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GENE RECOMBINATION IN THE BACTERIUM ESCHERICHIA COLI

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The study of inheritance in bacteria has, for the most part, been confined to the investigation of mutational changes in the course of clonal reproduction. With the exception of experiments on pneumococcus type transformations there have been few studies on the direct hereditary interaction of one bacterial type with another. The conception that bacteria have no sexual mode of reproduction is widely entertained. This paper will be devoted to the presentation of evidence for the occurrence in a bacterium of a process of gene recombination, from which the existence of a sexual stage may be inferred.

On the basis of mutation studies many investigators have concluded that the hereditary properties of bacteria are based on the existence of genes (Luria and Delbrück, 1943; Roepke *et al.*, 1944; Lwoff, 1941; Demerec and Fano, 1945; Gray and Tatum, 1944), although it is not clear whether these genes should be homologized with the Mendelian factors of higher organisms, or with the extranuclear factors which have been demonstrated in some microorganisms and higher plants (Sonneborn, 1943; Spiegelman *et al.*, 1945; Rhoades, 1943).

The genic basis of microbial inheritance does not depend on the demonstrability of a sexual phase in bacteria. However, more powerful genetic methods paralleling classical Mendelian analysis would be available if it were possible to follow the inheritance of characters in the products of a sexual fusion. The few examples of this approach thus far reported have provided no incontrovertible evidence for sexual reproduction in bacteria.

The phenomenon of paragglutination in the colon-typhoid-dysentery group might be regarded as an instance of bacterial hybridization, and was so interpreted by Almquist (1924). As reported by numerous authors, paragglutination refers to the development of new types which react with antisera for each of two distinct strains when these are grown together in mixed culture (Kuhn and Ebeling, 1916; Salus, 1917; Wollman and Wollman, 1925). The significance of these observations has been attacked by several authors (Breinl, 1921; Arkwright, 1930; Kauffmann, 1941), chiefly on the grounds that the paragglutination represents a nonspecific cross reactivity characteristic of "rougher" phases of these organisms. Hansen (1929) failed to obtain paragglutination in her experiments. In the light of more detailed recent information on the antigenic structure of this group, this problem certainly deserves a critical reinvestigation.

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Sherman and Wing (1937) have described experiments designed to detect recombinations of fermentative characters in mixed cultures of various *Escherichia coli* and *Aerobacter aerogenes* strains. Although new combinations of biochemical characters were found, similar types were found to an equal extent in pure cultures, so that these authors could not infer the occurrence of a sexual fusion. Their experiments are of the greatest interest, however, since they represent the first attempt to study this problem in bacteria by genetic methods using clear-cut characters. Gowen and Lincoln (1942) later performed similar experiments with strains of *Phytomonas stewartii*, using cultures differing in morphological and pigment characteristics. As in Sherman and Wing's studies, these authors were unable to differentiate the new types they found in their mixed cultures from types which arose spontaneously in single cultures. For this reason a definite conclusion could not be drawn from their results.

A discussion of hereditary processes in bacteria must take into account the extensive work on transformation of pneumococcal types, first described by Griffith (1928) and culminating in the isolation of the transforming principle in chemically characterizable form by Avery, MacLeod, and McCarty (1944). These studies have revealed that, under special experimental conditions, a product isolated from a serologically specific, smooth, pneumococcus culture will convert cells of a nonspecific rough culture to the smooth type characteristic of the source of the transforming principle. So far as is known, such transformations can be performed in only one direction (rough to smooth) and only under very special conditions. Boivin and Vendrely (1946) have reported a similar transformation involving the capsular polysaccharide of a strain of *E. coli*. There have been reported other instances of varying credibility (Kasarnowsky, 1926; Lommel, 1926; Legroux and Genevray, 1933; Frobisher and Brown, 1927; Burnet, 1925; Holtman, 1939; Cantacuzene and Bonciu, 1926). These studies have a direct bearing on recombination experiments, since transformations of this sort might be responsible for the occurrence in mixed cultures of some new types which are interpretable as recombination types. This will be discussed in more detail below.

