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Loss of Pigmentation in *Shigella flexneri* 2a Is Correlated with Loss of Virulence and Virulence-Associated Plasmid

ANTHONY T. MAURELLI,† BARBARA BLACKMON,‡ AND ROY CURTISS III§*

Department of Microbiology, University of Alabama in Birmingham, Birmingham, Alabama 35294

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In this study, we examined the relationship between the virulence of *Shigella flexneri* 2a and the ability of strains of *S. flexneri* 2a to absorb Congo red. Spontaneous nonpigmented (i.e., unable to bind Congo red [Pcr$^-$]) derivatives of a virulent, pigmented (Pcr$^+$) strain of *S. flexneri* 2a were isolated and assayed for virulence as determined by their ability to invade epithelial cells. All Pcr$^-$ mutants examined lost the ability to invade epithelial cells and were thus avirulent. Agarose gel electrophoresis of plasmid DNA from these avirulent, Pcr$^-$ mutants showed that the majority of these strains had lost a plasmid band corresponding to a virulence-associated plasmid, pSf2a140. In many of the mutants, concomitant loss of pigmentation, virulence, and pSf2a140 was accompanied by the appearance of a new plasmid, smaller than pSf2a140. We believe these new plasmids to be deletion derivatives of pSf2a140 and that loss of pigmentation and loss of virulence are associated with deletions in pSf2a140. We transduced Pcr$^-$ mutants to Pcr$^+$ and isolated transductants which suppressed the Pcr$^-$ phenotype. None of the Pcr$^+$ transductants regained the ability to invade epithelial cells. Several suppressors of the Pcr$^-$ phenotype were identified as mutations in cell wall biosynthesis. These results support our belief that although pigmentation is usually associated with virulence, genetic determinants unrelated to virulence can also affect the ability of the cell to bind Congo red. Therefore, the ability of *S. flexneri* 2a to bind Congo red does not necessarily imply the ability to invade epithelial cells. However, loss of ability to bind Congo red is accompanied by loss of virulence.

Genetic analysis of determinants of bacterial virulence is made difficult by the lack of easily scored markers which distinguish the virulent phenotype from the avirulent phenotype. Avirulent mutants of some pathogens have been recognized by their appearance as colonial variants in a background of virulent colonies (12, 16, 19, 24). For example, opaque variants arise from translucent colonies of virulent *Shigella flexneri* 2a on meat extract agar at a frequency of once in every 10^4 to 10^5 cell divisions, and these variants are avirulent (2, 16). However, such mutants were found to have pleiotrophic defects which accompanied the transition to the opaque colonial morphology (5). As such, genetic analysis to identify the specific defect responsible for loss of virulence is very difficult.

Another colonial phenotype which has been associated with the loss of virulence is the loss of ability to absorb the dye Congo red (24). Colonies of virulent strains of many different gram-negative pathogens, including *S. flexneri*, can absorb the dye from agar medium and thus appear pigmented (Pcr$^+$). Avirulent derivatives of these pathogenic strains are nonpigmented (Pcr$^-$ [21]). However, because the strain of *S. flexneri* tested by Payne and Finkelstein (21) was the same opaque colonial variant described earlier (16) which had multiple defects, one cannot determine whether loss of pigmentation alone is responsible for loss of virulence. The pleiotrophic nature of the mutation in this strain precludes the association of any one phenotype with loss of virulence.

