JOURNAL OF BACTERIOLOGY, Apr. 1977, p. 160-166
Vol. 130, No. 1

Printed in U.S.A.

Metabolic Characterization of the Viable, Residually Dividing and Nondividing Cell Classes of Recombination-Deficient Strains of *Escherichia coli*

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Received for publication 19 October 1976

The abilities of *rec*$^+$, *recB*$^-$ *recC*$^-$, *recA*$^-$, and *recA*$^-$ *recB*$^-$ *recC*$^-$ strains to support growth of bacteriophage T4, to take up oxygen, and to maintain cell integrity have been measured. (i) With respect to bacteriophage T4 growth, T4 phage is produced with identical lysis time in single-step growth curves with all strains tested. *rec*$^-$ strains show reduced phage production (fewer infected centers), but the average burst size per infected center is the same for all strains tested. Some *rec*$^-$ cells are unable to produce any phage, whereas the remainder of the *rec*$^-$ cells produce phage as rapidly and as efficiently as *rec*$^+$ cells. (ii) With respect to oxygen consumption, *rec*$^-$ strains are deficient relative to the *rec*$^+$ control to the same extent as the deficiency in phage production by the culture. The reduction in oxygen consumption is coordinate with reduction in rate of mass increase of the *rec*$^-$ culture. (iii) With respect to cell integrity, *rec*$^-$ cultures show increased lysis as measured by release of β-galactosidase into the medium. The kinetics of release suggest that *rec*$^-$ nondividing cells all eventually lyse. These results are most consistent with the idea that *rec*$^-$ residually dividing cells and viable cells are metabolically normal according to the parameters we have measured, whereas nondividing cells are metabolically inactive.

Strains of *Escherichia coli* K-12 with lesions in either the *recA*, *recB*, or *recC* genes exhibit a variety of phenotypic alterations when compared with isogenic *rec*$^+$ strains, including deficiencies in genetic recombination ability and high sensitivity to ultraviolet light (2, 9-12, 15, 17). In addition, mutants with lesions in any of these three *rec* genes grow more slowly and have significantly lower viability than isogenic *rec*$^+$ strains (6, 8, 9, 19). The growth defects of these *rec*$^-$ strains are explained by the existence of three classes of cells. Viable cells can divide more than 20 times; residually dividing cells can divide an average of 2.5 to 5.5 times; nondividing cells are incapable of a single division (5). The proportion of cells in each viability class is dependent on the *rec* genotype of the strain (5). The following model has been proposed to explain the formation of the three classes of cells. Viable cells of a *rec*$^-$ culture divide at the same rate as do *rec*$^+$ cells, but they suffer an unknown lethal event at a rate which is characteristic of the particular *rec* genotype. This lethal event probably consists of unrepaired damage in the deoxyribonucleic acid (DNA) of the cell, and it creates a residually dividing cell, which continues to divide at the rate characteristic of a *rec*$^+$ cell. However, the residually dividing cell can undergo an average of only 2.5 to 5.5 divisions, the exact number being characteristic of the *rec* genotype, before all of its progeny enter the nondividing cell class (5).

This study was undertaken to investigate the nature of the metabolic changes associated with the unknown lethal event. Since the residually dividing and nondividing cells contain the lethal damage, by further study of these cells we can learn about the kinetics and pleiotropy of expression of the lethal event. It has been shown that *rec*$^-$ nondividing cells are incapable of synthesizing DNA (4) and protein (3; Ramsey and Barbour, unpublished data). This could be the result of an inability to transcribe or replicate DNA that has been damaged by the lethal event. Here we report measures of three parameters of cell metabolism which are less immediately dependent on intact cellular DNA. We have measured the ability of various *rec*$^-$ strains to support the growth of bacteriophage and to take up oxygen and the rate and extent of cell leakage or lysis that occurs in *rec*$^-$ cultures.

