Mutation in *Shigella flexneri* Resulting in Loss of Ability to Penetrate HeLa Cells and Loss of Glycerol Kinase Activity

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A colonial variant of a virulent *Shigella flexneri* 2a has lost both virulence and glycerol kinase activity. It also has several other altered characteristics: lowered ability to oxidize tricarboxylic acid cycle intermediates, increased electrophoretic mobility, and decreased sensitivity to sodium lauryl sulfate. Genetic analysis has revealed that the gene governing glycerol kinase activity in *Shigella* has a different chromosomal locus than that from *Escherichia coli*. Furthermore, transduction of the *Shigella* glycerol kinase gene (*glp K*) into the avirulent *Shigella* strain can restore the ability to penetrate HeLa cells, whereas the gene from *E. coli* cannot. About half of the *glp K* mutants lose this ability, and only about half of the revertants of an avirulent *glp K* mutant regain it. This indicates that more than one gene affects glycerol kinase activity in *Shigella*, only one of which is associated with penetration. Glycerol kinase activity is closely correlated with changes in electrophoretic mobility, but does not appear to have any relationship to sodium lauryl sulfate sensitivity nor to the oxidation of tricarboxylic acid cycle intermediates.

A classical method for studying virulence of bacteria is to consider other alterations associated with the mutation from virulence to avirulence and try to relate them causally to the loss of virulence. We have studied an avirulent mutant of *Shigella flexneri* 2a. This mutant strain (24570) was obtained as a spontaneous opaque colonial variant of the virulent strain (M-42-43) which grows as translucent colonies on meat extract agar (9). Opaque colonies arise at the relatively frequent rate of one in 10⁴ cells from the virulent strain. This particular opaque strain has several pleiotropic alterations associated with the mutation. The strain has lost virulence by virtue of its inability to penetrate the intestinal epithelial cells (11). In model systems, it is also unable to cause keratoconjunctivitis in the guinea pig eye (19) and is unable to penetrate the HeLa epithelial cell line (17). In addition, the avirulent strain has become relatively resistant to sodium lauryl sulfate (SLS) (5), has a three- to fourfold increase in anodic electrophoretic mobility (EM) (6), and has an impaired ability to utilize certain tricarboxylic acid cycle intermediates (15).

Although it is unlikely that these pleiotropic effects are due to multiple point mutations, it is conceivable that they could be due to a deletion of several adjacent genes. Testing such possibilities by genetic analyses is difficult due to an inability to select for strains resistant to SLS or with an increased anodic EM. We were fortunate, therefore, to be able to take advantage of the observation of E. H. LaBrec (personal communication) that the avirulent strain does not ferment glycerol. With such a handle for selection, it was then possible to directly test by reversion analyses and other correlation techniques the relationship between the pleiotropic changes and the ability of organisms to penetrate epithelial cells.

This paper presents data to show that the mutated gene affecting glycerol utilization in the avirulent strain relates to glycerol kinase and is probably a different gene from the one governing glycerol kinase synthesis in *Escherichia coli*. It is also in a location far distant from the *kcp A* mutation which also governs the ability to penetrate the epithelial cells of the eye and produce keratoconjunctivitis (10). It will be shown that loss of glycerol kinase activity and changes in EM are related, but no observable correlation of glycerol kinase activity with SLS sensitivity or succinate utilization was detected. There is, however, some correlation between glycerol kinase activity and the ability to penetrate HeLa cells. The possibility that the lack of compete correlation with penetration may be related to polygenic control of glycerol kinase activity will be discussed.
MATERIALS AND METHODS

Bacterial strains. The strains used in this study are characterized in Table 1. S. flexneri 2a strain 24570 is a colonial variant which arose spontaneously from virulent strain M-42-43. Both strains were kindly provided by S. Formal. The 24570 glycerol-positive strains are spontaneous revertants of strain 24570. These arose at a rate of one in 10^4 cells. The M-42-43 glycerol-negative mutants were obtained by using a mutagenic procedure which will be described subsequently. The E. coli K-12 strain W1895 is an HfrC (met-). Strain 27p, an E. coli K-12 strain with a promoter mutation in the glycerol kinase gene, was kindly provided by D. Richey. Strain 76p is an E. coli K-12 strain which lacks glycerol kinase.

