JOURNAL OF BACTERIOLOGY, Jan. 1973, p. 133-144
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Vol. 113, No. 1
Printed in U.S.A.

Radiation-Resistant Mutants of *Salmonella typhimurium* LT2: Development and Characterization[1]

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Received for publication 23 September 1972

A series of repeated exposures to gamma irradiation with intervening outgrowth of survivors was used to develop radioresistant cultures of *Salmonella typhimuium* LT2. Stepwise increases in resistance to both ionizing and ultraviolet irradiation were obtained independently of the presence or absence of integrated P22 prophage. Single clonal isolates, representing parent and radioresistant populations, retained the general characteristics of the LT2 parent, including serological properties, phage typing, antibiotic sensitivities, mouse virulence, and most biochemical test reactions. Resistant cells were generally larger and contained 1.8 to 2.1 times more ribonucleic acid and protein than parent cells, but deoxyribonucleic acid (DNA) contents were similar. Heterogeneity in the populations with respect to release of H_2S, utilization of carbon sources, and growth on minimal medium is considered to be ancillary, rather than causally related, to increased radioresistance. The resistant isolates displayed an increased ability to reactivate gamma-irradiated P22 phage. DNA polymerase I and polynucleotide-joining enzyme activities were elevated in extracts of radioresistant cells relative to parent cells. It is suggested that the observed increases in radioresistance result from a selection of mutations leading to an increased capacity to repair DNA.

The exposure of microorganisms to continued environmental stress frequently results in the selection of strains resistant to the imposed conditions. When the stress agent is also mutagenic, as in the case of ionizing irradiation, an increase in general mutation rates, combined with the selective process, may accelerate the emergence of more radioresistant strains (44).

Several investigators have reported the development of increased radioresistance within bacterial populations when the survivors were repeatedly grown and reexposed to irradiation (12, 31, 45). Generally, for a given cycle of irradiation and growth, a moderate increase in radioresistance to a plateau level has been obtained (45). A modified growth-irradiation-cycle procedure, however, with the use of an increasing irradiation dose sequence, has been reported to result in the development of exceptionally high radioresistance in *Escherichia coli* (20). Subsequent studies of resistant cultures have revealed a reduced biosynthetic capacity (35), altered deoxyribonucleic acid (DNA) metabolism (39), and an increased pleomorphism with multinucleate filaments (34).

Clear interpretation of the relationship between the observed phenotypes and acquired radioresistance has been somewhat restricted, however, by the undefined genotype of the parent cells used and by failure to determine the heterogeneity of the resulting populations. The present work describes the application of the growth-irradiation-cycle procedure to a defined organism, *Salmonella typhimurium* LT2, and its lysogenic derivative containing P22 prophage, with the objective of obtaining, purifying, and characterizing highly radioresistant mutants. It was thought that such a system would provide a useful model for subsequent study of the genetics and mechanisms of radioresistance in microorganisms.

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A preliminary report of this study has appeared (R. Davies and A. J. Sinskey, Bacteriol. Proc., p. 38, 1970).

MATERIALS AND METHODS

Organisms and culture media. *S. typhimurium* strain DB21, a derivative of LT2 cured of known prophages, and strain DB20, a lysogen containing wild-type P22 phage, were obtained from D. Botstein's culture collection. Cultures were grown in Trypticase soy broth (BBL), containing 0.5% yeast extract (TSY broth), by reciprocal shaking at 37 C. Viable cells were enumerated on TSY agar or on M9 minimal medium agar (1) with appropriate supplements. Carbon sources, sterilized separately, were added aseptically at final concentrations of 0.2%. EM9 medium was prepared by supplementing M9 with 0.25% Casamino Acids.

Irradiation sources. Gamma irradiation was provided by a 30-kc, pool-type, U.S. Atomic Energy Commission ^{60}Co Mark I Food Irradiator. Dose rate, measured by Fricke dosimetry, was 5.17 ± 0.07 krad per min.