MATERIALS AND METHODS

Bacterial strains and phages. All bacterial strains used were *E. coli* K-12. Their *rec* genotypes and other phenotypic properties are listed in Table

1. The nomenclature conforms to that of Demerec et al. (7). T4D⁺ and T7 bacteriophages were kindly supplied by L. Astrachan.

Media. Luria medium (18), M9 medium (6), and EM9 glycerol medium (3) have been described. Where required, histidine was added to a final concentration of 100 μg/ml. AM9 medium consisted of M9 medium supplemented with 100 μg of every amino acid except tryptophan and tyrosine per ml. Tryptone medium consisted of tryptone (10 g/liter), NaCl (5.0 g/liter), and thiamine (0.5 mg/liter). It was used as a liquid medium, as soft agar (7 g of agar/liter), or as hard agar (12 g of agar/liter).

Antiserum to bacteriophage T4 was kindly supplied by L. Astrachan. In 5 min of adsorption with a suspension of free phage, 0.1 ml of a 1:50 dilution of antiserum was capable of inactivating at least 90% of 5×10^8 T4 plaque-forming units (PFU) present in 1.1 ml of tryptone medium. T4 antiserum was used at this concentration.

Pregrowth of strains. Each strain was grown overnight (37°C, on rotating wheel) at least two successive times in 5 ml of the medium to be used for the experiment. This stock culture was stored in the refrigerator. The night before an experiment, the stock culture was diluted 1:50 or 1:100 into 5 ml of fresh medium and incubated overnight, at 37°C, on a rotating wheel. To begin the experiment the overnight culture was diluted (between 1:25 and 1:200, depending on the strain and the medium) into fresh medium and grown with shaking to an optical density at 650 nm (OD_{650}) of 0.20 or 0.30.

OD measurements. The OD of cultures was measured at 650 nm by using a Beckman DU spectrophotometer with a Gilford digital absorbance meter, or a Cary recording spectrophotometer.

Bacteriophage experiments. Preparation of phage stocks, plating of bacteriophages, and one-step growth experiments were done as described by Adams (1) in tryptone media.

Infective center assays were performed as follows. Pregrown cells were grown to an OD_{650} of 0.30 (1.75×10^8 particles/ml) in tryptone medium. At 0 min, to 1.0 ml of culture was added 0.1 ml of T4 bacteriophage at the appropriate concentration, and the culture was incubated with shaking (200 rpm) at 37°C. At 10 min after infection, 0.1 ml of a 1:50 dilution of T4 antiserum was added. At 15 min after infection, the culture was diluted through tryptone broth and plated in tryptone soft agar on a lawn of *E. coli* K-12 strain JC4583 to measure PFU.

Oxygen uptake. Pregrown cultures were grown to an OD_{650} of 0.30. Oxygen uptake was measured at 30 or at 37°C on 5 ml of slowly stirred liquid culture with a Yellow Springs Instrument 5331 oxygen probe. Rate of oxygen uptake was constant under these conditions and was calculated from the slope of the chart recording of oxygen concentration in solution.

TABLE 1. *Bacterial strains*

Strain no.	*rec* genotype	Reference
JC4583[a]	+	2
JC4584[a]	B21 C22	6
JC4588[a]	A56	2
SDB1006[a]	A56 B21 C22	5
ED2024[b]	+	16

[a] These strains are F⁻ His⁻ Gal⁻ Thi⁻ Str^s EndA⁻.
[b] Lac-Pro$_{\Delta X111}$ His⁻ Trp⁻ T6^r Str⁻ Su$_i$⁻.

Lysis experiment. Pregrown cultures were grown in EM9 glycerol medium to an OD_{650} of 0.20. Five milliliters of each culture was induced with 10^{-4} M isopropyl-β-D-thiogalactoside (Sigma Chemical Co.) (this concentration of inducer gives near-maximal induction, but when diluted 1:50, as in the next step, the culture continues to be induced at less than 1% of its former rate) for 0.5 generation time. Each culture was then diluted 1:50 or 1:100 into fresh prewarmed EM9 glycerol medium, and samples were taken immediately and at 0.5 to 2.0 generation intervals. An uninduced culture was treated and sampled in parallel.