Growth of cells and preparation of extracts. The minimal medium used for growth culture, per liter of distilled water: K2HPO4, (10.5 g), KH2PO4, (4.5 g), MgCl2, (0.06 g), and (NH4)2SO4, (1 g). Carbon sources were added to give a final concentration of 0.02 M glycerol and 0.04 M DL-α-glycerophosphate (DL-α-GP). Casein acid hydrolysate (Difco, vitamin-free) was added to minimal medium to give a final concentration of 1%. Agar plates had, in addition, 15 g of agar per liter. Luria broth supplemented with 0.2% glucose and 5 mM CaCl2 (5) was used as the growth medium for the bacteria when the electrophoretic mobility or SLS sensitivity was tested. Nutrient broth (Difco) was used as the growth medium for studying biological oxidation. Growth was measured in a Bausch & Lomb Spectronic 20 spectrophotometer at a wavelength of 625 nm.

The cultures were incubated at 37°C on a rotary shaker operated at 200 rpm. The cells were harvested in the logarithmic phase, pelleted at 0°C, washed with cold 1% NaCl solution, and resuspended in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.4. The suspended cells were disrupted by four treatments in a 50-W sonicator (Heat Systems Ultrasonics, Inc., model W185) for a total of 80 s at 0°C. The resulting suspension was centrifuged at 30,000 × g for 20 min at 0°C, and the supernatant cell-free extract was used for enzyme assays.

Glycerol kinase assay. Glycerol kinase activity was assayed by measuring the formation of [14C]-α-GP from [14C]glycerol as described by Berman and Lin (2). Protein concentrations were determined according to the method of Lowry et al. (18).

Interrupted mating. Crosses between donor S. flexneri strain M-256, an Hfr strain formed by having received the F-lac portion of E. coli K-12 strain P4x-6, and S. flexneri 2a strain 24570 were performed as described previously (22). Selections were made on minimal glycerol or minimal rhamnose plates supplemented with 5 μg of aspartic acid per ml.

Transduction experiments. The conditions used for performing transductions have been reported in another paper (5).

Microelectrophoresis. EM was measured by the method of Corwin and Talevi (6).

Lysis by SLS. Lysis studies were performed as described by Corwin et al. (5).

Oxygen Uptake. Oxidation measurements were carried out essentially as described by Umbriet et al. (24) by using the conventional Warburg technique. The cells were grown in nutrient broth to stationary phase.

HeLa cell assay. The HeLa cell monolayers were maintained in milk dilution bottles in medium consisting of minimal essential medium (8), with Hanks balanced salt solution (13), supplemented with 10% fetal calf serum, 20 μmol of glutamine per ml, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. The cover-slip culture technique was used to prepare monolayers for penetration experiments (17).

Mutagenesis and penicillin selection. M-42-43 cells were grown in 10 ml of Luria broth to log phase (5 × 10^8 to 8 × 10^9 cells per ml). The cells were washed twice in saline and resuspended in 0.5 ml of 0.1 M citrate buffer (pH 5.6). To the suspension 0.1 ml of N-methyl-N′-nitro-N′-nitrosoguanidine (1 mg/ml) made freshly was added (1). The mixture was incubated for 20 min at 37°C. The cells were then added to 4.5 ml of Luria broth and grown for 3 h to allow the mutation to be expressed. A 5-ml amount of cells was washed twice and grown in 200 ml of minimal glucose for six generations to exclude auxotrophs. Penicillin selections were done three times in minimal glycerol to enrich for glyp K' cells by using a final concentration of 2,000 U of penicillin per ml and allowing the cells to incubate with the penicillin at 37°C for 90 min (12). The cells were then plated on minimal DL-α-GP plates, and the colonies that arose were tested for their ability to grow on minimal glycerol.

Glycerol-positive revertants of strain 24570 (glyp K') were isolated by plating exponential 24570 cells on minimal glycerol plates. The colonies were re-streaked on minimal glycerol plates for purification.

