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Multiplicity of Genome Equivalents in the Radiation-Resistant Bacterium *Micrococcus radiodurans*

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The complexity of the genome of *Micrococcus radiodurans* was determined to be $(2.0 \pm 0.3) \times 10^9$ daltons by DNA renaturation kinetics. The number of genome equivalents of DNA per cell was calculated from the complexity and the content of DNA. A lower limit of four genome equivalents per cell was approached with decreasing growth rate. Thus, no haploid stage appeared to be realized in this organism. The replication time was estimated from the kinetics and amount of residual DNA synthesis after inhibiting initiation of new rounds of replication. From this, the redundancy of terminal genetic markers was calculated to vary with growth rate from four to approximately eight copies per cell. All genetic material, including the least abundant, is thus multiply represented in each cell. The potential significance of the maintenance in each cell of multiple gene copies is discussed in relation to the extreme radiation resistance of *M. radiodurans*.

The bacterium *Micrococcus radiodurans* isolated from irradiated meat (1) ranges among the very most radiation-resistant organisms known. The radiation resistance of this strain has been attributed to its exceptional repair capabilities (12, 26, 32) rather than to an altered susceptibility to radiation of its genetic material per se. Thus, the ability to repair DNA double-strand breaks has been reported (4, 19). Involvement of multiple copies of the genetic information in each cell (redundancy) was ruled unlikely by Driedger (10) on the basis of nitrosoguanidine-induced death in the first generation after treatment. In the present investigation, the question of redundancy of genetic information was approached by measurements of complexity and content of DNA in each cell of *M. radiodurans*.

MATERIALS AND METHODS

Strains and growth conditions. The wild type of *M. radiodurans* was kindly provided by B. E. B. Moseley, Edinburgh. Strain MH 133 resistant to streptomycin (100 μg/ml) and requiring thymine was obtained after nitrosoguanidine mutagenesis (32) and trimethoprim selection (22). This strain has an absolute requirement satisfied by 2 μg of thymine per ml but not by uridine or deoxyuridine. Leakiness was less than 3% as judged by incorporation of deoxy[6-^{3}H]-uridine into alkali-stable material in the absence of thymine. No difference in DNA-to-mass ratio was found between wild type and MH 133 growing with 5 μg of thymine per ml. Thus, there is no indication that the velocity of replication forks is artificially limited by the availability of thymine (30) in MH 133 growing with 5 μg of thymine per ml.

Strains were grown at 37°C in tryptone-glucose-yeast extract and supplemented minimal and minimal media (22) with doubling times of 80, 165, and approximately 450 min, respectively. For some experiments, streptomycin (100 μg/ml) was added to cultures of MH 133. Growth was followed turbidometrically at 570 nm in a Zeiss Elko III colorimeter. All experiments were performed with exponentially growing cultures.

Cell numbers, DNA content, and incorporation of radiolabel. The number of cells was determined on the basis of colony counts on tryptone-glucose-yeast extract plates or in some cases by means of a Coulter Counter (34). DNA was measured on 20-ml samples of culture of optical density at 570 nm of 0.15 to 0.45 by the colorimetric assay of Burton (5) using deoxyribose as standard. Incorporation of [^{3}H]thymine was followed by trichloroacetic acid precipitation, collection on membrane filters, and liquid scintillation counting as previously described (17).

Preparation of DNA. A suspension of *M. radiodurans* was passed through a French pressure cell at 700 kg/cm^2 leading to extensive cell breakage. Sodium dodecyl sulfate was added to 0.1%, and the mixture was deproteinized at pH 9 and treated with ribonuclease essentially according to Miura (24) except that a mixture of phenol, chloroform, and isoamylalcohol (50:50:1) was used. Furthermore, a digestion with protease (from *Streptomyces griseus*, Sigma Chemical Co.) was included. *Escherichia coli* DNA was prepared in the same way except that lysis was obtained by heating to 60°C with 1% sodium dodecyl sulfate.

The DNA preparations were judged free from protein by analysis of their UV spectra (13). These indicated cytosine-plus-guanine contents of 64.9 and 49.8% for *M. radiodurans* and *E. coli* DNA, respectively, in agreement with previous estimates (16, 23, 28). No material of less than 10^7 daltons, and thus no RNA, was detected by agarose gel electrophoresis of the unsheared DNA preparations. The size of DNA frag-

ments after shearing by sonic disruption was determined by using restriction enzyme-generated fragments of phage λ DNA as markers. A distribution over the range from 1×10^6 to 7×10^6 daltons was established.