A General Electric germicidal 15-w lamp, type G8T5, operated in a light-proof chamber at ambient temperature, was used to provide ultraviolet (UV) irradiation. The incident UV dose rate at a distance of 30 cm was 18 ergs per mm^2 per sec as measured by a Blak-Ray UV intensity meter (Fisher Scientific Co.).

Growth-irradiation-cycle procedure. Cultures of *S. typhimurium* strains DB21 and DB20 were grown to stationary phase in 30-ml quantities of TSY broth in 250-ml Erlenmeyer flasks, shaken for 12 hr at 37 C. Samples (2 ml) were chilled, transferred to Pyrex tubes (75 by 10 mm), immersed in ice water, and exposed to gamma irradiation. Fresh flasks of TSY broth were inoculated with 0.5 ml of the irradiated broth cultures and incubated for a further 12 hr, at which time the entire procedure was repeated. The cyclic irradiation dose was selected on the basis of permitting 0.1% survival as determined by spreading 0.1 ml of diluted survivors on the surface of prepoured TSY agar plates and incubating for 2 days at 37 C. The 0.1% survival dose was reevaluated for each culture at weekly (14 cycle) intervals, and representative cultures were preserved by lyophilization and by stab culture in TSY agar.

Gamma irradiation survival. Cultures grown to early stationary phase in TSY broth (12 hr at 37 C) were chilled, centrifuged at 5,000 $\times$ *g* for 5 min, washed, and resuspended in chilled 0.067 M phosphate buffer to give 10^9 to 2×10^9 viable cells/ml. The cell suspensions were distributed as 2-ml samples in Pyrex tubes (75 by 10 mm) held in ice. Duplicate tubes were immersed in ice water and exposed to ^{60}Co gamma rays for the appropriate doses. Irradiated samples were maintained at 0 C until survivors were enumerated by TSY agar plate count. The elapsed time from irradiation exposure to plating did not exceed 30 min, and unirradiated controls were held for equivalent time periods.

UV irradiation survival. Suspensions of cells, prepared as described above, were serially diluted in chilled phosphate buffer. Duplicate 0.1-ml samples were spread on the surface of chilled plates of TSY agar which were exposed individually to the desired doses of UV irradiation. The plates were subsequently incubated for 2 days at 37 C, and surviving colonies were counted. Precautions were taken to avoid photoreactivation.

Nomenclature. The cultures were first identified with their parental origin, DB21 or DB20, followed by the symbol R(*n*) to imply that they had been exposed to *n* consecutive series of 14 growth-irradiation cycles. This designation referred to unpurified populations which were maintained as slant cultures on TSY media as described previously. Three further digits were appended to the designation to facilitate later identification of purified clonal isolates from the radioresistant populations.

General characters. Standard biochemical selective and serological tests were applied in accord with routine salmonella identification procedures (10). Representative cultures were forwarded to the Center for Disease Control for confirmatory testing. Susceptibility to penicillin, erythromycin, and chloromycin was determined by Difco standard disc assays. Mouse virulence was investigated by intraperitoneal challenge of male, 20-g, Swiss white mice (Charles River) with cultures representative of parent and elevated levels of radioresistance. The LD_{50} were calculated by the cumulative mortality method (6).

Relative cell size was determined by phase-contrast photomicrography and by comparing Petroff-Hausser total cell counts with absorbance at 550 nm and dry weight.

Distribution of phenotype with the radioresistant populations. The viability of the cells on minimal medium was determined as follows. Exponential cultures, grown in TSY broth for 3 hr at 37 C, were chilled, centrifuged, resuspended, and serially diluted in cold phosphate buffer. Samples (0.1 ml) were spread on the surface of triplicate plates of TSY agar and M9-glucose agar. Differences in recovery were determined and expressed as percent recovery on M9-glucose agar relative to TSY agar.

Tests were conducted to determine the utilization of carbon sources by the mutants. The carbohydrates tested were selected on the basis of the known locations of their utilization loci on the *S. typhimurium* genetic map (36). Carbon sources, filter-sterilized as 5% solutions, were added aseptically to M9 agar to give final substrate concentrations of 0.2%, and the media were dispersed into sterile petri dishes.