RESULTS

Growth characteristics of S. flexneri. Viru-
lent S. flexneri strain M-42-43 grows either on glycerol or \(\text{L}-\alpha\)-GP as a carbon source, whereas its avirulent mutant, strain 24570, can only utilize \(\text{L}-\alpha\)-GP. Strain 24570, after a long lag phase, grew almost as well as its virulent parent (Table 2). These growth characteristics suggested that a mutation had occurred in the glycerol kinase gene \((\text{glp } K)\) which codes for the first enzyme in the pathway required for the dissimilation of glycerol (16).

**Glycerol kinase activity of S. flexneri and E. coli.** The activity of glycerol kinase was measured in strains M-42-43 and 24570. As expected, strain 24570 showed no activity whether induced with glycerol or \(\text{L}-\alpha\)-GP (Table 3). Strain M-42-43 possessed glycerol kinase activity, but this was found to be lower (between 43 and 47\(^{\circ}\)) than that found in wild-type E. coli strain W1485.

**K\(_m\) and \(V_{\text{max}}\).** To determine whether the lower activity of the Shigella enzyme was due to a different enzyme than that of E. coli or to a lowered quantity of the same enzyme, or both, the \(K_m\) and \(V_{\text{max}}\) of glycerol kinase of extracts of these two strains were measured. The \(K_m\) of glycerol kinase of the Shigella strain was 2 \(\times\) \(10^{-4}\) M, whereas that of the E. coli strain was 1.5 \(\times\) \(10^{-3}\) M (Table 4). These differences were not considered to be significant. The \(V_{\text{max}}\) in these two strains, on the other hand, differed considerably. Since these data were obtained from crude extracts, the difference in \(V_{\text{max}}\) could have been due to either lower levels of enzyme or a different enzyme. It has been observed that the Shigella glycerol kinase formed a zone of precipitation with specific antiserum prepared against the crystalline E. coli glycerol kinase (21). If the two enzymes are indeed the same or very similar, then it seems likely that E. coli produces almost twice as much glycerol kinase as Shigella.

**Mapping.** Due to the close genetic homology between Shigella and E. coli (3), we determined whether the Shigella \(\text{glp } K\) gene mapped at the same chromosomal site as in E. coli. Since rhamnose \((\text{rha})\) maps next to \(\text{glp } K\) in E. coli at 76 min of the chromosome (7), rhamnose utilization was used as a cotransducible marker. Although S. flexneri 2a is \(\text{rha}\) negative, it can be reverted by mutagens. The \(\text{rha}\) locus in Shigella is located at the same genetic position as in E. coli (S. Falkow, personal communication).

Phage \(P_{\text{vir}}\) was used to transduce the \(\text{glp } K\) and \(\text{rha}\) genes from virulent Shigella strain M-42-43 \((\text{rha}\textsuperscript{-} \text{glp } K\textsuperscript{-})\) into avirulent strain 24570 \((\text{rha}\textsuperscript{-} \text{glp } K\textsuperscript{-})\). Selection was made for \(\text{glp } K^+\) or \(\text{rha}\textsuperscript{+}\) transductants. Of 300 \(\text{glp } K^+\) transductants, all were found to have remained \(\text{rha}\textsuperscript{-} \text{glp } K\textsuperscript{-}\) (Table 5). When \(\text{rha}\) was the selected marker, all of the transductants remained \(\text{glp } K\textsuperscript{-}\). This indicated that \(\text{rha}\) and \(\text{glp } K\) were not cotransducible in Shigella. The transduction between wild-type E. coli K-12 strain W1485 \((\text{rha}\textsuperscript{+} \text{glp } K\textsuperscript{+})\) and S. flexneri strain 24570 \((\text{rha}\textsuperscript{-} \text{glp } K\textsuperscript{-})\) was used to verify that \(\text{rha}\) and \(\text{glp } K\) were indeed cotransducible from E. coli (Table 5). Further evidence indicating that \(\text{rha}\) and \(\text{glp } K\) in Shigella were not cotransducible was the

### Table 2. Generation times of S. flexneri strains M-42-43 and 24570

<table>
<thead>
<tr>
<th>Growth medium (^a)</th>
<th>Generation time (min) (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M-42-43</td>
</tr>
<tr>
<td>Minimal glycerol</td>
<td>72</td>
</tr>
<tr>
<td>Minimal (\text{L}-\alpha)-GP</td>
<td>48</td>
</tr>
</tbody>
</table>

\(^a\) Growth media contained 0.05% casein hydrolysate plus 0.02 M glycerol or 0.04 M \(\text{L}-\alpha\)-GP.