Renaturation conditions. DNA was denatured in alkali and rapidly cooled to 0°C, and NaH_2PO_4 was added to give an Na^+ concentration of 1 M (33). Alternatively, denaturation was effected by boiling for 1 to 2 min in 0.01 M phosphate buffer at pH 7 followed by addition of concentrated buffer to give a final Na^+ concentration of 0.18 M. The solution was subsequently transferred to a thermostated cuvette in a Cary 15 spectrophotometer. The sample reached the temperature chosen for renaturation within approximately 1 min as monitored by a thermistor introduced through the stopper of the cuvette. The decline in optical density at 260 nm was continuously recorded.

RESULTS

Complexity of the genome of *M. radiodurans*. The difference in cytosine-plus-guanine content of DNA prepared from *M. radiodurans* and from *E. coli* (see above) was reflected by a corresponding (16, 23) difference of 6.3°C in their melting temperatures. Furthermore, a broad temperature optimum yielding the maximal rate of renaturation was confirmed (16, 33) to be located some 25°C below the melting temperature at the given salt concentration. All subsequent renaturation experiments were performed at optimum temperatures. The initial time course of renaturation of DNA from *M. radiodurans* and from *E. coli*, measured spectrophotometrically, was plotted by the method of Gillis et al. (16) (Fig. 1A). At a given DNA concentration, the initial rate of renaturation $[\Delta(c/c_t - 1)]/\Delta t$ is inversely proportional to the complexity of the DNA examined (16). For the DNA preparations used here, at any given concentration the rate of renaturation for *M. radiodurans* DNA was 1.4 times greater than that for *E. coli* DNA (Fig. 1B). Essentially the same results were obtained at high (1 M Na^+, Fig. 1) as at a low salt concentration (0.18 M Na^+, data not shown), where all rates were reduced approximately by a factor of six. Rates were reproducible within ±5%. One potential source of error is a minor variation in size distribution of fragments from different DNA preparations. An undetected difference, which could be as large as ±20% on the average size of fragments, would be of little consequence, however, because the renaturation rate is proportional to the square root of the fragment size (33) or even less size dependent (16). The complexity of *E. coli* DNA has been determined by a variety of methods to be around 2.8×10^9 daltons (6, 9, 16, 20). Consequently, the complexity of the genome of *M. radiodurans* was estimated to be $(2.0 \pm 0.3) \times 10^9$ daltons.

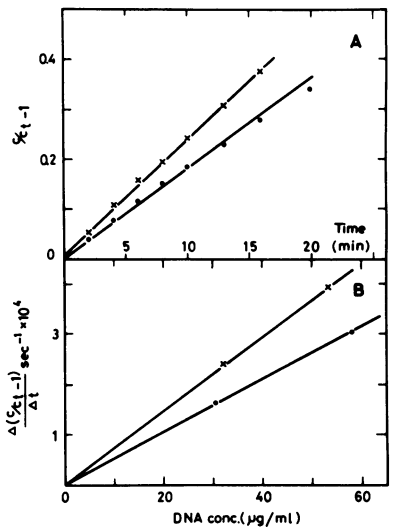

FIG. 1. *Renaturation rates of DNA from M. radiodurans and from E. coli. (A) The total DNA concentration (c) over the concentration of DNA remaining denatured at time t (c_t) was used to depict the initial kinetics of DNA renaturation (16). The temperature for renaturation was 83.3°C for M. radiodurans DNA (53.5 μg/ml) and 77.0°C for E. coli DNA (58.0 μg/ml) at an Na^+ concentration of 1 M. (B) Renaturation rate as a function of DNA concentration. Rates were determined as the initial slopes from plots like (A) Symbols:* (×) *M. radiodurans;* (●) *E. coli B/r.*

DNA content per cell. The DNA content of cultures of *M. radiodurans* growing at various rates in different media was determined colorimetrically (5), and the cell number was determined via colony-forming units. Actual number of particles as measured by a Coulter Counter agreed with the number of colonies formed (Table 1). Under the conditions used, *M. radiodurans* grew in a diplococcal state with only an insignificant fraction (<10%) of tetracocci. This was established in the phase-contrast microscope and from the monodisperse size distribution seen in the Coulter Counter. Consequently, to calculate the DNA content per single cell, the number of particles was multiplied by two to give the cell number. The DNA contents calculated in this way covered the range from 9×10^9 daltons/cell for very slow growing cells to 2.2×10^{10} daltons/cell for cultures growing on rich medium (Table 1, Fig. 2). The values determined here for the DNA content per cell are close to the lower estimates of the widely varying range previously reported (11, 14, 15, 29). A direct comparison is difficult, however, due to differ-

ences in growth conditions (11) and in the way of determining the number of monococcal cells (11, 14, 15, 29).