Morphologically unusual forms of various bacteria have been described by Mellon (1925) as zygospores, and been taken to imply a sexual fusion. It has been suggested by Dienes and Smith (1944) and by Smith (1944) that the "Large Bodies" which appear under certain conditions in cultures of *Bacteroides* may represent a sexual phase. There is as yet no evidence that the recombination phenomena in *E. coli* which will be discussed in this paper are related to any special structural form, such as those which have been described by these authors.

MATERIAL AND METHODS

Except as otherwise stated, the experiments reported on in this paper have all been performed with mutants of a single strain of *Escherichia coli*, K-12. This is a typical coliform bacterium originally isolated from human feces—a gram-negative rod, motile, lactose-fermenting, producing indole, and susceptible to each of the coli phages in the series T1 to T7 of Demerec and Fano (1945).

It has been used at Stanford University as a student laboratory strain for a number of years.

Mutant strains of *E. coli* characterized by specific growth factor requirements have been obtained after treatment with X-rays, ultraviolet light, and nitrogen-mustard. A single nutritional requirement is established at a single mutational step, and on the basis of studies on *Neurospora* is regarded as based on a change in a single gene. By successive treatments, multiple mutant strains with several genetically and biochemically independent nutritional requirements

TABLE 1

*Characteristics of Escherichia coli biochemical mutants**

STRAIN	REFERENCE†	REQUIREMENTS	SYMBOL	OBTAINED FROM	TREATMENT	DETECTION TECHNIQUE‡
58	1	Biotin	B−	K-12	X-ray	1
679	1	Threonine	T−	K-12	X-ray	1
58-161	2	Biotin, methionine	B−M−	58	X-ray	1
679-680	2	Threonine, leucine	T−L−	679	X-ray	1
Y10	3	Threonine, leucine, thiamine	T−L−B_1−	679-680	X-ray	2
58-278	2	Biotin, phenylalanine	B−ϕ−	58	X-ray	1
Y24	3	Biotin, phenylalanine, cystine	B−ϕ−C−	58-278	Ultraviolet	2
Y38	3	Arginine	A−	B/r§	Ultraviolet	2
Y39	3	Histidine	H−	B/r	Ultraviolet	2
Y44	3	Arginine, methionine	A−M−	Y38	Ultraviolet	2
Y45	3	Histidine, *p*-aminobenzoic acid	H−Pb−	Y39	Ultraviolet	2
679-183	2	Threonine, proline	T−P−	679	X-ray	1

* Mutants for resistance to phage T_1 have been obtained in strains 58-161, 679-183, Y10, and Y24, without detectable variation in nutritional requirements.

† References: (1) Gray and Tatum, 1944; (2) Tatum, 1945; (3) previously unpublished.

‡ Technique 1 is described by Gray and Tatum (1944); 2 by Lederberg and Tatum (1946).

§ This is a radiation-resistant mutant of *E. coli* B, isolated by Witkin (1946).

have been produced. The strains used in these experiments are described in table 1. In general, the nutritional characteristics of a strain are ascertained by inoculating media consisting of the basal medium plus various supplements; lack of visible growth in the absence of a given growth factor and optimal growth in its presence are the criteria for the determination of the nutritional requirements of a strain.

A mutant strain can be signified by suffixing a "−" sign to the initial of the substance in question; e.g., B−ϕ−C− refers to a strain which is deficient in the synthesis of biotin, phenylalanine, and cystine. On the other hand, particular emphasis can be placed on the ability of a strain to synthesize a particular growth factor by suffixing a "+". Thus, B−ϕ−C−T+L+B_1+

would refer to a strain deficient in the three factors mentioned above, but capable of growth in the absence of threonine, leucine, or of thiamine. The representation of a growth factor requirement by a minus sign is justified by the a priori consideration that a mutation establishing a growth factor requirement generally represents the loss of a function, and by the experimental finding of Beadle and Coonradt (1944) that wild are dominant to mutant genes in *Neurospora* heterocaryons. Strains which are "+" for all growth factors have been called prototrophs (Ryan and Lederberg, 1946), since this is the nutritional condition of the parental wild type *E. coli* strain from which all the mutants were ultimately derived.

In addition to these biochemical mutations, the character of resistance (abbreviated V_1^r) to coli phage T1 has been used. Spontaneous mutations for this character were selected by the method of Luria and Delbrück (1943) and are sufficiently rare (about 1 cell in 10^7 in a 24-hour culture from a small inoculum) to be negligible except when specifically selected for by applying excess phage to a large population of sensitive bacteria. There has been no indication in K-12 strains of any association between phage resistance and biochemical requirements, as reported by Anderson (1946) for mutants of another coli strain.