\(^b\) Slope of the straight line portion of the semilogarithmic curve of optical density at 625 nm versus time.

\(^c\) NG, No growth.

### Table 3. Glycerol kinase activity of S. flexneri and E. coli strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inducer</th>
<th>Sp act (^a) glycerol kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. flexneri</td>
<td>M-42-43</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>(\text{L}-\alpha)-GP</td>
<td>68</td>
</tr>
<tr>
<td>24570</td>
<td>None</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(\text{L}-\alpha)-GP</td>
<td>0</td>
</tr>
<tr>
<td>E. coli</td>
<td>W1485</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>(\text{L}-\alpha)-GP</td>
<td>129</td>
</tr>
</tbody>
</table>

\(^a\) Cells were grown for 6 to 10 generations as described in Materials and Methods in 1% casein hydrolysate ± inducer (0.02 M glycerol or 0.04 M \(\text{L}-\alpha\)-GP). Specific activity is expressed as nanomoles of \(\text{L}-\alpha\)-GP formed per minute per milligram of protein.

### Table 4. Comparison of \(V_{\text{max}}\) and \(K_m\) values of glycerol kinase from S. flexneri and E. coli\(^a\)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>(K_m) ((\text{M} \times 10^{-1}))</th>
<th>(V_{\text{max}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. flexneri 2a</td>
<td>Glycerol</td>
<td>2.0</td>
<td>93</td>
</tr>
<tr>
<td>E. coli K-12 W1485</td>
<td>Glycerol</td>
<td>1.5</td>
<td>190</td>
</tr>
</tbody>
</table>

\(^a\) All measurements were made by Berman and Lin's method (2).

\(^b\) Expressed as nanomoles per minute per milligram of protein.
transduction by P\textsubscript{vir} grown on Shigella strain M-42-43 (rha\textsuperscript{+} glp K\textsuperscript{+}) into E. coli strain DR 1 (rha\textsuperscript{+} glp K\textsuperscript{−}). All 20 glp K\textsuperscript{+} transductants remained rha\textsuperscript{+} (Table 5).

Conjugation studies were then used to get an approximate location of the glp K locus in Shigella. The male donor used was S. flexneri strain 256 (glp K\textsuperscript{−}), an Hfr strain formed by having received the F-lac portion of E. coli K-12 strain P4x-6, Hfr (22). As far as could be determined by genetic analyses for other selected markers, Hfr 256 retained only the F-lac region of the E. coli donor genome and otherwise exhibited the characteristics of a typical S. flexneri 2a strain. Furthermore, crosses with E. coli recipients showed a mating polarity similar to that of the P4x-6 strain from which it was derived, i.e., pro-thr-arg-his-gal-lac-F (22). In our hands, the thr marker could be transferred in 8 to 11 min, or about 3 to 6 min earlier than the results obtained by Schneider and Falkow (22), who studied a cross between Hfr 256 and an E. coli recipient strain. Our mating used a thr, arg, his multiauxotrophic derivative of 24570 S. flexneri 2a as a recipient for Hfr 256. We also observed a shallow initial slope, followed by a sharp upward inflection. The accuracy of extrapolating this shallow slope to the abscissa was limited, and the time of entry values are the range of three experiments selecting for threonine.

The results of an interrupted mating study using Hfr 256 and 24570 glp K are shown in Fig. 1. The glp K\textsuperscript{+} locus appears to be transferred at about 40 to 45 min from the origin. This places the Shigella glp K at approximately 55 to 60 min (Fig. 2) on the Taylor map (23). Although this experiment was repeated four times with essentially the same result, it is apparent that the scatter was significant at low recombinant levels. This was probably due to the relatively high rate of reversion of 24570 to glp K\textsuperscript{−}, which made the controls and experimental values variable. Therefore, these data are presented, not to indicate the precise location of the

![FIG. 1. Marker transfer in cross between Hfr Shi- gella flexneri M-256 and F- S. flexneri 24570. Selection for glp K\textsuperscript{+} recombinants was made after appropriate dilutions. S. flexneri strain M-256 is glp K\textsuperscript{−}, and strain 24570 is glp K\textsuperscript{+}.](http://iai.asm.org/)

Shigella glp K, but rather to support the transduction data indicating that the site of the Shigella glp K is not identical with E. coli at 76 min on the map and is far distant from the kcp A locus close to pur E (11).