Residual DNA synthesis. The thymine requirer MH 133 was used for measurements of residual DNA synthesis after inhibition of the initiation of new rounds of DNA replication. To ensure a constant specific activity of thymine incorporated throughout an experiment, cultures were labeled with [^{3}H]thymine for 10 min before addition of inhibitor. Rifampin (10 μg/ml) or chloramphenicol (100 μg/ml) was used to inhibit initiation of replication. There was no delay in the inhibition, and residual DNA synthesis was indistinguishable for the two antibiotics. Incorporation appeared to continue for about 70 min, allowing the DNA content to increase by 13% over the amount present at the time of addition of rifampin (Fig. 3A). The final increase in DNA content (ΔG_∞) ideally is a function only of the replication time (C) and the doubling time (τ) of the culture (3, 30). A replication time of C = 60 min was calculated from the measured values τ = 165 min and ΔG_∞ = 1.13. Alternatively, the replication time can be determined from the kinetics with which the final level of DNA is reached (3). This method yielded a value of 77 min for the replication time (Fig. 3B).

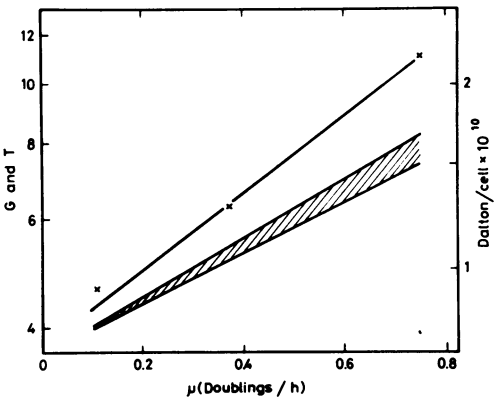

FIG. 2. *DNA content per cell and redundancy of termini as a function of growth rate (μ). The DNA content per cell of M. radiodurans MH 133 (×) is given in daltons as well as in genome equivalents per cell (G), the genome equivalent being taken as the complexity of the DNA. In addition, the number of termini per cell (T) (▨) calculated for a replication time between 60 and 80 min is shown (see text). All numbers refer to an individual cell of a diplococcal pair.*

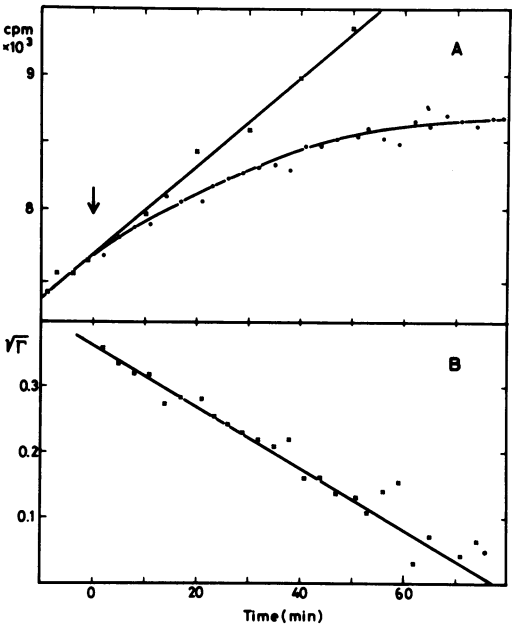

FIG. 3. *Residual DNA synthesis after inhibition of new rounds of replication. (A) Strain MH 133 growing with a doubling time of 165 min on supplemented minimal medium and 5 μg of thymine per ml was labeled at time −10 min with [methyl-^{3}H]thymine (5 μCi/μg). At zero time rifampin (10 μg/ml) was added (arrow). Counts have been corrected for the amount of DNA present at the onset of labeling so that counts shown are a measure of total DNA in the culture. Symbols: (×) Untreated control culture; (●) incorporation after addition of rifampin. (B) Replication time (C) determined from the plot of $\sqrt{\Gamma}$ against time. Γ is defined as $\Gamma_t = \Delta G_\infty - \Delta G_t$, where ΔG_t is the factor by which the amount of DNA has increased at time t after addition of inhibitor (3). The extrapolated intercept of this curve with the x axis gives the replication time (C).*