Several types of culture media were employed. The chemically defined minimal medium had the following composition, per liter: NH_4Cl, 5 g; NH_4NO_3, 1 g; Na_2SO_4, 2 g; K_2HPO_4, 3 g; KH_2PO_4, 1 g; $MgSO_4$, 0.1 g; glucose, 5 g; asparagine, 1.5 g; $CaCl_2$, trace; and trace element solution, 1 ml (as used for *Neurospora*; Beadle and Tatum, 1945). Agar was added at a concentration of 1.5 per cent when required, but to avoid flocculation the medium and the agar were autoclaved separately at twice the final concentration, and mixed before pouring plates. It was found that some lots of Difco agar were sufficiently free of the growth factors under consideration to make special washing unnecessary.

For nonsynthetic broths, "CC," consisting of peptone, yeast extract, and glucose, and "YB," the Difco product "yeast beef broth," were used.

One-ml inocula from broth cultures of distinct mutants (separately or in various combinations) were added to 50 ml YB in a 125-ml flask, and incubated for 24 to 48 hours at 30C with gentle shaking. After washing with sterile distilled water, samples of about 10^8 cells were inoculated into minimal agar pour plates, to which various supplements had been added as required. The plates were incubated for 48 hours and inspected for the presence of visible colonies. If these appeared, they were picked, suspended in sterile water, and tested for nutritional requirements. It was found that despite the heavy seeding of the plate, picking in this fashion ordinarily yielded homogeneous cultures, but for further study strains were subjected to serial single colony isolation on CC streak plates. Virus susceptibility was ascertained by cross-streaking the phage and the bacteria on a nutrient saline agar plate, as recommended by Demerec and Fano (1945), and recording whether lysis occurred at the intersection.

EXPERIMENTAL RESULTS

Spontaneous mutations of bacteria in pure culture were studied as a preliminary to the investigation of recombination. The over-all frequency of

random biochemical mutations in untreated cultures is less than 0.1 per cent, although samples totaling not more than 5,000 cells have been studied so that the precision of this measurement is doubtful. In view of the low rate and random occurrence of such spontaneous mutations, however, they may be regarded as a negligible factor in this study.

The spontaneous reversion of biochemical mutants to prototrophs is under detailed investigation (Ryan and Lederberg) and will be reported on more fully elsewhere. It has been found that most biochemical mutants will revert at a low rate, prototrophs being found in the proportion of 10^{-7} in 24-hour cultures of single mutants. Reversions of different factors are, so far as has yet been ascertained, entirely independent; as predicted from the low rate of reversion of the individual factors, in ca. 10^{10} cells examined no instance was found in which reversion had occurred at both loci of a double mutant. Such a coincidence would have led to the appearance of a prototroph in a culture inoculated with a double mutant such as T−L−. On the basis of these considerations, only double and triple mutants have been used in the study of recombination.

The frequency of spontaneous mutations to virus resistance is of the same low order of magnitude as nutritional reversion. Mutations from resistance (V_1^r) to susceptibility (V_1^s) have not been described owing to the lack of efficient techniques for the detection of such reversions.

Prototroph recombination types. Since coincidental spontaneous reversion at two or more loci does not occur at a sufficiently high rate to be detected, the presence of prototrophs in mixed cultures of multiple mutants is evidence for gene recombination. Each mutant is capable of synthesizing all the growth factors for which it is not deficient; therefore, different mutants should have "+" alleles for all but the two or three mutant genes that characterize each strain. The segregation of prototrophic alleles of every gene into one cell would result in a prototrophic cell. It would develop into a visible colony on minimal medium, whereas other mutant cells would be unable to proliferate owing to the absence in minimal medium of their nutritional requirements.

When washed samples of mixed cultures of B−M−P+T+ and B+M+ P−T− were plated into minimal medium, about 100 colonies developed for each billion (10^9) cells inoculated. No colonies appeared after inoculation of samples from the individual double mutants. One interpretation of the occurrence of prototrophs, designated as B+M+P+T+, is that the P+ and T+ genes of B−M−P+T+ and the B+ and M+ genes of B+M+P−T− have segregated into the same cell. This is a recombination hypothesis; alternatives will be discussed in the next section.