Cultures of strain B21 and its radiation-resistant derivative populations were plated on TSY agar and incubated overnight. Cells from 100 single colonies on the TSY plates were picked onto marked sectors on each carbon source test plate. The plates were incubated at 37 C for 72 hr and observed for colony formation.

For determinations of the distribution of radioresistance, overnight cultures of strain DB21 and its derivatives were plated on TSY agar to obtain single colonies. Cells from each of 100 single colonies of each

culture were transferred by wire loop into corresponding tubes (75 by 10 mm) containing sterile 1-ml quantities of TSY broth. The inoculated tubes were incubated for 12 hr at 37 C, chilled, and diluted with phosphate buffer to give approximately 10^8 viable cells per ml (as determined by absorbance at 550 nm). Disposable, sterile micropipettes (Micropet, Clay Adams) were used to transfer 0.01-ml quantities of the suspensions to 100 marked sectors on each of a series of chilled, prepoured TSY agar plates. Thus, approximately 10^6 cells grown from each single colony were deposited in each marked position on the recipient plates.

The inoculated plates were refrigerated and exposed individually to doses of gamma irradiation varying by increments of 25 krad. After incubation at 37 C for 48 hr, the plates were observed for surviving growth. For each inoculated position, it was possible to distinguish among doses that permitted confluent growth, doses that totally eliminated all 10^6 viable cells, or intermediate doses that permitted up to 30 mini-colonies of survivors (the limit of visual resolution). Thus, it was possible to estimate the 6 D dose (the dose necessary to destroy 10^6 cells) for each of 100 single-colony isolates obtained from each original culture.

Mutator activity. The time-dependent accumulation of *str* mutants within a growing population served as a basis for screening the wild-type strain DB21 and its radiation-resistant isolates for mutator activity.

Overnight TSY broth cultures of strains DB21, D21R1001, D21R6004, and D21R6008 were plated on TSY medium to obtain 100 to 200 colonies per plate. The plates were replicated onto fresh TSY agar and TSY agar containing 100 μg of streptomycin sulfate/ml. After 48 hr at 37 C, two representative colonies of each culture which were streptomycin-susceptible were picked from the TSY plates for the mutator experiment. The isolates were grown in TSY broth shake cultures at 37 C for a total period of 84 hr with media replenishment at 12-hr intervals. Samples were periodically removed and plated on TSY agar and TSY agar containing 100 μg of streptomycin/ml. The colony count in the presence of streptomycin compared to the TSY control was expressed as *str* mutation frequency per cell.

Host-cell reactivation. Cultures of strains DB21 and D21R6008 were grown for 15 hr at 37 C in TSY broth, chilled, centrifuged, suspended in 0.067 M phosphate at 10^9 cells/ml, and held in ice. Phage stocks of P22c^+ and P22c_2 were grown and titered to 2 $\times 10^{10}$ plaque-forming units (PFU)/ml in buffered saline as described by Botstein (3).

Serial dilutons of the phage suspensions were prepared in buffered saline, and 0.1-ml samples were spread on the surface of chilled prepoured plates of TSY agar. The plates were exposed to doses of 0, 1,000, 2,000, and 3,000 ergs/mm^2 of UV irradiation; the surviving phage were estimated by adding 2.5-ml quantities of melted, overlay agar containing 0.2 ml of the appropriate indicator host suspension. Plaques were counted after overnight incubation at 30 C.

In gamma host-cell reactivation experiments, the diluted phage stocks were added to chilled 2.5% nutrient broth (w/v, Difco dehydrated) or phosphate buffer to give approximately 10^8 PFU/ml. The suspensions of phage were distributed into Pyrex tubes, 76 by 10 mm (2 ml per tube), and were exposed to gamma irradiation (doses of 0, 100, 200, 400, and 800 krad) at 0 C. Surviving phage were enumerated by mixing 0.1 ml of the irradiated phage suspension with 0.2 ml of the appropriate indicator host in 2.5 ml of melted, overlay agar, and pouring the mixture onto the surface of the prepoured plates of TSY agar.