Other experiments to define more precisely the Shigella glp K site have been unsuccessful. Cotransduction of glp K with thymine or maltose markers, which are in the vicinity of glp K on the chromosome, has not been found. Selection of the maltose marker was based on the work of Formal et al. (10), showing that virulence could be restored to an avirulent S. flexneri 5 mutant by conjugal transfer of genes associated with the mal region. If the Shigella glp K is near mal, it nevertheless is not cotransduced with it.

**Relation of glp K to pleiotropic alterations of 24570.** To ascertain whether the presence or absence of the Shigella glp K gene is related genetically to the other alterations attributable

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**Table 5. Transduction of the glpK marker**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Selected marker</th>
<th>No. of colonies scored</th>
<th>Inheritance (%) of unselected markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-42-43 (rha\textsuperscript{+})</td>
<td>24570</td>
<td>glpK</td>
<td>300</td>
<td>—</td>
</tr>
<tr>
<td>M-42-43 (rha\textsuperscript{+})</td>
<td>24570</td>
<td>rha</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>24570</td>
<td>glpK</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>M-42-43 (rha\textsuperscript{−})</td>
<td>E. coli (glpK\textsuperscript{−})</td>
<td>glpK</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

*Except as otherwise indicated, the genotypes of the strains are as follows: S. flexneri 2a strain M-42-43 is glpK\textsuperscript{+} and strain 24570 is glpK\textsuperscript{−}. The rha\textsuperscript{+} M-42-43 is a revertant of M-42-43 which is normally rha\textsuperscript{−}. E. coli glpK\textsuperscript{−} was obtained from D. Richey.

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to the mutation from M-42-43 to 24570, additional glp K mutants were obtained from the virulent strain. In addition, a number of glp K revertants were isolated from 24570 as well as from one of the new glp K mutants of M-42-43 which proved avirulent. These revertants arose spontaneously at the rate of one in 10^4 cells. These strains were tested to see whether their electrophoretic mobility, biological oxidation, and virulence differed from those of the parent strains. If the mutation from M-42-43 to 24570 is a simple point mutation, then it should be able to revert, and the altered characteristics due to the mutation should revert back to the original state. If the mutation is a deletion of several genes, no true reversion should take place, although suppressor mutations might affect one or more of the phenotypic alterations. The possibility of simultaneous mutations, although usually unlikely, must be considered in Shigella 2a. To those working with this organism, the discovery of high rates of mutation of certain genes or phenotypes is not uncommon. For example, the colonial variation from translucent to opaque occurs at a rate of one in 10^4 cells. These could arise, of course, by mutations at different loci. The mal mutation in Shigella and the glp K gene also mutate at high frequency. Thus, the remainder of the results section will be concerned with the genetic relationships of the pleiotropic alterations in the avirulent 24570 strain.

**Microelectrophoresis.** The EM of the virulent strain M-42-43 and its avirulent mutant 24570 have been extensively studied in this laboratory (6). It has been shown that under similar conditions of growth (Luria broth with 0.2% glucose and 5 mM CaCl_2), strain M-42-43 showed an EM of 0.56 μm per s per V per cm, whereas strain 24570 was much more negative with a value of 1.87 μm per s per V per cm (6).

The EM of 24570 glp K* revertants as well as the M-42-43 glp K* mutants and its glp K* revertants were measured (Table 6). Also given are the values for 24570 transductants which received the glp K gene from E. coli strains 1895 or 27p. These data show a correlation between the presence of glycerol kinase and the EM in these Shigella strains. If a cell was glp K*, the EM values were less than 0.6 μm per s per V per cm; if the cell was glp K−, the EM was more negative, with values ranging from 1.2 to 2.0 μm per s per V per cm. This relationship did not hold in the E. coli strains tested. They had a very high negative EM (over 3.0 μm per s per V per cm) whether glp K− or glp K+. The Shigella transductants which received the glp K gene from E. coli donors all possessed a low EM, indicating that the high charge of E. coli was not due to a gene in the region of glp K. The charge of E. coli was probably due to another factor, such as fimbriae, which carries a negative charge (14).