We wished to determine whether the Pcr$^-$ phenotype was consistently accompanied by loss of virulence in *S. flexneri* 2a and whether all Pcr$^-$ mutants exhibited the same genetic defect. We wanted to test the reliability of using pigmentation as a marker to distinguish virulent strains from avirulent strains. The importance of being able to establish an association between pigmentation and virulence is apparent. It would allow us to use agar medium containing Congo red as indicator plates for detecting recombinants in gene transfer experiments to define and map genes involved in virulence. We therefore isolated spontaneous Pcr$^-$ mutants of a virulent Pcr$^+$ strain of *S. flexneri* 2a and assayed these strains for virulence as measured by the ability of the bacteria to invade and multiply within intestinal epithelial cells in tissue culture (7). In addition, we sought to reconstruct the virulent phenotype of avirulent Pcr$^-$ mutants by restoring the mutants to Pcr$^+$. Although pigmentation is an unselectable phenotype, a transposon inserted near the gene for pigmentation can serve as a selectable marker for mapping this gene (14). We generated random insertions of the transposon Tn*10* in a virulent Pcr$^+$ strain and prepared a transducing phage lysate on a collection of these Tn*10* insertion mutants. This lysate was then used to transduce avirulent Pcr$^-$ mutants to Pcr$^+$ by selecting for the transposon marker (tetracycline resistance) and screening for pigmented colonies. Pcr$^+$ transductants were then tested for acquisition of virulence.

Our results demonstrated that loss of pigmentation was consistently accompanied by loss of virulence and that the majority of avirulent Pcr$^-$ mutants analyzed had deletions in the virulence-associated plasmid (22) which we call pSf2a140. The Pcr$^-$ defect was suppressible in all mutants tested; however, none of the Pcr$^+$ recombinants isolated by transduction with the transducing lysate described above regained virulence. We discuss these results as they relate to the significance of pigmentation as a marker for virulence and its usefulness in future genetic studies on virulence in *S. flexneri* 2a.

* Corresponding author.

† Present address: Laboratorie des Entérobactéries, Institut Pasteur, 75724 Paris Cedex 15, France.

‡ Present address: Department of Microbiology, Clemson University, Clemson, SC 29631.

§ Present address: Department of Biology, Washington University, St. Louis, MO 63130.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1.

Media. L broth and L agar (17) were used as complete media for growing shigellae. For work with phage P1L4, L broth, L agar, and L soft agar (0.65% agar) contained 5 mM $CaCl_2$. Screening strains for pigmentation was done on tryptic soy agar (Difco Laboratories, Detroit, Mich.) to which Congo red (Sigma Chemical Co., St. Louis, Mo.) had been added to a final concentration of 0.003%. Selection for tetracycline resistance mediated by Tn*10* was done on Penassay agar (Difco) containing 0.8% NaCl and 12.5 μg of tetracycline hydrochloride (Sigma) per ml.

Tissue culture and assay for virulence. Strains of shigellae were assayed for virulence in an in vitro cell culture model. Henle 407 cells (embryonic human intestine [10]) were kindly supplied by T. L. Hale, Walter Reed Army Institute of Research, Washington, D.C. The cells were maintained in basal Eagle medium (Flow Laboratories, McLean, Va.) supplemented with 15% newborn calf serum (Flow) and 2 mM glutamine (Flow). Cells were plated at 1×10^5 to 2×10^5 per tissue culture dish (35 by 10 mm) (Lux Scientific Corp., Newbury Park, Calif.) and grown for 48 h before challenge with bacteria. Infection of Henle monolayers was as described by Hale and Formal (8) with the following modifications. Samples of bacteria (1×10^8 to 2×10^8 bacteria) in 1.0 ml of basal Eagle medium 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. 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 the presence of bacteria within the Henle cells.

Isolation and agarose gel electrophoresis of plasmid DNA. The rapid isolation procedure of Kado and Liu (11) was used to isolate plasmid DNA from 1.0 ml of L broth cultures of *S. flexneri* 2a. A 10-μl amount 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 (9) was carried out at 2.5 V/cm until the dye had entered the gel. Voltage was then increased to 5 V/cm for the rest of the run. A solution of 2 μg of ethidium bromide per ml was used to stain gels, and DNA in the gel was visualized by using a short-wavelength UV light source (Transilluminator model C61; Ultraviolet Products, Inc., San Gabriel, Calif.).

