SMALL-CELL SEGREGANTS FROM A POSSIBLY HOMOZYGOUS DIPLOID STRAIN OF ESCHERICHIA COLI

JAMES E. OGG AND RONALD D. HUMPHREY

Department of Microbiology and Pathology, Colorado State University, Fort Collins, Colorado

Received for publication 9 November 1962

ABSTRACT

Ogg, James E. (Colorado State University, Fort Collins) and Ronald D. Humphrey. Small-cell segregants from a possibly homozygous diploid strain of Escherichia coli. J. Bacteriol. 85:801–807. 1963.—Evidence was presented to show that a homozygous possibly diploid strain of Escherichia coli, when grown in a chemically defined medium under the conditions described in this report, will yield small-cell segregants that have haploid characteristics. The segregants have one-half as much deoxyribonucleic acid per cell as the diploid, give exponential survival curves when exposed to X rays, and have fermentative characteristics similar to the original haploid parent strain of the homozygous diploid strain. No detectable changes in surface antigens were associated with the segregation phenomenon. The haploidlike segregants can be converted to the diploid radiation-resistant state by growing them in the presence of camphor vapors.

Ogg and Zelle (1957) grew the adenine-dependent Escherichia coli strain 82/r in the presence of camphor vapors and isolated a stable large-cell, possibly diploid, organism. This strain, designated P6, was consistently stable and retained its large-cell characteristics in the absence of camphor after numerous subcultures in broth or on solid medium or both. E. coli P6 contained two to three times as much deoxyribonucleic acid (DNA) and twice the amount of ribonucleic acid (RNA) per cell as the parent strain 82/r. It was found to be about 2.5 times more resistant to the lethal effects of ultraviolet light than 82/r, and exhibited sigmoid survival curves following X- and γ-irradiation, whereas 82/r yielded characteristic exponential inactivation curves (Zelle and Ogg, 1957). These findings and other data presented by these two authors were compatible with the hypothesis that P6 was a stable diploid derivative of 82/r.

The rate of fermentation by P6 on a number of carbohydrates was reduced, in comparison with 82/r on the same substrates (Ogg and Zelle, 1957). While studying the mechanism of this reduced rate of fermentation, it was found that when P6 was grown under the special growth conditions described in this report small-cell, smooth-colony-forming segregants appeared in such cultures. In this paper, we present evidence indicating that the small-cell segregants of P6 have characteristics similar, if not identical, to 82/r, the original haploid parent of P6. This finding suggests that the homozygous diploid organism P6 will, under special conditions, undergo “haploidization.” We also show that the segregants can be converted back to the possibly diploid condition by growing them in the presence of camphor vapors.

MATERIALS AND METHODS

Strains. The strains used in these experiments were E. coli 82/r and P6. E. coli 82/r, isolated and described by Anderson (1951), is an adenine-requiring mutant of E. coli B/r. E. coli P6 (a large-cell, possibly homozygous diploid strain) was isolated after exposure of E. coli 82/r to camphor vapors (Ogg and Zelle, 1957). On nutrient agar, E. coli 82/r forms smooth, finely granular colonies, whereas P6 is characterized by colonies with a coarsely granular internal structure.

Media. In some phases of this study, the minimal medium used was that described by Brinton and Baron (1960), with the following changes: no amino acids were added to their basal medium, and adenine was added in a final concentration of 1 μg per ml to supply the nutritional requirements.
of *E. coli* 82/r, P6, and the P6 derivatives. The modified medium contained 14 g of Difco Noble agar dissolved in 420 ml of distilled water to which were added: physiological saline, 300 ml; KH₂PO₄ (80 g/liter), 40 ml; K₂HPO₄ (140 g/liter), 40 ml; (NH₄)₂SO₄ (20 g/liter), 40 ml; MgSO₄ (20 g/liter), 4 ml; adenine; and either 0.5 or 1.0% of appropriate carbohydrate. The ingredients, with the exception of the carbohydrate which was sterilized by filtration, were autoclaved separately and then mixed while hot. This medium without agar served as a chemically defined broth medium. Difco nutrient broth and agar containing 0.5% sodium chloride were used for routine cultivation and transfer of the *E. coli* cultures.