Cell composition determinations. Ribonucleic acid (RNA) was measured in triplicate samples of the cell suspensions by the Schmidt and Thannhauser method as modified by Munro and Fleck (32). The DNA content was measured in the same samples (after extraction of the RNA) by the diphenylamine method of Burton as modified by Giles and Meyers (14). Protein was estimated in duplicate samples of hydrolyzed cell suspensions (obtained by boiling for 5 min with 1 N NaOH) by the method of Lowry et al. (25). The sulfhydryl content of the cells was assayed in extracts of sonically disrupted cells by the bis (*p*-nitrophenyl) disulfide colorimetric method (11). Nonprotein sulfhydryl content was defined and measured as titratable mercaptan material soluble in cold 0.6 N perchloric acid. For dry weight estimations, triplicate 20-ml samples for each culture were centrifuged at 5,000 $\times$ *g* for 5 min. The sedimented pellets were resuspended in 2 ml of deionized distilled water, counted in a Petroff-Hausser chamber, and placed in tared aluminum drying dishes. The cells were dried at 105 C for 10 hr and weighed.

DNA enzyme activity. Cells of strains DB21, D21R1001, and D21R6008 grown on EM9-glucose medium were suspended in the appropriate buffer solutions and disrupted by sonic oscillation at 0 C. After mild centrifugation, the supernatant fluids were analyzed for DNA polymerase I activity by the method of DeLucia and Cairns (9) and for polynucleotide ligase activity by the method of Modrich and Lehman (29).

RESULTS

Growth-irradiation cycles. The development of radioresistance is reflected by the increase required in the 0.1% survival cyclic irradiation dose as shown in Table 1 for each strain. The cyclic dose was estimated at approximately weekly intervals by extrapolation of survival curve data obtained under equivalent conditions, namely, stationary-phase cultures irradiated in their growth media. Parallel increases in radioresistance were observed for both the lysogenic and nonlysogenic strains. The presence of an integrated viable prophage in the DB20 series of radioresistant mutants was confirmed by resistance to superinfection and by UV induction under an indicator lawn of DB21 cells. As the increases in radiation resistance were apparently independent of the presence or absence of pro-

TABLE 1. *Effect of increasing cyclic doses of irradiation and subculture of survivors on the radioresistance of lysogenic (DB20) and nonlysogenic (DB21) cultures of S. typhimurium LT2*

No. of cycles	Cyclic dose (krad) used for each series of 14 cycles	Designation of final culture obtained	Survival characteristics of the surviving culture of each 14 consecutive cycles			
			Mean LD_{90}[a] (krad)		3 D dose[b] (krad)	
			DB21	DB20	DB21	DB20
0		DB21	30	45	90	135
0–14	100	D21R1000	112	135	225	260
15–28	225	D21R2000	230	200	380	330
29–42	350	D21R3000	250	254	420	435
43–56	420	D21R4000	345	310	570	500
57–70	520	D21R5000	450	430	810	750
71–84	780	D21R6000	550	560	900	980

[a] The LD_{90} dose is the dose necessary to inactivate 90% of the initial viable cells.
[b] The 3 D dose is the dose necessary to inactivate 99.9% of the initial viable cells.

phage, the subsequent studies reported here concentrated on the nonlysogenic cultures.

Survival curves. The gamma irradiation survival characteristics for washed, stationary-phase cells irradiated in phosphate buffer at 0 C are shown in Fig. 1. The magnitude of the increases in gamma resistance displayed by the DB21 resistant populations may be appreciated by comparison with the survival data for *Micrococcus radiodurans* adapted from Serianni and Bruce (37) and also shown in Fig. 1. The gamma inactivation of the parent culture DB21 followed the exponential kinetics of classic, single-hit/single-target theories of cell death. The survival curves of the derivative populations, however, displayed progressively extended shoulder regions, together with stepwise decreases in asymptotic slope. These changes are reflected by the fact that the D_{10} and LD_{90} values increase up to maxima of 12.5 and 20.0 times the original, respectively.