TABLE 1. Bacterial strains

Strain	Relevant characteristics	Invasiveness in Henle assay	Source
2457T	Pcr$^+$ Mal$^-$ λ^r	+	S. Formal (4)
BS58[a]	Mal$^+$ λ *c*Itl^s	+	Spontaneous from 2457T
BS97	*pcr-14*[b]	−	This study[c]
BS98	*pcr-11*	−	This study
BS99	*pcr-22*	−	This study
BS100	*pcr-25*	−	This study
BS101	*pcr-12*	−	This study
BS102	*pcr-13*	−	This study
BS103	*pcr-24*	−	This study

[a] Mal$^+$ shigellae are resistant to λ *c*Itl but are sensitive to λ *c*Itlsh, a host range mutant of λ *c*Itl (6). Strain BS58 is sensitive to both phages.

[b] *pcr* mutants listed above are unable to bind Congo red when plated at 37°C on tryptic soy agar containing Congo red.

[c] BS97 through BS103 are spontaneous nonpigmented mutants of 2457T.

Preparation of pools of random insertions of Tn*10* in *S. flexneri* 2a. Bacteriophage λ561 (*b*221 *c*I857 *rex*173::Tn*10* *O*am29 *P*am80) was used to generate random Tn*10* insertions in *S. flexneri* BS58 by previously described methods (13). A total of about 4,000 independent tetracycline-resistant colonies were pooled and suspended in L broth containing 5 mM $CaCl_2$. A P1L4 lysate was prepared on the pooled cells.

Transduction methods. P1L4 transducing lysates were prepared by the confluent plate lysis technique (3). Recipients for transduction were grown at 37°C with aeration in L broth containing 5 mM $CaCl_2$. When the cells reached a density of 1×10^8 to 2×10^8/ml, they were infected with P1 at a multiplicity of 1 to 3. After adsorption for 20 min at 37°C, the mixture was plated directly onto plates to select for transductants.

RESULTS

Isolation of spontaneous Pcr$^-$ mutants of *S. flexneri* 2a. Strain 2457T, a virulent isolate of *S. flexneri* 2a (4) which is also pigmented on Congo red plates (21), was grown from a lyophilized stock and stored at −70°C in 1% peptone–5% glycerol. A culture of 2457T was grown to stationary phase at 37°C in L broth with aeration. This culture was stored at 4°C for 2 months and then plated on Congo red plates to isolate spontaneous Pcr$^-$ derivatives. Since virulent strains of bacteria are known to segregate spontaneous avirulent isolates on prolonged storage and subculture, we believed that examining a culture of 2457T after several months of storage at 4°C would improve our chances of finding Pcr$^-$ segregants. We were able to easily detect Pcr$^-$ colonies in a background of as many as 1,500 Pcr$^+$ colonies per plate. In this manner, we isolated 29 Pcr$^-$ mutants from 2457T and 20 Pcr$^-$ mutants from BS58, a virulent, Mal$^+$ derivative of 2457T. These strains were purified twice on Congo red plates at 37°C, and none of the Pcr$^-$ mutants segregated Pcr$^+$ colonies. Thus, the Pcr$^-$ phenotype appeared to be stable. All of the Pcr$^-$ mutants produced slightly larger and flatter colonies than the Pcr$^+$ parent strain on tryptic soy agar. To determine more precisely the frequency at which Pcr$^-$ segregants arose, we grew a culture of 2457T from a single Pcr$^+$ colony. Pcr$^-$ colonies appeared at a frequency of about 1.2×10^{-4} per CFU. This figure closely approximates the reported frequency of appearance of opaque colonial variants of *S. flexneri* 2a (16).

Assay of Pcr$^-$ mutants for virulence. 2457T had previously been shown to be capable of invading and multiplying within Henle intestinal epithelial cells in tissue culture (7), and such an assay correlates very well with demonstrated virulence in animals and human volunteers. We therefore tested our Pcr$^-$ mutants for virulence by assaying their ability to invade Henle cells in culture. All 29 Pcr$^-$ mutants from 2457T and the 20 Pcr$^-$ mutants from BS58 failed to invade Henle cells, whereas the parent strains, 2457T and BS58, were fully invasive. Usually 90 to 95% of the cells in a monolayer challenged with either 2457T or BS58 were found to have bacteria within the cytoplasm. Thus, the Pcr$^-$ phenotype was consistently associated with loss of virulence as measured by the ability to invade epithelial cells in culture.