**X-irradiation technique.** The cultures for the radiation-inactivation kinetic studies were grown for 22 hr in aerated nutrient broth at 37 C. The cells were sedimented by centrifugation, and the pellets resuspended and washed twice with 0.067 m phosphate buffer (pH 6.8). After the last washing, the cells were resuspended to original culture volume in the phosphate buffer. The washed-cell suspensions were placed in an ice bath and saturated with oxygen by bubbling with the gas for 15 min. These oxygenated suspensions remained in an ice bath until dilution and plating after radiation treatment. The X-ray source was a Picker Vanguard console model operated at 280 kv and 20 ma with aluminum (1.0 mm) and copper (0.5 mm) filtration. Viable cell counts of nonirradiated and irradiated samples were determined by plating appropriate dilutions made in phosphate buffer onto nutrient agar plates. The plates were incubated at 37 C for 48 hr.

**Serological techniques.** The various *E. coli* strains were grown in aerated nutrient broth for 20 hr at 37 C. Whole-cell antigens were prepared by suspending cells, washed twice with physiological saline, in a physiological saline solution containing 0.2% formaldehyde. The turbidity of the antigens was adjusted with formalized saline to correspond to tube no. 3 of a McFarland nephelometer scale. Antisera were prepared by injecting 1-ml quantities of the respective formalized antigens into rabbits, intraperitoneally, seven times during a 3-week period; 1 week to 10 days after the last injection, blood was collected, and the serum stored at 4 C. Reciprocal whole-cell cross-agglutination reactions and agglutinin-absorption tests were used to determine the serological relationship between antigens.

**Chemical methods.** Semiquantitative tests for the rate of utilization of various carbohydrates by the *E. coli* organisms were performed in Smith fermentation tubes containing minimal broth medium plus 1% of the appropriate carbohydrate. The amount of gas (in mm) that collected in the arm of the fermentation tube was measured at various intervals up to 72 hr of incubation.

With *E. coli* 82/r as the basis for comparison, the relative amounts of DNA and RNA per cell in 20-hr aerated nutrient broth cultures were determined by the technique described by Morse and Carter (1949). Total cell numbers were determined by counting cells suspended in 0.7% Noble’s Special Agar placed in a Petroff-Haussner counting chamber. Total cellular nitrogen was determined by the method of Johnson (1941). The technique of Ogg and Zelle (1957) was used in treating the *E. coli* P6 segregants with camphor vapors to convert them to the stable large-cell state. **Isolation of segregants from diploid *E. coli* P3.** The following method of cultivation was used for isolation of small-cell, smooth-colony-forming segregants from P6. Minimal medium (75 ml) containing 0.5% glucose and supplemented with adenine was placed in a 250-ml Erlenmeyer flask fitted with a rubber stopper. Cells from a 20-hr aerated nutrient broth culture of P6 were washed twice with 0.85% NaCl solution and resuspended in the saline solution. The flask received 0.1 ml of inoculum. Colony morphology of the inoculum was determined by plating appropriate dilutions on nutrient agar. The rubber-stoppered sealed flask was incubated under static conditions, and the gas pressure released at 24-hr intervals. At 48-hr intervals, samples were removed from the flask, plated on nutrient agar, and the colony morphology observed by the oblique-light method after 24 hr of incubation of the plates.

The above method of cultivation was used on 100 independent P6 cultures; after 96 hr of incubation, approximately 25% of the cultures yielded, in addition to typical P6 colonies, smooth colonies similar to the colonies of 82/r. These smooth colonies consisted of small cells identical in size to those of 82/r. Also appearing on some of these plates were colonies with smooth and rough sectors and colonies which appeared smooth on initial isolation but proved to contain a mixture of two types of cells, those which yielded a stable smooth-colony form like 82/r and those giving typical P6-type colonies. Even when single colonies of P6 were used as inoculum...
and grown in the above manner, small-cell segregants could sometimes be isolated.

Six segregants were selected at random from different cultures for characterization studies and designated P6S1 through P6S6. Two of these segregants, P6S2 and P6S5, were grown in the presence of camphor vapors to convert them to the diploid state. From the camphorated plates, two large-cell, possibly diploid strains (P6S2-P and P6S5-P) were isolated and used in the studies that follow.