**Lysis by SLS.** The sensitivity of S. flexneri and E. coli to SLS have been studied, and at

<table>
<thead>
<tr>
<th>Strain</th>
<th>EM type*</th>
<th>glpK</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Shigella flexneri 2a</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-42-43</td>
<td>Low</td>
<td>+</td>
</tr>
<tr>
<td>24570</td>
<td>Intermediate</td>
<td>-</td>
</tr>
<tr>
<td>24570 glpK* revertants</td>
<td>Low</td>
<td>+</td>
</tr>
<tr>
<td>(10)^6</td>
<td>Intermediate</td>
<td>-</td>
</tr>
<tr>
<td>M-42-43 glpK* mutants (8)</td>
<td>Intermediate</td>
<td>-</td>
</tr>
<tr>
<td>M-42-43 IS glpK* rever-</td>
<td>Low</td>
<td>+</td>
</tr>
<tr>
<td>tants (7)^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli K-12</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hfr C strain W1895</td>
<td>High</td>
<td>+</td>
</tr>
<tr>
<td>27p*</td>
<td>High</td>
<td>+</td>
</tr>
<tr>
<td>76p glpK mutant</td>
<td>High</td>
<td>-</td>
</tr>
<tr>
<td>glpK* transductants*</td>
<td>Low</td>
<td>+</td>
</tr>
<tr>
<td>(W1895) × 24570 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(27p) × 24570 (4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* EM is expressed in micrometers per second per volt per centimeter. Values are divided into three types: low, <0.6; intermediate, 1.2 to 2.0; and high, >3.0.

* Numbers in parentheses indicate the number of strains tested.

* These revertants are from 18, another avirulent glpK mutant of M-42-43.

* Strain 27p is a promotor mutant which increases the glycerol kinase activity twofold. It was obtained from D. Richey and E. C. C. Lin, as was 76p.

* Strain in the parenthesis is the donor strain in a phage P1, vir transduction.
least three genes are known to determine this characteristic. One gene near the lactose operon was demonstrated to govern sensitivity to SLS in E. coli (20). The two other genes are cotransducible with the arabinose operon (5).

It has been shown that the virulent S. flexneri strain M-42-43 and its avirulent mutant, strain 24570, differed in their relative resistance to the anionic detergent SLS. The avirulent mutant is far less sensitive to SLS than its virulent parent (Table 7). E. coli K-12 is much more sensitive to SLS than both of these Shigella strains (5). The percentage of lysis per 10 min of the avirulent strain ranged from 0 to 20%; the virulent strain had a lysis of 25 to 50%, and E. coli strain W-1895 lysed 50 to 75%. Based on these values, sensitivity to SLS can be divided into three classes: resistant, intermediate, and sensitive.

To establish whether SLS sensitivity was related to the mutation which made avirulent strain 24570 incapable of utilizing glycerol as a carbon source, the different mutants were tested. The 24570 glp K+ revertants remained resistant to SLS as its parent, but out of nine M-42-43 glp K− mutants tested, two remaining intermediate like M-42-43, and seven became sensitive to SLS. No glp K+ transductants from an M-42-43 to 24570 cross regained the SLS lysis characteristics of the M-42-43. Thus, the glp K gene does not affect SLS lysis characteristics.

**Biological oxidation.** Virulent Shigella strain M-42-43 has been shown to oxidize succinate, fumarate, and malate at a faster rate than its avirulent mutant. This has been shown in whole cells and in cell-free extracts (15). When the glycerol kinase mutants derived from these strains were studied, the oxygen uptake by these strains resembled that of the parents (Table 8). For example, strain 24570 has an uptake of 3.5 μl per 106 cells per 10 min, and its 24570 glp K+ revertants have a rate ranging from 1.6 to 3.6 μl per 106 cells per 10 min. Strain M-42-43 has a rate of 18.6 μl per 106 cells per 10 min, and its M-42-43 glp K− mutants have a rate ranging from 8.6 to 16.0 μl per 106 cells per 10 min. Thus, there appears to be no direct relationship between glp K and the oxidation of these tricarboxylic acid cycle intermediates.

