Characterization of Streptozotocin Induced Small Colony Phenotypes in Bacteria

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CHARACTERIZATION OF STREPTOZOTOCIN INDUCED SMALL COLONY PHENOTYPES IN BACTERIA

by

David M. Zimmer

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment
of the
Degree of Master of Arts

Western Michigan University
Kalamazoo, Michigan
April 1973
CHARACTERIZATION OF STREPTOZOTOCIN
INDUCED SMALL COLONY PHENOTYPES
IN BACTERIA

David M. Zimmer, M.A.
Western Michigan University, 1973

Streptozotocin-induced small colony variants of *E. coli* were characterized and found to have a genetic basis. Both *E. coli* and *S. typhimurium* were susceptible to the mutation causing small colonies. Small colony cell lines exhibited a greatly increased generation time and were shown to be non-motile, electron microscopy revealing a complete loss of flagella. The small colony phenotype was revertible by streptozotocin and nitrosoguanidine. The locus responsible for small colony phenotype in one strain of *E. coli* was found to map within a 19 minute segment of the chromosome between HfrH and HfrC.
ACKNOWLEDGMENTS

The author wishes to express thanks to Dr. Gyula Ficsor, under whose tutorage this work was accomplished, for his time and efforts. Thanks are extended as well to Dr. Walter Johnson and Dr. Joseph Engemann for their invaluable help.

Gratitude is also expressed to Dr. Darwin Buthala and Dr. Stephen Friedman, as well as to the Upjohn Co., who furnished the streptozotocin used in this investigation.

David M. Zimmer
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INTRODUCTION

While studying the genetic effects of the mutagenic antibiotic streptozotocin in bacteria, G. Ficsor observed the presence of small colony variants on nutrient agar plates. These small colonies were present in unusually high proportions (as high as 15 percent in some instances) and were essentially stable throughout repeated single colony isolations, indicating genetic alteration of the cells.

The investigation of the small colony variants was undertaken since the literature is not abundant with studies of small colony formers and the induction of any new type of mutant, especially a forward mutation, is of genetic interest. I have shown that the small colony variants considered here are indeed genetic in nature, that they can be induced by chemical mutagens other than streptozotocin, and that mutagen induced small colony phenotypes are common to more than one bacterial species. Genetic mapping of the mutant genes was accomplished, and electron microscopy was used to study cell morphology of small colony formers.

The possible genetic nature of the small colony mutants is discussed, as well as the relationship of streptozotocin induced smalls to previously reported small colony variants. The high frequency of induction of smalls and their possible future uses are also treated.
Streptozocin

Streptozocin (SZN), a fermentation product of *Streptomyces achromogenes* var. *streptozoticus*, is a broad-spectrum antibiotic exhibiting activity against a number of gram positive and gram negative organisms, both *in vivo* and *in vitro* (Vavra *et al.*, 1960). The following structure for SZN was proposed by Herr *et al.* (1967), indicating that SZN is a 2-deoxy-D-glucose derivative of N-methyl-N-nitrosourea, and this formula is now generally accepted for the compound.

![Structure of streptozocin](image)

Fig. 1. Structure of streptozocin. After Herr *et al.* (1967).

Deleterious effects of the drug were discovered and described throughout the 1960's and early 1970's, and at present, SZN is used only for investigational purposes.

Diabetogenic properties of SZN were first described by Rakieten *et al.* (1963), who showed that diabetes mellitus
was caused by exposure to SZN in both rats and dogs. In 1965, Evans et al. reported hyperglycemic effects of SZN in mice and rats, and Dulin et al. (1967) confirmed diabetogenicity of the drug, indicating that SZN interferes with beta cell function, resulting in decreased synthesis of insulin.

Evans et al. (1965) reported antitumor effects of SZN, in which they observed activity against the mouse tumors Ehrlich carcinoma, Sarcoma 180, and LS 178Y, and the rat tumor Walker 256.

Carcinogenicity of SZN was suggested by Arison and Feudale (1967), when 10 of 19 rats developed neoplasia of the renal cortex after single doses of SZN.

Mutagenic activity of SZN was demonstrated in 1968 by Gichner et al., and by Kolbye and Legator (1968), who reported that SZN with or without cofactor possesses a similar mutagenic spectrum to nitrosoguanidine, a known alkylating agent. Alkylating agents are a class of compounds which carry one or more alkyl groups and react within the cell by donating their alkyl groups to DNA, thus changing its molecular constitution and creating mutations (Freese, 1971). SZN is thought to be an alkylating agent because of behavioral similarities of SZN and other known alkylating agents.