Plasmid DNA profiles of Pcr⁻ mutants. Because of the association of a plasmid with virulence in *S. flexneri* 2a (22), we examined the avirulent Pcr⁻ mutants for alteration in their plasmid DNA. We divided the 29 Pcr⁻ mutants from 2457T into seven classes with respect to the virulence plasmid pSf2a140; representatives of these classes are listed in Table 1, and their plasmid profiles are shown in Fig. 1. The 2.6- and 2.0-megadalton (Mdal) cryptic plasmids present in the parent strain 2457T (15) were present in all of the Pcr⁻ mutants (data not shown). With the exception of BS97, all of the Pcr⁻ mutants examined were missing a plasmid band of 140 Mdal corresponding to pSf2a140. In the plasmid profiles of classes represented by BS98 through BS102, new bands which were smaller than 140 Mdal appeared. These new plasmids are probably deletion derivatives of pSf2a140. New plasmid bands in the Pcr⁻ mutants ranged in size from about 45 Mdal to about 110 Mdal (Table 2). These plasmids would represent a deletion of 30 to 95 Mdal of DNA from pSf2a140. BS103 was completely cured of pSf2a140, and no new plasmid band appeared in this mutant.

At this level of analysis, no loss of DNA from pSf2a140 was detectable in BS97. Because of the size of pSf2a140, small deletions in the plasmid would not be seen on a 0.7% agarose gel. It is possible that a small deletion or gene rearrangement on pSf2a140 was responsible for the loss of pigmentation in BS97, but further analysis of the plasmid is needed to test this hypothesis. However, the evidence from the other Pcr⁻ mutants examined very strongly suggests an association of loss of pigmentation with loss of DNA from pSf2a140. Additional evidence of this association comes from the plasmid profiles of the 20 avirulent Pcr⁻ mutants of BS58, all of which had lost a plasmid band of 140 Mdal (data not shown).

Transduction of Pcr⁻ mutants to Pcr⁺ with a P1 lysate grown on a pool of Tn*10* insertions. We wished to determine whether Pcr⁻ mutants could be restored to Pcr⁺ and, if so, whether the Pcr⁺ transductants would also regain the ability to penetrate Henle cells in the virulence assay. We generated random insertions of Tn*10* in BS58, a virulent Pcr⁺ derivative of 2457T, and prepared a P1L4 transducing lysate on a pool of these Tn*10* insertion mutants. This lysate was used to transduce the Pcr⁻ mutants (BS97 through BS103) to tetracycline resistance, and the transductants were screened for pigmentation on Congo red plates. All seven classes of Pcr⁻ mutants were successfully transduced to Pcr⁺, and the Tn*10*-linked Pcr⁺ phenotype was stably maintained. However, when the Pcr⁺ transductants were assayed for virulence in Henle cells, none of the transductants (0 of 39) had regained the ability to penetrate epithelial cells. It appeared either that the ability to bind Congo red was not sufficient for invasiveness or that the Pcr⁻ phenotype was suppressible by a Tn*10*-linked trait which was unrelated to the original mutation. Support for the latter view came from the observation that the Pcr⁺ transductants were pigmented at both 30 and 37°C, whereas the invasive, Pcr⁺ parent 2457T is known to display a temperature-dependent ability to bind Congo red, being Pcr⁺ at 37°C and Pcr⁻ at 30°C (A. T. Maurelli, B. Blackmon, and R. Curtiss, submitted for publication).