RESULTS

As the results of each of the following studies will show, the characteristics of the P6 segregants were similar to those of 82/r (the haploid strain), whereas the stable large-cell forms (P6S2-P and P6S5-P) were similar in every respect to P6 (the diploid type).

Morphological characteristics. The P6 segregants had smooth colonies that appeared to be identical to those of 82/r (Fig. 1). The large-cell types P6S2-P and P6S5-P had coarsely granular colonies similar to those of P6. It has been noted over the years that the diploidlike state of the 82/r strain is usually characterized by large-cell, coarsely granular colonies, whereas haploid forms appear as smooth colonies composed of small cells.

![Image of smooth-colony segregants](http://jb.asm.org/)

**Table 1. Serological relationships of Escherichia coli 82/r, P6, P6 segregants, and the diploidlike derivatives of the segregants**

<table>
<thead>
<tr>
<th>Whole-cell antigen</th>
<th>Antiserum&lt;sup&gt;*&lt;/sup&gt;</th>
<th>82/r</th>
<th>P6</th>
<th>P6S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>P6S2-P</td>
<td>640</td>
<td>640</td>
<td>2,560</td>
<td>2,560</td>
</tr>
<tr>
<td>P6S5</td>
<td>1,280</td>
<td>1,280</td>
<td>1,280</td>
<td>1,280</td>
</tr>
<tr>
<td>P6S2-</td>
<td>640</td>
<td>640</td>
<td>1,280</td>
<td>1,280</td>
</tr>
<tr>
<td>P6S2</td>
<td>1,280</td>
<td>1,280</td>
<td>1,280</td>
<td>1,280</td>
</tr>
<tr>
<td>P6S5-P</td>
<td>320</td>
<td>320</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>P6S5-</td>
<td>2,560</td>
<td>2,560</td>
<td>5,120</td>
<td>5,120</td>
</tr>
</tbody>
</table>

<sup>*</sup> Agglutinin titer expressed as reciprocal of the serum dilution.

However, colony morphology alone is not a criterion for the identification of the diploid or haploid state in the 82/r strain, since the senior author has isolated very rough granular colony-mutant forms from 82/r which appear to have cells identical to the parent 82/r in most characteristics, i.e., small cells, equal DNA content, etc.

Serological relationships. The cross-agglutination reactions between the whole-cell antigens and antisera listed in Table 1 indicate that no gross change occurred in the surface antigenic structure when P6 segregated to the small-cell smooth-colony type. Neither did any alteration of the surface antigens take place when the P6 segregants were converted by camphor treatment to diploidlike forms. However, a considerable variation was noted in the agglutination titers of any one serum when tested against several whole-cell antigens. For example, the limiting dilution titer of a serum was lowest with P6S5 antigen and highest with P6S5-P antigen. This result probably reflects a difference in the antigen preparations and inherent errors in the agglutination test rather than gross differences in the surface antigenic structure of the cells. This conclusion was supported by the data obtained from reciprocal adsorption tests. All agglutinating antibodies were removed from the three antisera by adsorption with any one of the whole-cell antigens (i.e., P6S2 whole-cell antigen will adsorb all agglutinins from its homologous antiserum as well as from the antisera of P6 and 82/r, and vice versa).

Rate of fermentation. The rate of fermentation of the P6 segregants in the synthetic medium containing glucose, lactose, or sodium pyruvate
was similar to that of 82/r (Table 2). As reported by Ogg and Zelle (1957) and again as shown by the data in Table 2, the rate of fermentation of the diploid P6 was slower than that of its haploid parent 82/r. After 36 hr of incubation, 82/r and the P6 segregants produced approximately the same amount of gas from pyruvate, whereas the diploid P6 and the apparent diploids P6S2-P and P6S5-P did not produce gas from this carbohydrate at this time. It will be noted from an examination of the data that P6, P6S2-P, and P6S5-P showed a reduced rate of fermentation on both glucose and lactose as determined by total gas production. However, after approximately 72 hr of incubation, the amount of gas produced by the diploids approached that of 82/r and the P6 segregants. This also applied to the metabolism of pyruvate. Apparently, a change to the diploid state repressed the ability of the cell to ferment the carbohydrates that were tested.