In 1970, Bhuyan reported inhibition of DNA synthesis in mammalian cells by SZN, and Reusser (1971) found that SZN induced rapid degradation of cellular DNA in vivo.
(apparently not \textit{in vitro}) in either resting or dividing \textit{Bacillus subtilis} cells. Reusser also stated that SZN exhibits specificity for cytosine containing mononucleotides, but that no interaction was apparent between SZN and isolated DNA or synthetic polynucleotides. Reusser concluded that SZN preferentially inhibits DNA synthesis in \textit{B. subtilis} as compared to RNA or protein synthesis.

Small Colony Variants

Small colony variants among microbial species are derived from cells which have a reduced growth rate on agar plates. The basic premise in the genetical study of small colony variants is that by investigating these deviations from the norm, we can extrapolate to the mechanisms of the normal situation. Despite the magnitude of mutagenicity studies in bacteria, references to small colony variants in the literature are infrequent.

"Microcolonies" of several species of bacteria were described (Hoffman and Frank, 1964 and 1965; Chan, 1963), but these microcolonies were "micro" merely due to the young age of the colony.

In 1931, Kuhn and Sternberg isolated small colony variants of several microbial species after exposure to ammonia or phenol. Hadley, Delves, and Klimek (1934) used lithium chloride to induce small colony variants in species of \textit{Shigella}, which they isolated by filtration, and they
erroneously thought that the small form represented a stage in the life cycle of the bacteria.

Swingle (1934) also used lithium chloride as an inducer of small colony variants which were antigenically similar to the parentals, but could not confirm Hadley, Delves, and Klimek's claim of filterability as an isolation technique. Youmans (1937) reported results similar to Swingle's, and suggested that the smalls were caused by a decrease in metabolic rate caused by exposure to the inhibitory lithium chloride. In 1946, Colwell induced small colony variants in \textit{E. coli} with 2-methyl-2,4-naphthoquinone. Her smalls were stable if plated on nutrient agar containing quinone, but were unstable over long periods of time when quinone was removed.

All the small colony variants reported from 1931 to 1946 were highly unstable, invariably reverting to normal size upon removal of the inducing agent, and thus are not considered to be genetically determined variants.

Wise and Spink (1954) isolated stable small colony variants in \textit{Staphylococcus aureus} from patients with a history of antibiotic therapy, and were able to demonstrate induction of small colony formers by \textit{in vitro} antibiotic treatments. Weed and Longfellow (1954) reported the induction of small colony variants in \textit{E. coli} by low concentrations of copper ions in broth cultures. Clowes and Rowley (1955) confirmed Weed and Longfellow's observations,
and reported that although 98-99 percent of their own smalls were unstable, 2 strains did not revert to normal upon removal of the copper. These stable small colony variants had a 20 percent greater generation time than parentals and showed reduced motility, but were otherwise identical to their parents.

Clowes and Rowley (1955) attempted to map the small colony gene with F+ recombination mapping, and were able to transfer the colony size gene about 1 percent of the time in one of the small colony strains. However, if norleucine resistance (nl \( ^{r} \)) was transferred, the small colony gene was also transferred in 35 percent of the recombinants, thus showing strong linkage to nl \( ^{r} \). In a previous study, Clowes and Rowley (1953) were unable to map conclusively the nl \( ^{r} \) locus, but were able to demonstrate strong linkage of nl \( ^{r} \) to arabinose (ara). Therefore, the gene for small colony variance in that particular strain is probably linked closely to the ara operon. Mapping data from the other small colony isolate were not identical, and were not as readily interpretable. Clowes and Rowley (1955) concluded that their two small colony strains were probably the result of nonallelic, single-gene mutations, and that since colony growth on various media was variable but still slow, permeability of some essential nutrient(s) may have been affected.
Small colony variants of *Saccharomyces cerevisiae* (baker's and brewer's yeast) called *petites* were first described by Ephrussi and Hottinguer (1950), who demonstrated that the small colony nature of the *petites* was due to mutation of cytoplasmic genes, and therefore exhibited genetic characteristics of that mode of inheritance. Subsequent research on *petites* has revealed that they lack certain respiratory enzymes of the mitochondria necessary for aerobic respiration (Jinks, 1964). *Petites* which phenotypically resemble those described by Ephrussi and Hottinguer (1950) but are genotypically different, have also been described. The other varieties of *petite* mutants consist of segregational *petites*, which give Mendelian segregation ratios when crossed to wild type strains and are the result of chromosomal mutations, and suppressive *petites*, which do not follow patterns of either Mendelian or cytoplasmic inheritance (Jinks, 1964).