At each sampling time, 1 ml of culture was taken for an OD_{650} reading. Two 1-ml samples of culture were taken into tubes containing 1 drop of toluene and 1 drop of 5% Sarkosyl (CIBA-Geigy), vortexed, and iced immediately. β-Galactosidase was assayed according to the procedure of Pardee et al. (13). Two 2-ml samples of culture were taken into cold centrifuge tubes containing 1 ml of a culture of *E. coli* strain ED 2024 (which had been grown to an OD_{650} of 0.60 in Luria medium, washed and resuspended in EM9 glycerol, and iced for use as carrier cells). (Enzyme units in the supernatant were therefore multiplied by 1.5 to correct for the volume of the carrier culture supernatant.) Tubes were centrifuged at 6,000 rpm, for 10 min, at 4°C. About 1.5 ml of supernatant was carefully removed into another tube with a Pasteur pipette. This supernatant was vortexed, and 1.0 ml was taken into a tube containing Sarkosyl and toluene and was assayed for β-galactosidase as above.

The data were treated as follows. Enzyme units from uninduced culture and uninduced supernatant were subtracted from the induced values at each time point, and the amount of lysis was expressed as the percentage of culture enzyme that was present in the supernatant at each time point.

RESULTS

Growth of bacteriophage T4. We measured the ability of an isogenic series of *rec*⁺ and *rec*⁻ strains to support the growth of bacteriophage. To find out whether *rec*⁻ cultures are slower or less efficient in production of bacteriophage, we did one-step growth curves. Results of these one-step growth curves with phage T4 on *rec*⁺ and *rec*⁻ strains are shown in Fig. 1. For all strains tested, the lysis time (35 min) and the burst size (200 PFU per infected cell) are the same. The four strains are also identical to one another in rates of intracellular phage accumulation, and in one-step growth curves of bacteriophage T7 (data not shown). From these results we conclude that any *rec*⁻ cell that is capable of supporting phage growth does so at the same rate and with the same efficiency as a *rec*⁺ cell.

To ascertain if all *rec*⁻ cells are capable of

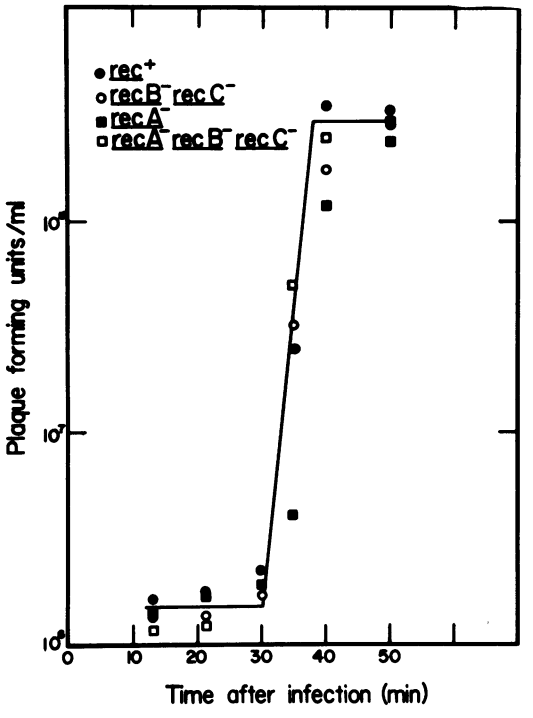

FIG. 1. *One-step growth curve of bacteriophage T4 on rec⁺ and rec⁻ strains of E. coli K-12. The number of input phage for each strain was adjusted to give about the same number of productively infected cells per culture. Bacterial hosts were rec⁺ (JC4583)* (●); *recB⁻ recC⁻ (JC4584)* (○); *recA⁻ (JC4588)* (■); *recA⁻ recB⁻ recC⁻ (SDB1006)* (□).