Relative amounts of nucleic acids and total nitrogen per cell. A comparison of the relative amounts of DNA, RNA, and total nitrogen per cell of E. coli 82/r, P6, and the P6 segregants supports the idea that the P6 segregants are haploid (refer to data in Table 3). These segregants contained approximately the same amount of nucleic acids and nitrogen as 82/r, and about one-half as much of these substances as P6. The values obtained for DNA, RNA, and N₂ indicate that, regardless of the size of the cells in the stationary phase of growth, the ratio of these substances to the other was approximately equal. These data also show that, as the ploidy (DNA) increases, the RNA and N₂ of the cell increase in proportion, to maintain a constant nucleocytoplasmic ratio. The figures given for P6 represent some 20 independent analyses and do not support the conclusion previously reached by Ogg and Zelle (1957) that P6 has approximately 2.75 times more DNA than 82/r. Since their mean figure was based on four determinations, our figure of 2.0 ± 0.30 would appear to be statistically more reliable because of the greater number of samples. The stable large-cell types (P6S2-P and P6S5-P) isolated after camphor treatment of P6S2 and P6S5 contained...
about twice as much nucleic acids as the parent strains (refer to bottom two lines of Table 3). Since P6S2-P and P6S5-P contained approximately the same amount of DNA per cell as P6, they would appear to be diploid on the basis of the DNA analysis.

**Kinetics of ionizing radiation-inactivation.** The segregation of P6 gave small-cell forms that were radiation-sensitive; that is, they gave essentially a first-order, exponential X-ray survival curve similar to that of the haploid strain 82/r (Fig. 2). For a given X-ray dose, the six segregants exhibited practically identical values for the surviving fraction; therefore, the data from these radiation runs were consolidated, and only the mean surviving fractions are plotted in Fig. 2. The large-cell derivatives P6S2-P and P6S5-P gave sigmoid-type survival curves similar to that of the diploid P6. This difference in threshold dose between the small-cell forms (82/r and P6 segregants) and the large-cell types (P6, P6S2-P, and P6S5-P) may be interpreted according to the multitarget theory: the large-cell forms require a number of "hits" before the exponential rate of inactivation is established. Thus, the radiation data are consistent with the hypothesis that the small-cell segregants of P6 are haploid and the large-cell types obtained by camphor treatment of the segregants are diploid.

**Discussion**

The large-cell homozygous diploid *E. coli* P6 strain, when grown in chemically defined medium under the conditions described in this report, will yield small-cell segregants that have characteristics similar to the haploid organism 82/r (the original parent strain of the diploid organism). These segregants were found in aged cultures and formed smooth colonies, whereas the diploid was characterized by the rough granular form. As yet, the physical and chemical conditions that bring about the segregation of P6 to the small-cell form are unknown. It is thought to be associated with the semiaerobic growth conditions; under aerated conditions, a number of P6 cultures grown in the basal medium failed to yield segregants when incubated for up to 5 days.

The conditions that stimulate or trigger the haploidization process in the diploid may be similar, in some respects, to those reported by Braun and co-workers for inducing population changes in *Brucella*. [Refer to the publications of W. Braun (1953, 1958) for reviews describing environmental conditions that induce population changes in *Brucella* species.] When smooth-colony-forming (S) cells of *Brucella abortus* were grown in a closed-culture system, they found that rough-colony-type (R) cells appeared after 5 days of incubation. They attributed this change in the population to specific modifications of the environment (selective environmental conditions) which favored the multiplication of the mutant R cells arising spontaneously in S-cell cultures. Further aging of the cultures resulted in the appearance of an S' type, phenotypically identical to the initial S-cell type. The cultural phases that occurred can be expressed as S → R → S'. Changes in antigenicity and virulence of the cultures were associated with cell-type changes.

In the system described in this paper, however, a change in colony morphology from R to S accompanied changes in cell size and nucleic acid content of the cell as well as in radiation resistance. Also, no gross differences in surface antigenicity were detected between the haploid and diploid cell forms; each cell type was able to adsorb all agglutinins from an antiserum produced against the opposite cell types. The data
given in Table 1 also indicate a serological relationship between the two cell types.