Bottstein and Jones (1969) reported the presence of nitrosoguanidine induced small colonies on minimal media. Bottstein and Jones did not investigate their smalls, and no other reports of small colony variants induced by common chemical mutagens have been found. Howard Carr (personal communication) has indicated that they are not unique to this laboratory, stating that SZN induced small colony variants of *E. coli* have been observed by him and Herbert S. Rosenkranz, and that nitrosomethylurethane and methyl-
azoxy-methanol acetate are also capable of inducing similar small colony variants in bacteria.
MATERIALS AND METHODS

Strains

All strains used are listed in Table I along with information on genotype, phenotype, species, sex type, and derivation. Strains 202 and 203 are SZN mutagenized derivatives of 188 that were selected for their small colony phenotype. They were selected from separate mutagenic treatments to reduce the possibility of common parentage. Both were tested for phenotype and marker stability (before and after SZN treatment) by plating 0.1 ml of an overnight nutrient broth culture on appropriately supplemented minimal media and scoring for reversion every day for four days.

Media

The nutrient medium was Bacto nutrient broth (Difco) solidified with 2 percent Bacto agar (Difco).

Minimal media consisted of a salt solution containing 9.85 g K$_2$HPO$_4$, 4.22 g KH$_2$PO$_4$, 0.94 g (NH$_4$)$_2$SO$_4$, 0.44 g sodium citrate, and 0.05 g MgSO$_4$ per liter, with a carbon source (autoclaved glucose or filter-sterilized lactose) added to give a 0.2 percent final solution. Supplemented media were obtained by adding 1.0 ml of 2.0 mg/ml solution of the required L-amino acids to 100 ml of minimal media.
<table>
<thead>
<tr>
<th>Strain #</th>
<th>Genotype</th>
<th>Colony Phenotype</th>
<th>Sex type</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>188</td>
<td>arg&lt;sup&gt;-&lt;/sup&gt;, met&lt;sup&gt;-&lt;/sup&gt;, leu&lt;sup&gt;-&lt;/sup&gt;, his&lt;sup&gt;-&lt;/sup&gt;, trp&lt;sup&gt;-&lt;/sup&gt;, lac&lt;sup&gt;-&lt;/sup&gt;, gal&lt;sup&gt;-&lt;/sup&gt;, mtl&lt;sup&gt;-&lt;/sup&gt;, xyl&lt;sup&gt;-&lt;/sup&gt;, sm&lt;sup&gt;r&lt;/sup&gt;, λ&lt;sup&gt;r&lt;/sup&gt;, λ&lt;sup&gt;-&lt;/sup&gt;, mal&lt;sup&gt;-&lt;/sup&gt;, t&lt;sub&gt;6&lt;/sub&gt;</td>
<td>Normal</td>
<td>F&lt;sup&gt;-&lt;/sup&gt;</td>
<td>E. coli</td>
<td>Coli Genetic Stock Center, Yale University</td>
</tr>
<tr>
<td>201</td>
<td>met&lt;sup&gt;-&lt;/sup&gt;, uridine&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Normal</td>
<td>HfrC</td>
<td>E. coli</td>
<td>S. Friedman, Western Michigan University</td>
</tr>
<tr>
<td>183</td>
<td>his&lt;sup&gt;-&lt;/sup&gt;, gal&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Normal</td>
<td>HfrA</td>
<td>S. typhimurium</td>
<td>K. Sanderson, University of Calgary, Canada</td>
</tr>
<tr>
<td>184</td>
<td>pro&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Normal</td>
<td>HfrB2</td>
<td>S. typhimurium</td>
<td>K. Sanderson</td>
</tr>
<tr>
<td>130</td>
<td>met&lt;sup&gt;-&lt;/sup&gt;, arg&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Normal</td>
<td>HfrH</td>
<td>E. coli</td>
<td>G. Ficsor, Western Michigan University</td>
</tr>
</tbody>
</table>
**TABLE I (Continued).**