The possibility must be considered that the prototrophs consist of some sort of association of the unaltered mutants. In a classical illustration of nutritional symbiosis, or syntrophism (Lederberg, 1946), Valentine and Rivers (1927) showed that *Hemophilus canis* and *Hemophilus parainfluenzae*, which require X and V factor, respectively, would grow in mixed culture in media lacking these substances. They concluded that an exchange of these growth factors, synthesized by the bacteria, occurred through the medium. While there is no good reason to doubt this conclusion, these authors did not, in fact, conclusively

demonstrate that this was the mechanism of the interaction. It is possible that cells were present in their mixed cultures which, as a result of gene recombination, required neither of the two factors. The situation is obscured by the use by these authors of serial transfers of large numbers of bacteria.

Syntrophism has been shown to occur with *E. coli* mutants (Lederberg, 1946). It is not likely, however, that it plays a significant role in the appearance of prototrophic colonies. Washed cells inoculated into minimal medium do not show syntrophism unless small quantities of their required growth factors are added. In minimal agar plates heavily inoculated with a washed mixed culture, a uniform turbidity does appear, which is ascribable to a limited exchange of factors and subsequent syntrophic growth.

Evidence of several sorts has been obtained for the homogeneity and uniqueness of prototrophs isolated from mixed cultures. Most significant, they are at least relatively stable, and attempts to detect mutant cells by an efficient selective technique (Lederberg and Tatum, 1946*a*, 1946*b*) have been unsuccessful. Massive doses of ultraviolet light, killing all but 10^{-5} of the cells in the culture, were no more successful in breaking up the supposed associations. In addition, prototrophs obtained from B−M−P+T+V_1^r and B+M+P−T−V_1^s were studied. Both susceptible and resistant cultures were obtained. Although one of the parental strains is resistant, the susceptible cultures were uniformly lysed upon application of the phage; on the other hand, there was no change in the nutritional behavior of cultures of resistant prototrophs subsequent to the application of the virus, which would be expected, in an association of the original mutants, to lyse the susceptible B+M+P−T−V_1^s cells and leave only B−M−P+T+V_1^r. A nicotinicless mutant has been obtained by ultraviolet irradiation of a prototroph derived from P−T− and B−M−. The prototroph in which this mutation occurred could have been neither a heterocaryon nor an association of diverse types, since in either case the absence of nic+ genes in the mutant would require the simultaneous mutation of more than one representative of this gene. This coincidence is highly improbable. The microscopic examination of seeded agar supported the conclusion that the cells of strain K-12 are well dispersed, so that most of the colonies that appear would be derived from single cells when only a few hundred cells are inoculated per plate, as was done after the initial isolation of prototrophs. Single cell isolations from a "recombination prototroph" strain have been made by Dr. M. Zelle of the National Institute of Health; all of the single cell cultures tested were of the same nutritional and virus-resistance type as the culture from which they were isolated.

Transformation. The evidence just presented points to the conclusion that the prototrophs are a new type of cell, which did not arise by spontaneous changes in a single double-mutant strain. Gene recombination, which was postulated above, is, however, not the only interpretation for the origin of these new types which would fit the evidence that has been presented. Alternatively, transforming principles, analogous to those demonstrated for pneumococcus serotypes, might participate in these genetic changes. The postulated transforming factors would be produced in one cell by genes in the "+" state, and after dif-

fusion through the medium would convert mutant genes in another cell into their active, prototrophic alleles.

Since the conditions of the recombination experiments require that any transforming substance be present in the medium, an attempt was made to modify a nutritional mutant with a culture filtrate from another mutant. $B+M+P-T-V_1^s$ was grown in YB broth, and samples of 12- and 36-hour cultures were freed of cells by centrifugation and filtration through an ultrafine sintered glass filter. The filtrate was diluted with an equal volume of YB and inoculated with $B-M-P+T+V_1^r$. As a control, $B-M-P+T+V_1^r$ cells were inoculated with $B+M+P-T-V_1^s$ into filtrate broth. After the cultures were incubated for 48 hours, they were analyzed for prototrophs by the methods described above. None were found in the $B-M-P+T+V_1^s$ cultures grown in the presence of $B+M+P-T-V_1^r$ filtrate, indicating the absence of an active transforming principle in the medium under these conditions. On the other hand, the growth in mixed culture of $B-M-P+T+V_1^s$ and $B+M+P-T-V_1^r$ cells resulted in the appearance of numerous prototrophs.