supporting bacteriophage growth we performed both direct plating assays and infective center assays. In both types of experiments, *rec⁻* cultures show a reproducible deficiency in the fraction of cells that support bacteriophage growth (Table 2). Numerically identical results (with respect to the normalized values for efficiency of plaque formation) are obtained with bacteriophage T7 (data not shown). The results of infective center assays are identical at low and high multiplicities of infection; this implies that the defect in the cells that do not support phage growth is not in adsorption of phage. The proportion of *rec⁻* cells that do not support phage growth is comparable, although not identical, to the proportion of nondividing cells in each *rec⁻* strain as determined by Capaldo et al. (5). Since the one-step growth experiment shows that any *rec⁻* cell that supports phage growth does so at the *rec⁺* rate and burst size, we conclude from these data that it is probably the nondividing cells that are incapable of supporting bacteriophage growth, and therefore that the residually dividing cells are indistinguishable from *rec⁻* viable cells or *rec⁺* cells in this respect.

Oxygen uptake. We used rate of oxygen uptake as a criterion of respiration in the *rec⁻* strains. The data from seven sets of experiments are shown in Table 3. Rate of oxygen uptake is reproducibly deficient in the *rec⁻* strains as compared with the *rec⁺* strains. The mean values of the normalized oxygen uptake

TABLE 2. *Relative efficiency of plating of bacteriophage T4 on rec⁺ and rec⁻ hosts*

Expt	PFU/ml of infected culture			
	rec⁺[a]	*recB⁻ recC⁻*	*recA⁻*	*recA⁻ recB⁻ recC⁻*
I[b]	3.90	2.99	3.63	2.90
II[b]	3.87	2.66	3.35	2.38
III[b]	3.29	2.12	2.68	2.39
IV[c]	2.07	1.62	1.62	1.23
V[c]	2.99	2.06	2.45	1.81
VI[d]	1.57	1.12	1.40	1.10
VII[d]	1.61	1.27	1.42	1.09
Normalized[e] mean value ± standard error	100	73±2	86±2	67±2

[a] Host genotype.
[b] Direct plating assay: multiplicity of infection (MOI) = 10^{-6} PFU/microscopically visible cell. PFU/ml × 10^{-3}.
[c] Infective cancer assay: MOI = 0.16 PFU/microscopically visible cell. PFU/ml × 10^{-7}.
[d] Infective center assay: MOI = 3 PFU/microscopically visible cell. PFU/ml × 10^{-8}.
[e] Normalized to *rec⁺* value.

TABLE 3. *O_2 uptake by rec⁺ and rec⁻ strains*

Expt	Rate of O_2 uptake (μmol of O_2/min per OD_{650} unit per ml of culture)			
	rec⁺[a]	*recB⁻ recC⁻*	*recA⁻*	*recA⁻ recB⁻ recC⁻*
I[b]	0.093	0.081	0.089	0.074
II[b]	0.118	0.095	0.088	0.078
III[b]	0.113	0.092	0.088	0.080
IV[c]	0.071	0.052	0.064	0.051
V[d]	0.141	0.069	0.107	0.085
VI[e]	0.154	0.141	0.133	0.137
VII[f]	0.093	0.070	0.087	0.054
Normalized[g] mean value ± standard error	100	77±5	85±3	71±4

[a] Genotype.
[b] Luria medium, 30°C.
[c] M9 medium + histidine, 100 μg/ml, 30°C.
[d] AM9 medium, 37°C.
[e] Luria medium, 37°C.
[f] M9 medium + histidine, 100 μg/ml, 37°C.
[g] Normalized to *rec⁺* value.