<table>
<thead>
<tr>
<th>Strain #*</th>
<th>Genotype</th>
<th>Colony Phenotype</th>
<th>Sex type</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>202, 203</td>
<td>Same as 188.**</td>
<td>Small</td>
<td>F-</td>
<td><em>E. coli</em></td>
<td>Derived and selected from SZN treatments of 188.</td>
</tr>
<tr>
<td>130 small</td>
<td>Not tested.</td>
<td>Small</td>
<td>HfrH</td>
<td><em>E. coli</em></td>
<td>Derived from 130.</td>
</tr>
<tr>
<td>172A</td>
<td>his-</td>
<td>Normal</td>
<td>Unknown</td>
<td><em>S. typhi-murium</em></td>
<td>Bruce N. Ames, University of California, Berkeley, California</td>
</tr>
<tr>
<td>204</td>
<td>Not tested.</td>
<td>Small</td>
<td>Unknown</td>
<td><em>S. typhi-murium</em></td>
<td>Derived from 172A</td>
</tr>
<tr>
<td>127</td>
<td>lac-</td>
<td>Normal</td>
<td>Unknown</td>
<td><em>E. coli</em></td>
<td>G. Ficsor, Western Michigan University</td>
</tr>
<tr>
<td>127 small</td>
<td>lac-</td>
<td>Small</td>
<td>Unknown</td>
<td><em>E. coli</em></td>
<td>Derived from 127.</td>
</tr>
</tbody>
</table>

*Strain numbers used by G. Ficsor.

**Only those markers used in conjugations were tested. For method of testing see Materials and Methods.
Chemicals

Streptozotocin (Lot #U9889 62 HKI-81A) was donated by the Upjohn Co., Kalamazoo, Michigan. N-methyl-N-nitro-N-nitrosoguanidine (MNNG) was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin; diethylsulfate (DES) and ethylmethanesulfonate from Eastman Organic Chemicals, Rochester, New York; and 2-aminopurine (2-AP) from Nutritional Biochemicals Co. (N.B. Co.), Cleveland, Ohio. Captan (98.9 percent, technical grade) was obtained from Stauffer Chemical Co., Richmond, California. ICR-191 was contributed by Dr. H. Creech, University of Pennsylvania.

Conjugation Mapping

The technique for conjugation mapping was adapted from that described by Sanderson and Demerec (1965). All mapping was Hfr-mediated, gradient of transmission recombination mapping. A 12 ml mixture of a log phase, nutrient broth culture of donor (3 ml) and a similar culture of recipient (9 ml) were drawn onto a Millipore filter (0.45 μm). The filter was then transferred to a nutrient plate and incubated for 5 minutes at 37°C. Bacteria were resuspended in 10 ml fresh nutrient broth by placing the filter in the liquid and swirling gently for about 10 seconds.

All conjugations were allowed to proceed uninterrupted for 1 hour at 37°C, followed by plating of 0.3 ml of the
suspension directly onto selective medium containing 0.2 percent lactose, 1200μg/ml streptomycin sulfate (N.B. Co.), and supplemented with tryptophan, methionine, arginine, and histidine.

Unless otherwise noted, recombinants were apparent after 2 days incubation at 37° C. Single colonies were taken from selective plates and streaked for single colony isolation on selective media. These isolated single colonies were then restreaked on nutrient agar, and scored after 48 hours for size phenotype. The intermediate streaking was necessary to insure that no donor or recipient was picked up from the original selective plates, since these would grow on nutrient agar and prevent accurate scoring of recombinants.

Reversion

Qualitative reversion studies of small colony strains were accomplished using the spot test method (Ames, 1971). This assay consists of plating a lawn of approximately 10^8 cells on an agar plate, then placing a mutagen on the agar, incubating, and observing the plate for the presence of reversions.

Doubling Time

Doubling time of small colony strains was measured by change in percent transmittance of 500 mμ of a log
phase nutrient broth culture over a 5 hour period. Readings were taken every 30 minutes using a Spectronic 20 Spectrophotometer (American Optical).

Mutagenic Treatments

Mutagenic treatment for both generation of smalls and dosage curves were made by resuspending an overnight nutrient broth culture in fresh nutrient broth, adding mutagen at the proper concentration, and incubating for 1 hour at 37° C with shaking. Cells were then washed 3 times in minimal media salts without glucose, and serial dilutions from the third resuspension were plated. When generating smalls, 3μg/ml of SZN was found to be the optimum concentration of mutagen. Other dosages were 0, 1, 2, and 4μg/ml of SZN, and 0, 10, 15, 20, and 25μg/ml of MNNG for the dosage curves.

Motility

Motility of cell lines was established by a modification of the hanging drop technique. A drop of bacterial suspension was placed on a slide, covered with a cover slip, and observed at 630x magnification for motility.