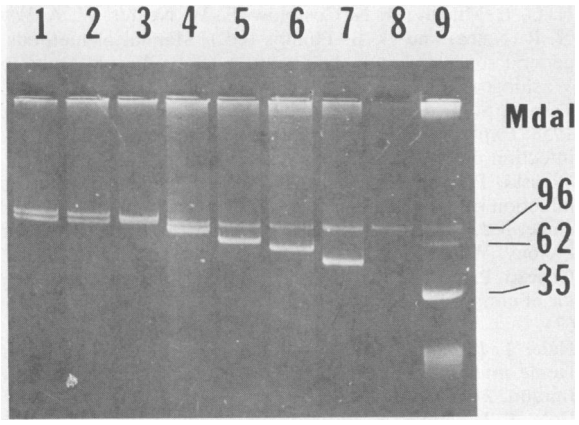

FIG. 1. Plasmid DNA profiles of Pcr⁻ mutants of 2457T. DNA was isolated by the procedure of Kado and Liu (11) and electrophoresed on a horizontal 0.7% agarose gel. Lane 1, 2457T; lanes 2 through 8, BS97 through BS103; lane 9, molecular weight standards: 35 Mdal of plasmid from *Escherichia coli* V517 (18), 62 Mdal of plasmid Rldrd19 (20), 96 Mdal of plasmid R40a (1).

TABLE 2. Characterization of plasmid DNA in Pcr⁻ mutants

Strain	Approx size of new plasmid (Mdal)
BS97	—[a]
BS98	110–120
BS99	85–90
BS100	65
BS101	55
BS102	45
BS103	—

[a] —, No new plasmid bands were detected.

We wished to identify some of the Tn*10*-linked suppressors of the Pcr⁻ phenotype to determine which genetic determinants could affect the ability of the organism to bind Congo red. In light of the simultaneous loss of pigmentation and loss of DNA from pSf2a140, we looked for Tn*10*-linked suppressors of *pcr* which mapped to the plasmid. If the plasmids present in BS97, BS98, and BS99 represent deletion derivatives of pSf2a140, it should be possible to transduce donor DNA large enough to cover the size of the presumed deletions in pSf2a140 in these mutants. Since the transducing capacity of P1L4 is about 60 Mdal (3), we would not expect to be able to repair deletions of greater than 60 Mdal (e.g., mutants BS100 through BS103) because of the absence of regions of homology for recombination of donor DNA with plasmid DNA.

Analysis of the plasmid profiles of all 39 Pcr⁺ transductants failed to show any alteration in size of the presumptive deletion derivatives of pSf2a140 in the recipients. It therefore appeared that none of the suppressors we isolated mapped to these plasmids.

By using conjugational and transductional mapping techniques, we succeeded in mapping one suppressor and identified it as a Tn*10* insertion in the gene for glucose 1-phosphate uridyltransferase (manuscript in preparation). When this mutation (*galU*:Tn*10*) was transduced, by selection for tetracycline resistance, into any Pcr⁻ mutant, pigmentation was restored but the avirulence of the strain was unaltered. Other suppressors of *pcr* were examined and found to map at different locations in the chromosome of *S. flexneri* 2a. Therefore we can conclude that, at least in the cases of suppression of *pcr* which we examined, restoring the ability to bind Congo red does not restore virulence to Pcr⁻ mutants. The available evidence, however, is not sufficient to exclude the possibility that pigmentation is necessary for virulence and that our experimental design precluded the isolation of transductants which would restore both pigmentation and virulence.

DISCUSSION

We have isolated spontaneous Pcr$^-$ mutants from virulent Pcr$^+$ *S. flexneri* strains 2457T and BS58. All Pcr$^-$ mutants tested had lost the ability to penetrate epithelial cells in the assay for virulence. This confirms and extends the earlier report of Payne and Finkelstein on the association of pigmentation with virulence (21). We have demonstrated that loss of virulence is a consistent phenotype of Pcr$^-$ strains of *S. flexneri* 2a. Loss of virulence which accompanies loss of pigmentation appears to be related to deletion of DNA from pSf2a140. Nearly all of the Pcr$^-$ mutants which we examined (48 of 49) had lost a plasmid band corresponding to pSf2a140. BS97 still had a plasmid of about 140 Mdal, but we cannot rule out the possibility that a small deletion had occurred in pSf2a140 in BS97 and could not be resolved on a 0.7% agarose gel. In this regard, BS97 resembles 24570, the opaque colonial variant of 2457T described earlier (16) which is also avirulent and does not appear to have lost any DNA from pSf2a140 (15).