It would be of interest to determine whether DNA-breakdown products could stimulate the segregation of P6 to the small-cell smooth-colony form. It was observed by Braun and Whallon (1954) and Braun, Firshem, and Whallon (1957) that DNA + deoxyribonuclease could promote the R → S population change when added to Brucella cultures. Also, we have not yet attempted to induce the segregation of P6 by radiation treatment, as used with success by Beckhorn (1950) and Lederberg et al. (1951) to induce haploidization in heterozygous diploid E. coli K-12 prototrophs.

As can be seen from an examination of Fig. 2 (X-ray survival curves), the segregation of the diploid P6 to the apparent haploid state resulted in a loss of radiation resistance. Radiation resistance was regained when the haploid segregants were converted to the diploid condition by the camphor treatment. These observations, together with the data with DNA, indicate that radiation sensitivity or resistance in this particular system can be correlated with the ploidy of the organisms. This confirms in a more direct manner the observation of Zelle and Og (1957) that the sigmoid radiation-inactivation curve obtained for P6 when it was exposed to X- or γ-irradiation was due to a doubling of the chromosomal number. These radiation data also support the idea that radiation resistance is due largely to the action of nuclear genes in E. coli. It has been suggested that radiation resistance in E. coli B derivatives depends on the action of one or more genes (Withkin, 1946, 1947; Hill, 1958; Hill and Simson, 1961). Adler and Copeland (1962) reported that radiation-resistance characteristics may be transferred from a radiation-resistant donor strain to a radiation-sensitive recipient strain during conjugation between E. coli K-12 derivatives. They suggested that several genes located on a linkage group were involved in conferring radiation resistance to the prototrophs. In an earlier report by Zelle and Og (1957), radiation resistance was found to be transferable from the diploid P6 strain to a haploid E. coli B derivative, and the radiation-resistant prototrophs obtained in such matings had P6 characteristics, that is, coarsely granular colonies and large cells. In their experiments, the contribution of a whole chromosome or a partial chromosomal transfer by P6 during conjugation could have accounted for such a result. Thus, it appears that more than one mechanism may be responsible for the radiation resistance exhibited by certain E. coli strains: (i) the action of one or more genes on one chromosome, (ii) a homozygous or heterozygous diploid condition where a double set of genes would have to be inactivated before the cell loses viability, and (iii) a heterogeneous condition where radiation-resistant gene(s) may be either in an episomic particle in the cytoplasm or attached to the recipient cell's chromosome.

Data are presented which show that the rate of fermentation of various carbohydrates was influenced by the change from the haploid to diploid condition (Table 2). The diploid cells exhibited a delayed fermentation reaction in comparison with the haploid strains. In homozygous diploid bacteria, one could expect a greater rate of metabolism since there are two copies of each gene present in a cell and, hence, the potential to produce twice as much enzyme. Yet, in these experiments, the homozygous diploids appeared to be repressed in their fermentative ability, whereas the apparent haploid segregants were unpressed. A similar observation was reported by Lederberg et al. (1951) in heterozygous diploid prototrophs obtained through mating of various E. coli K-12 derivatives. They found these heterozygous diploids to be slightly delayed in fermentative capabilities. The metabolic changes observed in P6 would not appear to be the result of a drastic alteration of the nucleocytoplasmic ratio, since this factor remained constant in cells in the stationary phase of growth regardless of the cell type (Table 3). The possible mechanism(s) responsible for this repression of fermentative ability in homozygous diploids is being investigated, and the results will be published in a later paper.

In the system recorded in this paper, we have available for comparative studies the original haploid parent strain, a homozygous diploidlike strain obtained from the parent by doubling the chromosome number of the haploid bacterium, a haploid segregant from the diploid cell, and its diploid counterpart. Such strains should be of value in the contemplated study of gene-dosage effect and gene interaction.

Acknowledgments

This study was supported by research grant G-16940 from the National Science Foundation.
The senior author expresses his sincere gratitude to Max R. Zelle of the Division of Biology and Medicine, Argonne National Laboratory, for his encouragement during the course of these investigations.

LITERATURE CITED