Electron Microscopy

Electron microscopy was generously donated by Dr. Darwin Buthala, Western Michigan University.
Overnight nutrient broth cultures were fixed in 3 percent gluteraldehyde for 1 hour at room temperature, centrifuged at 1000 x g for 30 minutes, washed twice in distilled water, and resuspended to approximately $10^{10}$ cells/ml. One drop of bacterial suspension was placed on a form bar, carbon coated grid, and bacteria were allowed to attach to the grid, then excess fluid was removed. Cells were shadow cast with platinum-palladium at approximately a 30° angle and observed with a Siemens 1A electron microscope.

Alternately, cells were fixed in 3 percent gluteraldehyde for 30 minutes, and washed in distilled water. Staining was accomplished by placing equal volumes of phosphotungstic acid and cell suspension on the grid; cells were allowed to adhere to the grid, and excess fluid was removed. Cells were then observed as above.
RESULTS

Dose Response

Tables II and III show that the occurrence of small colony phenotypes is increased by increasing the dosage of both SZN and MNNG, and that both *E. coli* and *S. typhimurium* are susceptible to the mutation resulting in small colony phenotypes. *E. coli* 130 and *S. typhimurium* 184 were chosen for dosage data because they were to be used for mapping as well. Small colony lines of 130 and 184 were not used for mapping because it was decided that wild type colonies would be easier to select against a background of smalls than to select small colonies from a background of wild type colonies. Hence, the Hfr strains used in conjugation were wild type for colony size, and dosage data were not taken for the eventual recipient (188 or its SZN mutagenized derivatives).

Attempts to induce small colony variants in *E. coli* 188 with the two known alkylating agents EMS (0.1 M) and DES (0.1 M) as well as 2-AP were unsuccessful.

Cell Types

Cells taken from SZN induced small colonies are invariably non-motile as shown by a modification of the hanging drop technique. Electron microscopy revealed that
<table>
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<tr>
<th>Concentration of SZN (in μg/ml)</th>
<th>E. coli</th>
<th>S. typhimurium</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 (100%)</td>
<td>0 (100%)</td>
</tr>
<tr>
<td>1</td>
<td>0.80 (95%)</td>
<td>0.68 (35%)</td>
</tr>
<tr>
<td>2</td>
<td>No data</td>
<td>2.04 (21%)</td>
</tr>
<tr>
<td>3</td>
<td>9.9 (2.6%)</td>
<td>1.56 (27%)</td>
</tr>
<tr>
<td>4</td>
<td>17.5 (11%)</td>
<td>2.71 (41%)</td>
</tr>
</tbody>
</table>

*Mutation frequency: \( \frac{\text{No. small colonies/plate}}{\text{No. colonies/plate}} \times 100. \)
### TABLE III

Effect of increasing dosage of NG on incidence of small colony phenotypes.

<table>
<thead>
<tr>
<th>Concentration of NG (mg/ml)</th>
<th>Frequency* of small colony phenotypes per $10^2$ survivors (survival)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>E. coli</strong></td>
</tr>
<tr>
<td>0</td>
<td>0 (100%)</td>
</tr>
<tr>
<td>10</td>
<td>2.5 (55%)</td>
</tr>
<tr>
<td>15</td>
<td>5.7 (19%)</td>
</tr>
<tr>
<td>20</td>
<td>3.3 (14%)</td>
</tr>
<tr>
<td>25</td>
<td>3.4 (15%)</td>
</tr>
</tbody>
</table>

*Mutation frequency: $\frac{\text{No. small colonies/plate}}{\text{No. colonies/plate}} \times 100.$
the only gross morphological change in the cells was the absence of flagella on small colony formers (Figures 2, 3, 4, and 5). In one electron photomicrograph of strain 202, one flagellum was present on one bacterium. The strain is thought to have either reverted or become contaminated, and the datum was disregarded. Stock cultures of both smalls and parentals were routinely stored in nutrient broth at $4^\circ\text{C}$, and were only stored after at least 3 single colony isolations to insure purity. Inocula from these stored cultures were used to start the overnight broth cultures for electron microscope studies.

Cells from normal sized, SZN treated colonies were not examined with either test for motility. If flagella-less cells were observed in normal colonies after SZN treatment, a "curing" effect of SZN would be implied. However, this is considered unlikely since all smalls were extensively isolated and purified, and any non-permanent alterations of cell morphology caused by exogenous agents would have been restored to normal by dilution of the agent.

Doubling Time

Since small colonies were present on nutrient agar after a minimum of 48 hours, and broth cultures of smalls required about 20 hours to grow to turbidity (compared to 12-15 hours for wild type), it was suspected that small
Fig. 2. Electron photomicrograph of E. coli 127 parental, x 14,800.
Fig. 5. Electron photomicrograph of *S. typhimurium* 204, x 20,000.