rates are numerically very similar to the data on phage growth. Since respiration is required for support of bacteriophage growth, it appears that the population of cells that do not respire is the same as the population of cells which do not support phage growth. By comparison with the percentages of cells in the various viability classes as estimated by Capaldo et al. (5), it is likely that the fraction of cells that is unable to support phage growth or consume oxygen is the nondividing cell fraction. This conclusion is independently corroborated by the plot of the oxygen uptake data shown in Fig. 2. There exists a linear relationship between culture growth rate (as measured by doubling of the OD_{650}) and oxygen uptake rate. This is independent of strain genotype and, therefore, of the fraction of viable cells in the culture. This implies that each cell contributes to the mass increase of the culture at the same rate at which it takes up oxygen. Therefore, the residually dividing cells of *rec*⁻ cultures, which contribute to the mass increase of the culture (5), respire, and the nondividing cells do not. It appears that residually dividing cells are metabolically indistinguishable from viable cells, according to the two parameters we have measured. In addition, we believe that our values for the percentage of viable plus residually dividing cells as measured by oxygen uptake and support of phage growth are probably more accurate than those published by Capaldo et al. Their data are derived from particle counts of penicillin-treated cells (3). The penicillin particle count method may overestimate the percentage of nondividing cells because some of the residually dividing cells which are close to the end of their residual divisions elongate so little in penicillin that they are counted as nondividing cells.

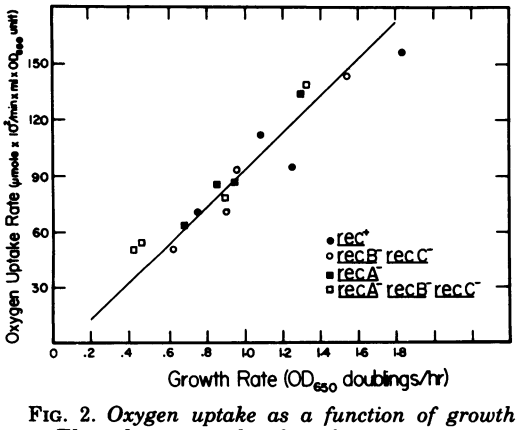

FIG. 2. *Oxygen uptake as a function of growth rate. These data were taken from four experiments in Table 3. Strain symbols are the same as in Fig. 1.*

Our revised estimates for the percentage of cells in each viability class are shown in Table 4. The fraction of cells that support phage growth and take up oxygen was taken to represent viable plus residually dividing cells and the remainder of the culture was therefore assumed to consist of nondividing cells. The percentages of cells in the viable cell class are the same as reported by Capaldo et al. (5) because they were obtained by direct plating methods.

Lysis. Until now no evidence of lysis in *rec*⁻ cultures has been reported. If the nondividing cells do not respire, however, it seems possible that they could become leaky and even lyse (14). Therefore, we measured lysis in *rec*⁻ cultures, using as a criterion the release of β-galactosidase into the supernatant of a culture pulse induced with isopropyl-β-D-thiogalactoside. The results from one such experiment are shown in Fig. 3. Under these experimental conditions, a few generation times postinduction, all *rec*⁻ cultures begin to show evidence of lysis. The rate of lysis is constant over three to four generation times, and characteristic of the particular *rec* genotype. The results in Fig. 3 have been consistently obtained in several experiments in which appearance of β-galactosidase in the culture supernatant is the criterion of lysis. These results have been corroborated by experiments in which the appearance of trichloroacetic acid-precipitable ribonucleic acid (RNA) label in the culture supernatant is the criterion of lysis. Over longer time periods, the rate of appearance of enzyme in all culture supernatants slows down, and after seven to eight generation times, the extent of lysis has reached nearly 100% in the *recB*⁻ *recC*⁻ and *recA*⁻ *recB*⁻ *recC*⁻ cultures (Fig. 4). At that time the extent of lysis in the *recA*⁻ culture is still increasing. It is not possible to follow lysis in the *recA*⁻ culture further because the culture

TABLE 4. *Revised estimates of the classes of cells in Rec⁻ cultures*

Relevant genotype	Fraction of cells in:		
	Viable cell fraction[a]	Nondividing cell fraction[b]	Residually dividing cell fraction[c]
rec⁺	1.00	0	0
recA⁻	0.66	0.15	0.19
recB⁻ *recC*⁻	0.34	0.25	0.41
recA⁻ *recB*⁻ *recC*⁻	0.18	0.31	0.51

[a] From Capaldo et al. (5).

