. 4. Reversal of low-temperature-induced avirulence by growth at 37°C. *S. flexneri* 2a strain 2457T was grown at 30°C overnight with aeration, diluted 1:100 into L broth, and grown at 30°C with aeration. Cultures were shifted to 37°C for different lengths of time and then assayed for invasiveness in Henle monolayers. The percentage of infected cells was scored after 3 h of incubation of bacteria with monolayers. A control sample, subcultured from a 37°C overnight culture and grown at 37°C, infected 95% of the Henle cells in the monolayer.

with 1 to 2 × 10^8 bacteria in 1.0 ml of basal Eagle medium. The culture grown at 30°C was fully invasive after growth at 37°C for 2 h (Fig. 5, sample B). In contrast, the culture grown at 30°C did not express invasiveness after incubation at 37°C for 2 h in the presence of chloramphenicol (sample A). These results indicated that protein synthesis is required for a phenotypically avirulent strain grown at 30°C to express virulence when shifted to 37°C. We should also point out that chloramphenicol treatment of a culture grown at 37°C did not abolish its virulence potential in the Henle assay (sample C). Thus, we have demonstrated that de novo protein synthesis is required to restore virulence after the temperature shift.

DISCUSSION

In the experiments reported in this paper, we have shown that expression of virulence genes in *S. flexneri*, *S. sonnei*, and *S. dysenteriae* is regulated by growth temperature. The loss of virulence at 30°C does not involve a true genetic alteration but is completely reversible by adjusting the growth temperature to 37°C. This was established by the observation that virulent shigellae grown at 30°C are noninvasive in the Henle assay for virulence but reexpress the ability to invade Henle cells after a period of growth at 37°C. Replica-plating experiments for pigmentation, a property associated with virulent *Shigella* strains, demonstrated that the entire population of bacteria can reexpress the ability to bind Congo red after a shift in growth temperature from 30 to 37°C. If we assume that pigmentation is a phenotype associated with expression of factors required for invasion of epithelial cells by shigellae, then these results also indicate that reexpression of virulence determinants occurs throughout the bacterial population after a temperature shift up to 37°C. Therefore, it is not likely that clonal expansion during growth at 37°C of a small fraction of the bacteria previously grown at 30°C is responsible for the high levels of infected Henle cells observed in the temperature upshift experiments. Restoration of virulence, as measured by the ability of the bacteria to invade Henle cells, requires protein synthesis after the shift from 30 to 37°C. Consequently, it is reasonable to conclude that temperature-regulated expression of one or more virulence genes in *Shigella* spp. is controlled at the transcriptional or translational level.

In the Henle assay for virulence, we observed a consistently low level of infected cells in monolayers challenged with virulent shigellae grown at 30°C. This is probably the result of the gradual reexpression of virulence of the bacteria during the course of the assay, which involves incubation of the bacteria with the monolayer for 3 h at 37°C. In experiments in which the challenged Henle monolayers were incubated at 30°C for 3 h immediately after centrifuging the bacteria onto the cells, virulent shigellae grown at 30°C failed to invade the cells and little or no background of infected cells was detected (less than 1%). Under these same conditions, virulent shigellae grown at 37°C still were able to invade greater than 90% of the cells in the monolayer. Thus the low background of infected cells in monolayers challenged with bacteria grown at 30°C represents progressive reexpression of components necessary for invading Henle cells during the 3-h incubation period at 37°C.

Virulent *Shigella* strains which are grown at 37°C penetrate the corneal epithelium of a guinea pig in the Sereny test, and the first symptoms of conjunctivitis appear within a few hours. When grown at 30°C, these strains fail to penetrate the epithelium and do not induce conjunctivitis. Because these phenotypically avirulent strains can reexpress the virulent phenotype after growth at 35°C, one might have expected conjunctivitis to develop after an initial lag period if the surface temperature of the eye was 35°C or higher.

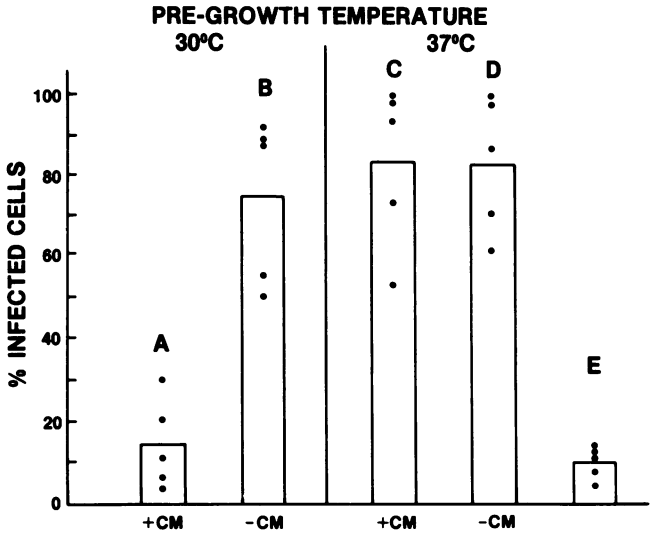

FIG. 5. Effect of inhibition of protein synthesis on reexpression of virulence at 37°C. Columns A and B represent *S. flexneri* 2a cultures grown at 30°C and then shifted to 37°C in the presence (A) or absence (B) of 1.25 μg of chloramphenicol per ml. Cultures represented by columns C and D were grown at 37°C and then incubated with (C) or without (D) chloramphenicol at 37°C in parallel with cultures A and B. Column E represents *S. flexneri* 2a cultures grown at 30°C without a subsequent shift to 37°C and in the absence of chloramphenicol. All columns represent the mean value of five independent experiments, the individual values of which are indicated by solid dots.