Note absence of flagella.
colony strains had an increased generation time. Figures 6 and 7 show that *E. coli* 130 and *S. typhimurium* 184 divided in 4.5 and 2.5 hours, respectively, while smalls derived from these strains failed to divide in 5 hours, even though they were in log phase.

Although increased generation time can account for the small colony phenotype after 48 hours, it cannot explain the fact that small colonies do not attain normal colony size even after 7 days incubation. If increased generation time were the only factor involved in forming small colonies, increased incubation time should allow the slow growers to attain normal colony size.

**Qualitative Reversion of Smalls**

While performing routine isolation procedures on small colony formers, several isolates were found that were unstable and segregated normal sized colonies. These revertant colonies grew right over the small colonies and attained normal size. These unstable lines were not considered contaminants since small colony isolates from a given unstable clone remained unstable upon further restreaking. Characteristics of reversion of smalls allowed the use of the spot test assay of Ames (1971) to determine susceptibility of smalls to reversion.

As shown in Table IV, SZN, MNNG, DES, EMS, captan, 2-AP, and ICR-191 were used to effect reversion. Good numbers of
Fig. 6. Doubling time. Doubling time of \textit{E. coli} 130 small (●●) compared to its parental, \textit{E. coli} 130 (○○).
Fig. 7. Doubling time. Doubling time of *S. typhimurium* 184 small (●●) compared to its parental, *S. typhimurium* 184 (○○).
TABLE IV
Reversion of small colony cell lines with the spot-test assay. A + indicates reversion.

<table>
<thead>
<tr>
<th>Strain</th>
<th>EMS</th>
<th>DES</th>
<th>SZN</th>
<th>NG</th>
<th>Captan</th>
<th>ICR-191</th>
</tr>
</thead>
<tbody>
<tr>
<td>127</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>184</td>
<td>-</td>
<td>-</td>
<td>+*</td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>202</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*After 3 weeks incubation, the last two at room temperature.
reversions were observed with SZN and MNNG in 127 small
and 184 small. No reversion occurred in 202, although
this strain was more susceptible to the growth inhibiting
effects of the mutagens as evidenced by a larger clearing
around the spot of the chemical. After 3 weeks of incuba-
tion, the last 2 weeks at room temperature, DES and ICR-191
elicted a positive response in 184 small. No spontaneous
reversions were observed in any negative control in this
experiment, though they have been observed in other
experiments.
Conjugation Mapping

Gradient of transmission conjugation mapping of the small colony gene was attempted to determine its approximate position on the chromosome. In all conjugations, streptomycin sensitivity \( (\text{sm}^S) \) was the contraselected marker, and lactose \( (\text{lac}) \) was the selected marker. Both \( \text{lac} \) and \( \text{sm}^S \) were exceptionally stable in the four strains used, since not a single colony appeared in any control where donor or recipient alone was plated on selective media.

Initial attempts to map the small colony gene utilized interrupted mating conjugation, in which the time of entry of the mutant gene after initiation of conjugation is measured. Though much effort was made to adapt this technique, the quantity of streptomycin \( (200\mu\text{g/ml}) \) in the selective medium was insufficient to inhibit growth of the streptomycin sensitive strains when only the \( Hfr \) were plated. Since conjugation techniques depend on media constituents to allow growth of only exconjugants, the presence of colonies on control plates rendered these experiments useless. The concentration of streptomycin finally used for gradient of transmission mapping \( (1200\mu\text{g/ml}) \) was that recommended by Sanderson and Demerec (1965).

Gradient of transmission mapping appears to be superior to the interrupted mating technique when the locus in question can be anywhere from 0-90 minutes on the linkage.

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map (90 minutes are required to transfer the entire genome in *E. coli*).

In all crosses, the donor (*HfrH* or *HfrC*) was wild type for colony size genes, and the recipient was of mutant phenotype (small colony). Therefore, if wild type colonies can be obtained from a given cross, the area of the mutant gene(s) must lie within the genome transferred by the *Hfr* strain. An *Hfr* strain is capable of transferring that segment of its chromosome lying between the first marker transferred during mating and the contra-selected marker. *HfrH* for example, can transfer all the genes between 86 minutes and 64 minutes. See Fig. 8. (The contra-selected marker can be physically transferred but exconjugants receiving it do not grow, since streptomycin sensitivity is now imparted to the exconjugants.)