This failure to demonstrate a transforming principle cannot be regarded as rigorous proof of its absence, since there may have been subtle, undetected variations in the specific environmental conditions required for its activity. There is, however, further indirect evidence in a following section that such factors do not play an important role.

Combinations of other E. coli strains and conditions for recombination. Prototrophs have been obtained consistently in "crosses" of a variety of multiple mutants derived from K-12. There has been no exhaustive attempt to define the most favorable conditions for recombination. The best results have been obtained in rich, well-buffered media, cultured at 30C for at least 6 hours with moderate shaking. High salt concentrations, low and high temperature, diluted media, low buffering capacity, and low pH appear to be inimical to the recombination process even at levels which affect growth but slightly. Since *E. coli* strain B (Luria and Delbrück, 1943) has been used extensively in studies of bacterial mutation, an attempt was made to demonstrate the occurrence of a recombination process in this strain. Mutants were obtained which are summarized in table 1. Prototrophs were not found in mixed cultures of the double mutants of B/r. Similarly prototrophs were not found in mixtures of $A-M-P+T+$ (from B/r) and $A+M+P-T-$ (from K-12). There may be specific genetic requirements which must be fulfilled before recombination will occur.

Other recombination types. If prototrophs arise from the segregation into the same cell of + alleles of various genes, there should be present in the same cultures cells into which combinations of − alleles, different from those of the parental types, have segregated. In looking for these types, $B-\phi-C-T+L+B_1+V_1^s$ and $B+\phi+C+T-L-B_1-V_1^r$ were used, so that there were available 7 markers, some of which might be expected to segregate independently and give rise to new recombination types.

In order to detect these types, mixed cultures were plated into a minimal medium to which various supplements had been added, in different combinations.

In order to suppress the parental types, at least one of the requirements of each of the parental strains was withheld, and the supplements consisted of at most four factors, such as B, ϕ, T, B_1; B, ϕ, L, B_1; etc. An attempt was made at first to calculate the proportions of different recombination types simply from the number of colonies that appeared on different media. This method proved unsatisfactory with the markers used, since the variations in total numbers were so much greater than the differences found from one combination to another. Therefore, colonies were picked from various plates and suspended in water; growth after inoculation into a series of tubes containing all the supplements

TABLE 2

Relative proportions of various nutritional cell types in a mixed culture of $B-\phi-C-T+L+B_1+V_1^s$ and $B+\phi+C+T-L-B_1-V_1^r$

TYPE	NUMBER OF THIS TYPE ISOLATED*	NUMBER OF PROTOTROPHS	RATIO OF THIS TYPE TO PROTOTROPHS	REMARKS
$B-\phi-C-T+L+B_1+V_1^s$	(Parental type. Present in large excess)			
$B+\phi+C+T-L-B_1-V_1^r$	(Parental type. Present in large excess)			
$B+\phi+C+T+L+B_1+$	86		1.00	Prototrophs
$B+\phi+C+T+L+B_1-$	36	37	0.97	Thiamineless
$B+\phi+C+T-L+B_1+$	2	31	0.06	Threonineless
$B+\phi+C+T+L-B_1+$	4	55	0.07	Leucineless
$B-\phi+C+T+L+B_1+$	5	56	0.09	Biotinless
$B+\phi-C+T+L+B_1+$	1	52	0.02	Phenylalanineless
$B+\phi+C-T+L+B_1+$	1	19	0.05	Cystineless
$B+\phi+C+T+L-B_1-$	3	16	0.19	Possible single-reversion type
$B-\phi-C+T+L+B_1+$	2	41	0.05	Possible single-reversion type
$B-\phi+C+T+L+B_1-$†	3	28	0.11	
$B-\phi+C+T-L+B_1+$†	(Isolated in a similar experiment)			
$B-\phi+C+T+L-B_1+$†	(Isolated in a similar experiment)			

* These figures do not include results of tests of virus resistance. Of 49 prototrophs tested, 20 (41%) were resistant. Seven out of 20 thiamineless (35%) were resistant.