[b] From Tables 2 and 3, average fraction of metabolically inactive cells.

[c] 1 − (viable + nondividing fractions).

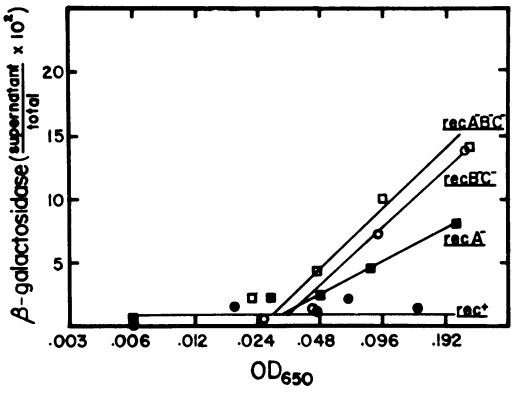

FIG. 3. *Appearance of β-galactosidase in culture supernatant. Each point represents the amount of β-galactosidase activity present in the culture supernatant at that time point expressed as a percentage of the amount of β-galactosidase activity present in the same volume of total culture at that time point. Strain symbols are the same as in Fig. 1.*

must be diluted 10-fold every three generations, and this eventually dilutes the β-galactosidase to an undetectable level. It is for this same reason that the data points for all the strains become less precise toward the end of the experiment.

The rate of lysis for each strain is comparable to the rate at which residually dividing cells segregate into the nondividing cell class in each strain (Fig. 4). Residually dividing cells have been shown to be inducible for β-galactosidase (3) and to synthesize RNA and protein at the wild-type level (Capaldo, Ramsey, and Barbour, unpublished data). After pulse induction (or pulse labeling), β-galactosidase (or labeled RNA) which was synthesized in residually dividing cells, should immediately begin to segregate into nondividing cells. However, there is no evidence of lysis until a few generations later, which indicates a lag before the nondividing cells begin to lyse.

The time after induction at which evidence of lysis begins to appear depends on the particular batch and type of medium used (both EM9 glycerol medium and tryptone broth have been used) and on the OD_{650} of the culture, and not simply on the number of doublings which have taken place since induction. This may reflect the exhaustion of a particular nutrient in the medium. Although we can draw no conclusions from the time at which lysis begins, we believe that it is significant that the rate of lysis is: (i) increased in the *rec*$^-$ strains as compared to the *rec*$^+$ strain; (ii) characteristic of the particular *rec* genotype; and (iii) predictable from the rate of formation of nondividing cells from residually dividing cells in each strain.

DISCUSSION

Previous results (3-5) have shown that: (i) nondividing cells are incapable of synthesizing DNA or protein, and in some cases contain damaged DNA or no DNA at all. (ii) Dividing cells, comprised of the residually dividing and viable cell classes, synthesize DNA and protein at the *rec*$^+$ rate and appear to contain intact DNA. (iii) Residually dividing cells contribute to the mass increase but not to the viability increase of a *rec*$^-$ culture. These results leave unclear the answers to the following questions: (a) Is there some residual metabolic activity in nondividing cells which is not directly dependent on intact DNA? (b) Is there any detectable deficiency in residually dividing cells, which may contain lethal damage in their DNA, but in which (so far) no effects of that damage have been found? The answers to these questions are important because they could help us to determine what specific lesion leads to cell death in the absence of the *recA*, *recB*, and *recC* gene products.