Results of the mapping are presented in Table V. In cross 130 x 202, it can be seen that of 36 recombinants taken from selective plates, all 36 exhibited normal colony phenotype on nutrient agar after 48 hours incubation. This indicates that the gene(s) for colony size lie somewhere between *HfrH* and *sm*, or an area of about 77 percent of the chromosome (Fig. 8).

Since cytoplasmic genes are at present not known to be transferred during *Hfr* mediated conjugation, the fact that the small colony genes are transferred by this type of mating all but excludes cytoplasmic mutants as a cause of small colony phenotype in 202.
Fig. 8. Linkage Map of *E. coli*. Only those markers used in conjugation are shown. Numbers are map units in minutes from the origin (O90). \(\text{Sm}^+\) = streptomycin resistance (\(\text{Sm}^\text{r}\)) or sensitivity (\(\text{Sm}^\text{s}\)). \(\text{Lac}\) = lactose. \(\text{Ara}\) = arabinose. \(\text{HfrH}\) and \(\text{HfrC}\) show the sites of integration of the F-factors in these strains; the arrow indicates the direction of transfer. After Hayes (1968).
### TABLE V
Results of conjugation mapping.

<table>
<thead>
<tr>
<th>Cross</th>
<th>No. recombinants selected</th>
<th>Phenotype of recombinants restreaked on nutrient agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>130 x 202</td>
<td>36</td>
<td>All Normal</td>
</tr>
<tr>
<td>201 x 202</td>
<td>46</td>
<td>26 Normal, 20 Normal and Small</td>
</tr>
<tr>
<td>130 x 203</td>
<td>40</td>
<td>All Normal</td>
</tr>
</tbody>
</table>
In order to reduce the size of the region in which the small colony gene resides, a second cross was necessary. Strains 201 (HfrC) and 130 (HfrH) transfer a 14 minute segment of the chromosome that is common to both, but 201 transfers the region in the opposite direction of 130 (Fig. 8). Therefore, if wild type colonies can be obtained from 201 x 202, HfrC is capable of transferring the small colony gene(s), and it must lie between HfrH and HfrC. Table V shows that normal sized recombinants were obtained from cross 201 x 202, indicating that the gene(s) under investigation do indeed lie in the 19 minute segment between HfrH and HfrC. However, not all the recombinants from this cross were of normal size. Some recombinants showed a mixed phenotype (small and normal sized) on nutrient agar. Since this is a pattern expected if donor or recipient were accidentally carried over from the original selective plates, single colonies from streaks exhibiting the mixed phenotype were restreaked on selective media to ascertain that they were true recombinants. All colonies tested grew on selective media, indicating that they were indeed true recombinants. The cause of the mixed phenotype is unknown and was not investigated, and the possibility exists that the colony size genes investigated here are unstable under the conditions of certain genomes.
Strain 203 is a small colony, separate isolate of SZN treated 188. The small colony genes of 203 were mapped to determine whether any other small colony strains carry allelic mutations. The results of cross 130 x 203 gave results identical to 130 x 202 (Table V) indicating that the small colony genes of both 202 and 203 map in the same region of the chromosome.

However, although the behavior on nutrient agar of recombinants selected from 130 x 203 was identical to those from 130 x 202, their behavior on selective media was different. Recombinants from 130 x 203 required a minimum of 3 days incubation, and were difficult to pick up even after 4 days (130 x 202 recombinants required only 2 days). Selective media restreaks also required 4 days growth for easy isolation. There exist then, some undefined differences in the genomes of 202 and 203, yet by the criteria used in this investigation (colony size on nutrient agar after 48 hours incubation), they behave identically.

In brief, the gene responsible for small colony phenotype in 202 is within the 19 minute segment of the circular linkage map from HfrH and HfrC, and in all probability, the small colony genes in 203 map in the same region.
DISCUSSION

The relationship of the small colony variants investigated in this study to those mentioned in the literature is largely unknown. Most of the small colony variants cited in the literature review section are unrelated to those presented here since their small colony nature is not permanent. Only Clowes and Rowley's (1955) study is analogous to the present one, since their smalls were clearly due to genetic alteration of the cells. In both studies, phenotypic curing is excluded as a cause of reduced colony size since cell lines are stable through innumerable single colony isolations. Mutation of cytoplasmic genes is not considered likely since cytoplasmic entities are not known to be transferred during either F-factor of Hfr-mediated conjugation (Hayes, 1968). Thus the small colony genes of both Clowes and Rowley (1955) and those reported here are thought to reside within the circular bacterial linkage group.