† It should be noted that these types represent double-requirement recombination types.

but one was used to classify the types. Unusual types were checked several times. For those plates so supplemented as to permit the development of colonies of a given type, the number of that type obtained was compared with the number of prototrophic colonies picked. This method permits a comparison of the relative numbers of prototrophs and of various other types. The results of such an analysis are summarized in table 2.

In crosses between $B-\phi-C-T+L+B_1+$ and $B+\phi+C+T-L-B_1-$ the recombination types $B-\phi+C+T+L-B_1+$, $B-\phi+C+T-L+B_1-$, and $B-\phi+C+T+L+B_1-$ have been found. In addition, $B-\phi+C+P-T+$ was found in a mixture of $B-\phi-C-P+T+$ and $B+\phi+C+P-T-$. Furthermore, there have been numerous instances of recombination types consisting of a single biochemical requirement coupled with resistance or with susceptibil-

ity, when that same requirement in the parental type was coupled with the alternative response to the virus. To account for these recombinants, we postulate a sexual phase in this strain of *E. coli:* a cell fusion which allows the segregation of genes in new combinations into a single cell.

The alternative to this conclusion is that transforming substances, capable of inducing mutations *in both directions*, are present in the medium. No analogous system of transformations has been described. The possibility that spontaneous mutations for biochemical requirements occur in cells which are being transformed for other factors in one direction has been examined by looking for thiamineless ($B+\phi+C+T+L+B_1-$) mutants in a mixed culture of $B-\phi-C-T+L+B_1+$ and $B+\phi+C+T-L-B_1+$. None was found among 80 colonies which appeared on thiamine-supplemented agar, although they occur at a considerable rate in combinations of $B-\phi-C-T+L+B_1+$ and $B+\phi+C+T-L-B_1-$, as already pointed out. Bilateral transformations cannot be distinguished from a sexual process by the genetic analysis of a population. However, the relative frequencies of various types, as shown in table 2, provides indirect evidence on this point. In the cross between two triple mutants, the prototrophs are the most frequent type. This suggests that if transformation occurs at all, it is likely to affect all three "−" loci. A priori, one would expect the types in which only a single locus had been transformed to be the most frequent. These data on frequencies of various classes must, however, be interpreted with caution since selective differentials, which might operate even in a richly supplemented medium, have been neither completely controlled nor extensively studied.

DISCUSSION

A complete analogy cannot be drawn at present between the inheritance of bacterial characters and the Mendelian processes of higher forms. The occurrence in mixed cultures of bacterial mutants of new types, which can be represented as recombinations of the characteristics of the original strains, suggests very strongly that hybridization and segregation take place. However, the details of the genetic structure of these bacteria have not been ascertained as yet. We do not know how many alleles governing a particular character are present in each cell, how they divide in relation to cell division, nor their relationship to the nuclei which have been described in bacteria (Robinow, 1945). Further studies on the factors contributing to the relative proportions of different types may lead to the solution of these problems. Disparities in these proportions have already been mentioned; the apparent nonrandom segregation of phenotypic characters may be due to factor linkages, to cytoplasmic transmission, or to other processes. A possibly similar situation has been reported in interspecific hybrids of yeasts by Lindegren (1945). Although the isolation of ascospores should allow precise tetrad analysis, in most instances there was not a clear-cut segregation of vitamin requirements, but, instead, prototrophs predominated. This was ascribed to "a cytoplasmic mechanism . . . obscuring the Mendelian ratios."

The utilization of nutritional requirements in the study of recombination

illustrates the principles of a more general method. Any mutation which can be selected for (or against) in competition with the parental types could be used. Comparable results should then be obtained with such characters as virus resistance, resistance to antibiotics and other chemical agents, sugar utilization, and agglutination with antibody or lysis in the presence of complement. Such reagents may extend the general approach of recombination study to organisms which are not well adapted to the application of nutritional methods.

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SUMMARY

Evidence has been presented for the occurrence of character recombination in the bacterium *Escherichia coli*. This suggests the existence of a sexual phase. Recombinations of genes controlling several growth factor requirements and resistance to a specific bacteriophage have been found.

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