**Virulence.** The ability of virulent strains to penetrate HeLa cells (4) and to cause keratoconjunctivitis correlates well with the ability to invade the intestinal epithelial cells of starved guinea pigs and monkeys (17). Using the method of LaBrec et al. (17), we verified the fact that virulent S. flexneri strain 24570 did not invade or multiply within HeLa cells. In contrast, virulent S. flexneri strain M-42-43 heavily infected the HeLa cells, with most of the bacteria appearing in the cytoplasm. By using glycerol kinase as the marker to distinguish between the parent strains, mutants, and their derivative strains, penetration of HeLa cells by these strains was tested. "Virulence" will be used in the narrow sense to describe this ability to penetrate HeLa cells.

Table 8 shows that 50% of the glp K+ revertants derived from avirulent strain 24570 regained the ability to penetrate HeLa cells. Similarly, 44% of the glp K− strains derived from virulent strain M-42-43 (glp K+) via mutagenesis were incapable of penetration. One of these, avirulent M-42-43 glp K− strain I8, was able to revert to glp K+, and all four colonies tested had regained virulence as measured by HeLa cell penetration. Conjugants receiving the glp K marker from E. coli K-12 strains 27p or W1895 were not capable of making the avirulent strain 24570 regain virulence. Transductants receiving the glp K marker from virulent S. flexneri strain M-42-43 also showed that 39% had been converted from avirulent (24570) to virulent strains (Table 10). Thus, there is a partial correlation between the glp K phenotype and penetration. This puzzling result seems to indi-

Table 7. Sensitivity to sodium lauryl sulfate

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lysis class*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. flexneri M-42-43</td>
<td>I</td>
</tr>
<tr>
<td>S. flexneri 24570</td>
<td>R</td>
</tr>
<tr>
<td>E. coli W1895</td>
<td>S</td>
</tr>
<tr>
<td>M-42-43 glpK mutants (9)</td>
<td>2F + 7 S</td>
</tr>
<tr>
<td>24570 glpK+ revertants (10)</td>
<td>10 R</td>
</tr>
</tbody>
</table>

* Abbreviations: S, sensitive (>55%); I, intermediate (25 to 50%); R, resistant (0 to 20%).

* Numbers in parentheses represent number of strains tested.

Table 8. Succinate oxidation by stationary-phase cells of S. flexneri 2a

<table>
<thead>
<tr>
<th>Strain</th>
<th>Oxygen uptake*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-42-43</td>
<td>18.6</td>
</tr>
<tr>
<td>M-42-43 glpK (8)*</td>
<td>8.6 to 16.0*</td>
</tr>
<tr>
<td>24570 glpK</td>
<td>3.5</td>
</tr>
<tr>
<td>24570 glpK+ revertants (10)</td>
<td>1.6 to 3.6*</td>
</tr>
</tbody>
</table>

* Cells were grown in nutrient broth without glucose and with vigorous shaking. Uptake is expressed as μl per 106 cells per 10 min.

* Numbers in parentheses represent number of strains tested.

* Range of values obtained.
TABLE 9. HeLa cell infection by strains of S. flexneri 2a

<table>
<thead>
<tr>
<th>Strain</th>
<th>glpK</th>
<th>Penetration*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-42-43</td>
<td>+</td>
<td>1/1</td>
</tr>
<tr>
<td>M-42-43 glpK mutants</td>
<td>-</td>
<td>5/9</td>
</tr>
<tr>
<td>M-42-43 18, glpK</td>
<td>-</td>
<td>0/1</td>
</tr>
<tr>
<td>B8 glpK+ revertants</td>
<td>+</td>
<td>4/4</td>
</tr>
<tr>
<td>24570 glpK</td>
<td>-</td>
<td>0/1</td>
</tr>
<tr>
<td>24570 glpK+ revertants</td>
<td>+</td>
<td>5/10</td>
</tr>
</tbody>
</table>

*Expressed as number of virulent strains/total strains tested.