Clowes and Rowley (1955) showed that one of their smalls was strongly linked to \( n_{1}r \), which was in turn strongly linked to the \( ara \) operon. The possibility exists, therefore, that the mutations studied in both cases are allelic.

In Clowes and Rowley's (1955) other stable cell line, mapping data did not offer a reliable genetic interpretation, and they inferred that this indicated non-allelism.
of the small colony genes in their two isolates. The mapping data presented in the current study are more consistent with an allelic hypothesis, since the genes in 202 and 203 map in the same region of the chromosome. Although indicating allelism in 202 and 203, these mapping data neither prove nor disprove the allelic hypothesis.

Cells from old broth cultures (late stationary phase) usually lack flagella, and since a loss of flagella is characteristic of "sick" cell lines (S. Friedman, personal communication), the lack of flagella in this case is probably a secondary effect of the small colony mutation. Since the known genes for motility map at 36 and 37 minutes on the E. coli map (Hayes, 1968) (outside the area transferred by HfrH and HfrC), it is unlikely that loss of flagella is due to a direct mutation of a motility gene, though the possibility exists that a previously unmapped motility gene may be affected.

The high rate at which small colony phenotypes are induced is of interest, since the phenotype has been shown to have a genetic cause, and mutation at the observed rates is very rare. At a concentration of 3μg/ml of SZN, about 10 percent of the cells surviving treatment are of small colony phenotype (Table II), while a lactose amber mutant reverts at a frequency of only 0.01 percent. A possible explanation of the difference in mutation rate is that SZN (and MNNG) has an affinity for cytoplasmic
entities, since in petite induction in yeast, acriflavines exhibit such an affinity and cause a high mutation rate (Strickberger, 1968). However, DNA-containing cytoplasmic organelles (such as mitochondria in eukaryotes) are not known to exist in bacteria. In addition, mapping results in the present study indicate a chromosomal nature for the small colony genes.

An alternative explanation of the high frequency of small colony induction is that of a "hot spot", or an area of the chromosome that is particularly susceptible to the mutagenic effects of SZN. Non-random mutagenicity of chromosomal genes has been reported (Hayes, 1968, and Bottstein and Jones, 1969), and a chromosomal segment containing such a hot spot could conceivably result in the high frequency of small colony mutants. However, the hot spots discussed by Hayes (1968) were spontaneous reversions of T4 phage, and it may not be valid to compare a forward mutating system such as the one studied here, to a reverse mutating system (with which the vast majority of mutagenic work has been done). Since in a reverse mutating system, genetic damage is already present in the form of an altered codon, a specific type of mutation is required to revert the effects of that codon (either generation of a suppressor mutation or exact reversal of the altered codon). A forward mutating system, however, is capable of detecting any mutation occurring within a
given gene (assuming, of course, that the affected codon modified function of the protein encoded), a fact which must certainly increase the apparent sensitivity of the gene to mutation.

Freese (1971) estimated that the average bacterial gene contains 1000 nucleotides, an estimate which is probably low, since regulatory elements of the gene were not included. It might be expected then, that a gene containing 1000 nucleotides would exhibit a 1000 times greater mutation frequency than a reverse mutating system in the same gene, where essentially one nucleotide is being monitored. (In practice, the forward mutating system would not be expected to realize a mutation rate equal to the full 1000 times, since not all mutations result in impaired protein function.) The possibility also exists that a mutation in any one of a number of genes can result in the small colony phenotype (multigenic effect). Such a case would cause an even higher mutation rate. It is possible then, to explain the frequency of smalls in terms of a forward mutating system and multigenic effects, but whether both or either are in fact causes of the high induction rate of small colony phenotypes is yet to be determined.

Further characterization of the small colony phenomenon is required before the biochemistry of the mutants or their biological significance can be determined. Their
high frequency of induction may indicate great sensitivity to chemical mutagens, though with present techniques, few smalls were observed at low doses of a mutagen as potent as streptozotocin. Greater knowledge and improved techniques for isolating and identifying smalls may render the system useful as an indicator in screening procedures for identification of chemical mutagens.
CONCLUSIONS

1. The induction of small colony variants in bacteria is neither mutagen nor species specific.

2. The frequency with which small colony variants are induced in *E. coli* and *S. typhimurium* by SZN is dosage dependent.

3. The small colony isolates investigated are the result of a mutagenic event or events on the circular linkage group of the bacteria which maps within the 19 minutes segment of the chromosome between HfrH and HfrC.

4. The small colony variants investigated are probably the result of non-allelic single gene mutations.

5. Cells from small colonies lack flagella, but show no other gross morphological differences from their parentals.
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