Although no measure of the corneal temperature of the guinea pig appears in the literature, it is reasonable to assume that the temperature would be close to those reported for rabbits and humans; i.e. 35.7 and 34.8°C, respectively (21). However, the bacteria which do not quickly invade the epithelium are destroyed by the large numbers of polymorphonuclear neutrophils which are found in the conjunctival fluid after inoculation of the bacteria onto the eye (26). This rapid clearance of bacteria from the eye would preclude any delayed development of conjunctivitis due to reexpression of virulence determinants in situ.

We found that *S. flexneri* 2a strain 2457T can be grown at temperatures between 35 and 39°C and still retain its capacity for invading Henle cells. Bacteria grown at 33°C exhibited a very poor ability to invade Henle cells. This shift in phenotype over a 2°C range may indicate that the expression of virulence is sharply defined between 33 and 35°C and activated within this narrow range. However, it will be necessary to identify and quantitate temperature-inducible virulence factors to determine whether this interpretation is correct.

The influence of growth temperature on expression of virulence genes may prove to be a common characteristic of bacterial pathogens of mammals. Production of adhesion antigens by some enterotoxigenic *Escherichia coli* strains is temperature dependent. For example, a large number of calf and lamb isolates produce a proteinaceous surface antigen called K99 which promotes colonization of the small intestine (4, 19, 27). Optimal amounts of K99 antigen are produced at 37°C, and little or no K99 is produced at temperatures less than 32°C (4, 5). Invasive strains of *E. coli* which carry the Vir plasmid attach to intestinal brush borders when grown at 37°C. Vir^+ strains grown at 18°C do not attach to brush borders, nor do they react with antisera raised against the same strain grown at 37°C and preabsorbed with an isogenic Vir^- strain (17). Mannose-resistant hemagglutination is expressed in *Salmonella typhimurium* cultures grown at 37°C but not in cultures grown at 18°C (13). Mannose-resistant hemagglutination is also correlated with the ability of salmonellae to attach to HeLa cells, an attachment which is reduced in cultures grown at 18°C. The K1 capsular polysaccharide antigen of *E. coli*, which plays an important antiphagocytic role in bacterial meningitis, is also produced at 37°C but not at lower temperatures (2). A characteristic of virulent strains of *Yersinia pestis* is calcium dependency at 37°C (3). Recent evidence indicates that calcium dependency is a virulence factor and that genes required for calcium dependency in *Y. pestis* show increased transcription at 37°C compared with the level of transcription at 26°C (J. D. Goguen, J. Yother, and S. C. Straley, manuscript in preparation). Virulence of *Y. pestis* is also greater at 37 than at 26°C (18). A unique outer membrane protein has been observed in *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* strains grown at 37°C (1). This protein, which is present in reduced amounts in strains grown at 30°C, appears to be associated with the virulence plasmid. However, because the virulence of these strains in contrast to that of *Y. pestis* is greater at 26 than at 37°C (1), the role of this temperature-inducible protein in virulence is unclear.

The reliance on temperature as a signal to trigger expression of virulence genes would work very well for an organism which moves back and forth from the biosphere to the relatively constant temperature environment of a mammalian host. It is reasonable to speculate that a pathogen would have no need to express virulence determinants such as adhesive appendages until it was in a host. Therefore, a control system which could sense the host environment (via temperature) is an efficient way of regulating expression of cellular products until they are required. This type of control circuit is analogous to those found in the operons for carbohydrate utilization in *E. coli* such as the *lac* operon. In this system, expression of the enzymes for metabolizing lactose is regulated by the presence or absence of the sugar substrate (29). Thus, high temperatures (37°C) may act as an inducer for expression of virulence genes just as lactose is an inducer for expression of the *lac* operon.

We would like to identify *Shigella* proteins which are made at 37 and not at 30°C and to determine which of these proteins are essential for virulence. Our observation that shigellae grown at 30°C do not even attach to Henle cells suggests that an outer membrane or cell wall component necessary for attachment and invasion may not be produced at this temperature. The failure of shigellae to absorb Congo red when plated at 30°C also indicates that some components on the surface of the bacteria are not present at the lower temperature. Pigmented strains of *Y. pestis* have been shown to possess a 140,000-molecular-weight outer membrane protein which is not present in nonpigmented mutants (28), and it is possible that pigmentation in shigellae may also be associated with a unique outer membrane protein.

Growth temperature may act to affect the expression of a single gene or a group of genes. These genes may, in turn, act directly or indirectly to control the expression of the ability of the organism to invade epithelial cells. Our current efforts are directed toward determining the mechanism by which temperature regulates virulence gene expression in *S. flexneri* 2a and also toward attempting to identify the gene products affected. In a separate paper, we will describe the isolation of a Mu *d*1 (Ap^r *lac*) insertion in a virulence gene which demonstrates that growth temperature control acts at the transcriptional level (A. T. Maurelli and R. Curtiss III, manuscript in preparation). This approach will also enable us to identify specific virulence determinants whose expression is regulated by growth temperature.

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