The disappearance of a 140-Mdal plasmid and the appearance of smaller plasmids in strains BS98 through BS102 suggested that the smaller plasmids arose as a result of spontaneous deletion of DNA sequences from pSf2a140. Although we cannot unequivocally conclude that the small plasmids which appear in these strains did indeed derive from pSf2a140, this is the most reasonable explanation. The other plasmids in the mutants are unchanged, and it is unlikely that the 105-Mdal cryptic plasmid gave rise to the new plasmids. The simultaneous loss of pSf2a140 and appearance of a single new plasmid in each Pcr$^-$ strain is consistent with the hypothesis that the loss of pigmentation is due to the deletion of DNA from pSf2a140.

Our ability to restore Pcr$^-$ mutants to Pcr$^+$ without restoring invasiveness demonstrates that pigmentation alone does not distinguish a virulent strain from an avirulent strain. Although Pcr$^-$ mutants of *S. flexneri* 2a were always noninvasive in Henle cells, not all Pcr$^+$ strains were invasive. These observations also raised the question of the relevance of pigmentation to virulence in *S. flexneri* 2a. Since loss of pigmentation is always accompanied by loss of virulence, there would seem to be more than just a coincidental association of pigmentation with virulence. The suppression of the Pcr$^-$ phenotype without restoration of virulence reflects the fact that certain genes which affect cell wall or outer membrane biosynthesis can also affect the ability of the organism to bind Congo red. Suppression of Pcr$^-$ mutants by the *galU*::Tn*10* mutation is an example of this, since *galU* mutants are blocked in the attachment of O-specific side chains in the lipopolysaccharide of the cell wall (23). Pigmentation, then, may be a phenotype which can be due to a variety of different genetic determinants, including one or more necessary for the ability of *S. flexneri* 2a to invade epithelial cells. However, a precise demonstration of a role for pigmentation in virulence is needed if we are to consider Pcr$^+$ to be a necessary phenotype for virulent strains of *S. flexneri* 2a.

Our inability to transduce Pcr$^-$ strains to Pcr$^+$ and cotransduce the invasive phenotype may be due to limitations in the transducibility of genes located on pSf2a140. If the defect in Pcr$^-$ strains is due to deletion of genetic information from pSf2a140, restoration of both Pcr$^+$ and invasiveness would require transduction of DNA homologous to the deleted region and recombination of that DNA into the deletion derivative of pSf2a140 in the recipient. Some of the Pcr$^-$ mutants had apparent deletions in pSf2a140 which would be too large to be repaired by P1L4-mediated transduction. This may explain the failure to transduce invasiveness with Pcr$^+$ in strains BS100, BS101, BS102, and BS103. It is not known why the other Pcr$^-$ mutants could not be transduced to Pcr$^+$ and invasiveness. It is possible that P1L4 is very inefficient at packaging pieces of pSf2a140, or the problem may be in recombination of the transduced DNA into the homologous region in the recipient. Alternatively, what we presume to be deletion derivatives of pSf2a140 in the Pcr$^-$ mutants may be plasmids which arise from a different source, perhaps from the cryptic plasmids in *S. flexneri* or from the chromosome. If so, then we would not expect to be able to transduce DNA from pSf2a140 into these strains because of the lack of DNA sequence homology in the recipients. Therefore, although the loss of DNA from pSf2a140 is associated with loss of both properties, we have not been able to identify a genetic locus which is responsible for both pigmentation and virulence.

We had originally set out to determine whether or not Pcr$^-$ was a consistent phenotype of avirulent strains and whether or not it could be used as a marker to identify avirulent strains in a population. It is clear that Pcr$^-$ mutants are avirulent, but simply screening mutagenized cultures for Pcr$^-$ mutants may yield only mutants of the type which we described in this study. These mutants may ultimately be shown to suffer from the same defect, although large amounts of DNA from pSf2a140 were lost in many of the strains. It is likely that other mutations which alter virulence may not affect the ability of the strain to bind Congo red, and in screening just for Pcr$^-$ colonies other avirulent mutants will go undetected. So, although screening for the Pcr$^-$ phenotype was useful in isolating the avirulent mutants we described, additional methods need to be developed to identify other types of avirulent mutants.