TABLE 10. HeLa Cell penetration by S. flexneri

<table>
<thead>
<tr>
<th>Method</th>
<th>Donor</th>
<th>Recipient</th>
<th>Penetration*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transduction</td>
<td>27p</td>
<td>24570</td>
<td>0/4</td>
</tr>
<tr>
<td>Transduction</td>
<td>M-42-43</td>
<td>24570</td>
<td>7/18</td>
</tr>
<tr>
<td>Conjugation</td>
<td>W-1895</td>
<td>24570</td>
<td>0/5</td>
</tr>
</tbody>
</table>

*Expressed as number of virulent strain/total strains tested.

cate that there may be two different genes affecting glp K, one of which is related to HeLa cell penetration and the other not.

DISCUSSION

It has been demonstrated that the virulent S. flexneri 2a strain M-42-43 has mutated to an avirulent strain, 24570, which no longer ferments glycerol, due to a loss of glycerol kinase (glp K) activity. Reversion of the mutant to glp K+ restores the ability to penetrate HeLa cells to only half of the revertants. Furthermore, selection of additional glp K mutants of M-42-43 only results in a loss of virulence in half of these strains. The strains are classified as virulent if they can penetrate HeLa cells. One conclusion from such results might be that more than one gene affects glycerol kinase activity in Shigella, but only one is involved with the ability of the bacteria to penetrate HeLa cells.

The first clue leading to this hypothesis was the fact that virulent S. flexneri produced pink colonies on 1% glycerol-MacConkey agar, whereas E. coli strains formed dark-red colonies. This indicated at the very least a difference in the quantity of glycerol kinase produced. Richey and Lin (21) have presented evidence comparing the enzyme from our strains of Shigella with that from E. coli indicating that the enzymes are essentially identical. The Shigella enzyme reacts with antiserum prepared against the crystalline E. coli enzyme by using the Ouchterlony double-diffusion technique, is sensitive to fructose diphosphate, and has nearly identical Km values. Thus, it would seem that there might be a difference in the level of derepression of glp K in the two species, although a comparison of turnover numbers of both enzymes was not possible in the absence of a purified preparation of the Shigella enzyme.

The evidence presented in this paper is not consistent with the simple explanation of a difference in derepression of the synthesis of the enzyme. First, the glp K genes of the two genera appear to be at different loci on the chromosome. The E. coli glp K gene cotransduces with rha, whereas the Shigella glp K gene does not. Interrupted mating studies also places the rha and glp K genes in Shigella apart. It does not appear that this is simply a matter of translocation of genes. When the Shigella glp K is introduced into E. coli, it does not cotransduce with rha. Perhaps a more important distinction between the genes in the different strains is their relation to virulence. Transduction of the glp K+ gene from E. coli into the avirulent Shigella strain 24570 (producing dark-red colonies on glycerol-MacConkey agar) does not restore virulence. Transduction of glp K from the virulent Shigella into 24570 does give rise to virulent transductants, but not always. Similarly, only some glp K+ revertants of the avirulent strain regain virulence, and only some glp K mutants of the virulent Shigella lose virulence. Thus, there is an indication that at least two genes affecting glycerol kinase activity exist.

The complete correlation of glycerol kinase activity with changes in electrophoretic mobility of Shigella remains unexplained. This correlation is observed whether the enzyme in Shigella is from Shigella or donated from E. coli. The three- to fourfold increase in negative charge upon the loss of the enzyme activity may be due to the necessity of the enzyme for the synthesis of certain cell envelope components, i.e., phospholipids, but this cannot relate to virulence per se since only the gene in Shigella has any correlation with virulence. Rather, it would seem that perhaps some specific cell wall state may affect glycerol kinase activity and once the enzyme is present it causes the alteration in electrophoretic mobility.

The mutation of the virulent M-42-43 to 24570 was selected on the basis of an alteration in colonial morphology, not the ability to ferment glycerol. The lack of correlation of succinate oxidation and SLS sensitivity to glycerol kinase does not necessarily mean that these characteristics might not correlate, at least partially, with colonial morphology. Thus, if a par-
ticular cell wall mutation which results in colo-
mial morphology changes also causes a loss of
glycerol kinase in Shigella, then the altered
characteristics of 24570 might correlate with
glycerol kinase. Interestingly, we have obtained
a glp K mutant of M-42-43, I8, which upon reversion
to glp K+ did restore HeLa cell penetration
in four out of four cases.

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