TABLE 1. *Cell numbers and DNA contents*[a]

Growth rate (μ) (doubling/h)	CFU/(ml × OD_{570}) (×10^8)	Particles/(ml × OD_{570}) (×10^8)	DNA/(ml × OD_{570}) (μg)	DNA/cell (dalton × 10^{10})
0.13	1.7 ± 0.2		5.1 ± 0.3	0.90 ± 0.15
0.36	1.3 ± 0.1	1.3 ± 0.1	5.5 ± 0.2	1.27 ± 0.14
0.75	0.86 ± 0.12	0.95	6.3 ± 0.3	2.20 ± 0.40

[a] Values for colony-forming units (CFU) and for DNA per milliliter × optical density at 570 nm (OD_{570}) are based on 10 or more determinations at each growth rate. The number of particles was determined for one culture at μ = 0.75 and for three cultures at μ = 0.36. Values for DNA per cell refer to an individual cell of the diplococcal pair.

DISCUSSION

The DNA content per bacterial cell decreases with the growth rate of the culture to approach a lower limit for stationary cells (9, 20, 31). In those cases previously investigated, this minimum value of DNA per cell is, within experimental error, equal to the complexity of the particular DNA species as determined by renaturation kinetics (16). Therefore, bacteria are generally considered haploid organisms with only one copy of their genetic information in each resting cell.

In *M. radiodurans*, however, the amount of DNA per individual cell of the diplococcal pair does not fall below 8×10^9 daltons even in extremely slowly growing cells (Fig. 2). As the complexity of the DNA from this organism was determined to 2×10^9 daltons (Fig. 1), it is concluded that a minimum of four genome equivalents of DNA is maintained and no haploid stage is attained. It should be kept in mind that the determination of the number of genome equivalents per cell is a composite of the results from renaturation kinetics, chemical assays for DNA, and cell numbers. Thus, an accumulated uncertainty of ±30% on the final estimate is arrived at from the reproducibility of the individual measurements. Even a generous allowance for uncertainty could not, however, alter the basic conclusion that multiple copies of the genetic information are maintained in *M. radiodurans*. This is the case also for the least-abundant genetic material located close to the site of termination of DNA replication. The redundancy of termini (T) can be calculated (3) from the number of genome equivalents per cell (G), the replication time (C), and the generation time (τ): $T = (C \ln 2/\tau)(2^{c/\tau} - 1)^{-1}G$. The replication time ($C$), determined to be within the range of 60 to 80 min (Fig. 3), constitutes only a very minor fraction of the total doubling time for slowly growing cells. For this reason the lack of a more definite value for C does not dramatically influence the number of termini calculated (Fig. 2). This would be true also assuming a moderate variation in C with changing τ as suggested by some studies (8) rather than a constant C (7, 20). The redundancy of genetic material in *M. radiodurans* thus applies to the entire chromosome, with even the terminal markers being multiply represented in each cell.

Driedger (10) concluded that death of individual cells in the first generation after treatment with nitrosoguanidine was consistent with the presence of only one genome per cell. No direct evidence for this state of affairs was obtained however, and further a later report (12) established that 90% of those X ray-exposed cells that were rendered unable to eventually form a colony, did indeed undergo from one to six divisions before dying. Such behavior would not be difficult to reconcile with redundant genetic information as found in the present study.

Redundancy of genetic information in conjunction with an efficient recombination mechanism would provide a most attractive basis for the unusually high resistance of *M. radiodurans* toward agents deleterious to DNA. In contrast to the efficient excision mechanism previously demonstrated in this organism (2), a recombination mechanism could conceivably cope with those damages that involve both of the complementary DNA strands at a given site. *M. radiodurans* has been shown to be exceptionally resistant to this type of damages. These lesions include double-strand breaks produced by X or γ irradiation (4, 18) and interstrand cross-links caused by mitomycin C (32) or 8-methoxypsoralen (M. T. Hansen, unpublished data). Evidence for an efficient recombination system in this organism has been obtained from studies with mutant strains (25, 27). The direct genetic demonstration of recombination, e.g., after exposure to γ radiation, has been hampered by the lack of adequately developed genetics in this organism. A physical demonstration based on density labeling is confronted with the dilemma that bromodeoxyuridine greatly increases the radiation sensitivity of *M. radiodurans* (21).

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