LITERATURE CITED

1. **Bukhari, A. I., J. A. Shapiro, and S. L. Adhya.** 1977. DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
2. **Cooper, M. C., H. M. Keller, and E. W. Walters.** 1957. Microscopic characteristics of colonies of *Shigella flexneri* 2a and 2b and their relation to antigenic composition, mouse virulence and immunogenicity. J. Immunol. **78**:160–171.
3. **Curtiss, R., III.** 1981. Gene transfer, p. 243–265. *In* P. Gerhardt, R. G. E. Murray, R. N. Costelow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
4. **Formal, S. B., G. J. Dammin, E. H. LaBrec, and H. Schneider.** 1958. Experimental shigella infections: characteristics of a fatal infection produced in guinea pigs. J. Bacteriol. **75**:604–610.
5. **Gemski, P., and S. B. Formal.** 1975. Shigellosis: an invasive infection of the gastrointestinal tract, p. 165–169. *In* D. Schlessinger (ed.), Microbiology—1975. American Society for Microbiology, Washington, D.C.
6. **Gemski, P., Jr., J. A. Alexeichik, and L. S. Baron.** 1972. Behavior of coliphage lambda in *Shigella flexneri* 2a. J. Virol. **10**:668–674.
7. **Hale, T. L., and P. F. Bonventre.** 1979. Shigella infection of Henle intestinal epithelial cells: role of the bacterium. Infect. Immun. **24**:879–886.
8. **Hale, T. L., and S. B. Formal.** 1981. Protein synthesis in HeLa or Henle 407 cells infected with *Shigella dysenteriae* 1, *Shigella flexneri* 2a, or *Salmonella typhimurium* W118. Infect. Immun. **32**:137–144.
9. **Hayward, G. S., and M. G. Smith.** 1972. The chromosome of bacteriophage T5. I. Analysis of the single-stranded DNA fragments by agarose gel electrophoresis. J. Mol. Biol. **63**:383–

395.
10. **Henle, G., and F. Deinhardt.** 1957. The establishment of strains of human cells in tissue culture. J. Immunol. **79**:54–59.
11. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
12. **Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85**:1274–1279.
13. **Kleckner, N., D. F. Barker, D. G. Ross, and D. Botstein.** 1978. Properties of the translocatable tetracycline-resistance element Tn10 in *Escherichia coli* and bacteriophage lambda. Genetics **90**:427–461.
14. **Kleckner, N., J. Roth, and D. Botstein.** 1977. Genetic engineering *in vivo* using translocatable drug-resistance elements. New methods in bacterial genetics. J. Mol. Biol. **116**:126–159.
15. **Kopecko, D. J., J. Holcombe, and S. B. Formal.** 1979. Molecular characterization of plasmids from virulent and spontaneously occurring avirulent colonial variants of *Shigella flexneri*. Infect. Immun. **24**:580–582.
16. **LaBrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. **88**:1503–1518.
17. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
18. **Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **i**:417–420.
19. **Mazigh, D., J. M. Alonso, and H. H. Mollaret.** 1983. Simple method for demonstration of differential colony morphology of plasmid-associated virulent clones of *Yersinia enterocolitica*. J. Clin. Microbiol. **17**:555–557.
20. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529–1537.
21. **Payne, S. M., and R. A. Finkelstein.** 1977. Detection and differentiation of iron-responsive avirulent mutants on Congo red agar. Infect. Immun. **18**:94–98.
22. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852–860.
23. **Simmons, D. A. R.** 1971. Immunochemistry of *Shigella flexneri* O antigens: a study of structural and genetic aspects of the biosynthesis of cell surface antigens. Bacteriol. Rev. **35**:117–148.
24. **Surgalla, M. J., and E. D. Beesley.** 1969. Congo red agar plating medium for detecting pigmentation in *Pasteurella pestis*. Appl. Microbiol. **18**:834–837.