rec$^-$ cultures are metabolically deficient with respect to support of bacteriophage growth and uptake of oxygen to approximately the same extent as they contain nondividing cells. A key finding in these studies is that *rec*$^-$ strains show reduced phage production (fewer infected centers), but the average burst size per infected center is the same for all strains tested. Clearly, some cells are unable to produce any phage, whereas the remainder of the *rec*$^-$ cells produce phage as rapidly and as efficiently as *rec*$^+$ cells. The fact that oxygen uptake is directly related to culture doubling time, independent of what fraction of cells in the culture is dividing, shows that the ability to take up oxygen is likewise an all-or-none property of a *rec*$^-$ cell, and that respiration goes on only in cells that divide. These results indicate that there is a class of metabolically inactive cells and that there is not a class of cells that is partially active in either respect. From the correlation between the fraction of cells that are inactive and the fraction of cells in each strain which do not divide, and from our knowledge of the inactivity of the nondividing cells with respect to DNA and protein synthesis, the most logical conclusion is that the metabolically inactive class corresponds to the nondividing cell class. In addition, *rec*$^-$ cultures show evidence of lysis at the rate at which cells segregate into the nondividing cell class of each particular

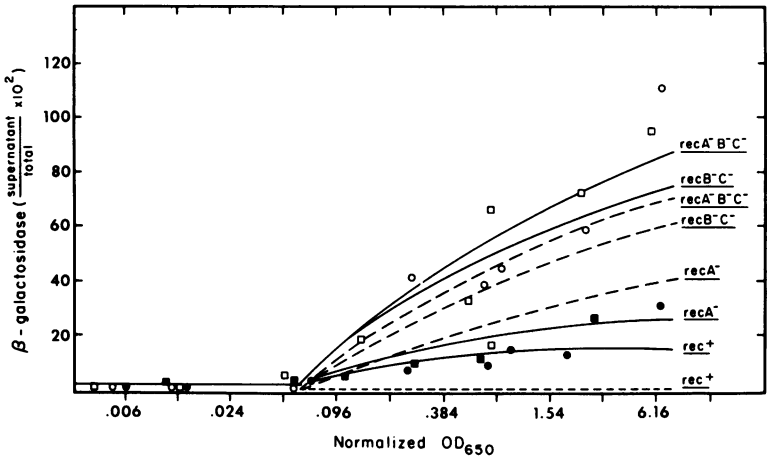

FIG. 4. *Long-term appearance of β-galactosidase in culture supernatant. Strain symbols are the same as in Fig. 1. Data points were calculated as explained in the legend to Fig. 3. Solid lines (——) represent the experimentally determined appearance of β-galactosidase in the culture supernatant. Broken lines (- - - -) represent the rate of formation of nondividing cells in each strain. The formation of nondividing cells has been plotted with the origin arbitrarily chosen to coincide with the point at which evidence of lysis begins to appear. This has been done to facilitate comparison of the experimental and theoretical curves. In fact, however, the nondividing cells which are lysing were formed three or so generations previously. In plotting these theoretical curves, we have used our recalculations of the percentage of cells in each viability class (Table 4) to derive new rates of segregation from one cell class into another (for method of calculation, see Capaldo et al. [5]). These recalculated segregation rates have been used to derive the theoretical curves. Normalized OD_{650} represents the OD of the growing culture corrected for dilution.*

strain. Therefore, it appears that nondividing cells are metabolically inactive and that they eventually lyse.

It is probable that the residually dividing cells and the viable cells are metabolically normal (i.e., behave like *rec*⁺ cells) with respect to support of bacteriophage growth and uptake of oxygen. The only defect of residually dividing cells, and the only characteristic that distinguishes them from *rec*⁻ viable cells and *rec*⁺ cells, is that they eventually cease to divide. This characteristic makes it necessary to postulate the existence of a primary lethal event, which causes a viable cell to become residually dividing (5). The fact that this lethally damaged residually dividing cell continues to divide and metabolize normally is consistent with the hypothesis that the primary lethal event consists of damage to DNA. According to this type of model, this damage may be expressed as lethal after several generations when, due to the DNA damage, something becomes limiting and further divisions are not possible.

ACKNOWLEDGMENTS

This investigation was supported by U.S. Public Health Service research grant GM17329 and Public Health Service research career development award GM38140, both from the National Institute of General Medical Sciences (S.D.B.), and a National Science Foundation Graduate Fellowship (J.E.M.).

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