The UV irradiation survival curves (Fig. 2) show that small but significant increases in UV resistance were also developed concomitantly with those observed for gamma irradiation. However, unlike the latter, these increases (particularly in terms of shoulder extension) occurred primarily in the transition from DB21 to the D21R1000 level of resistance.

General characteristics. The resistant-derivative cultures displayed essentially all of the identification characteristics of *S. typhimurium* (Table 2). The cultures retained their virulence to mice, with LD_{50} doses varying from 1.8×10^7 to 3.7×10^7 viable cells/mouse.

Cell volume and cell mass data, however, showed that for all levels of radioresistance the average cell size of the resistance cultures was approximately double that of the parent. This

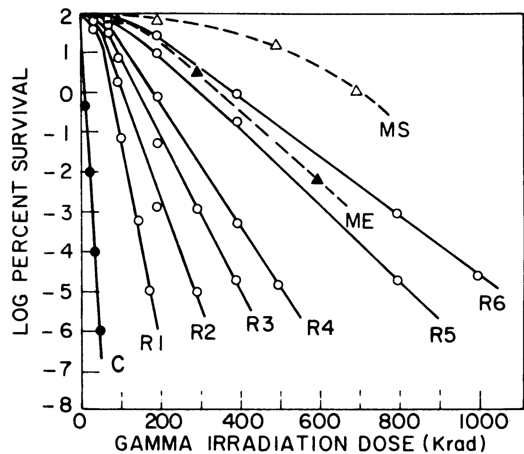

FIG. 1. *Gamma irradiation survival curves for radioresistant and parent cultures of S. typhimurium LT2. Cells previously grown to early stationary phase in TSY broth were irradiated in 0.067 M phosphate buffer at 0 C. Curve C (●) represents the survival of the nonlysogenic parent strain, DB21; curves R1 to R6 (O) represent the mean survival data of the mutant populations R1000 to R6000 derived from DB21. The survival curves (broken lines) for Micrococcus radiodurans are from Serianni and Bruce (37): curve MS (Δ) for stationary-phase cells; curve ME (▲) for exponential-phase cells.*

increase was associated with morphological changes leading to a predominance of swollen, oval, or almost spherical cells similar to those reported for radioresistant *S. newport* by Licciardello et al. (24).

The difference in cell size is also reflected in the dry weight, protein, and RNA content of radioresistant cells, as shown in Table 3 for strain D21R6008. No detectable differences in cellular DNA content were observed. The radi-

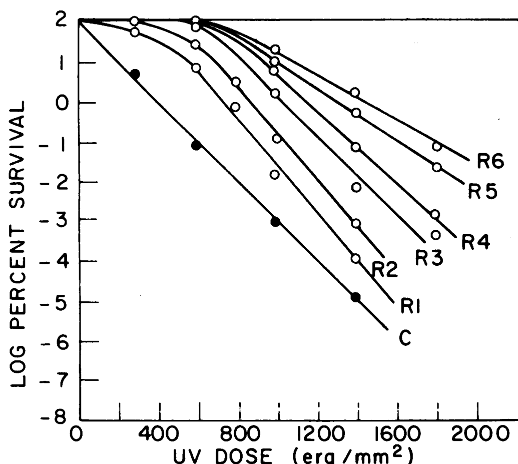

FIG. 2. *UV irradiation survival curves for radioresistant and parent cultures of S. typhimurium LT2. Cells from stationary-phase TSY broth cultures were suspended in chilled 0.067 M phosphate buffer, spread on the surface of prepoured TSY agar plates, and exposed to UV irradiation at 25 C. The symbols used are identical to those described in Fig. 1.*

TABLE 2. *Summary of general characters of parent and radioresistant cultures of S. typhimurium DB21*

Characters that remained unchanged	Characters that changed with increased gamma resistance
Growth in TSY media	Increased resistance to UV irradiation
Gram stain	Cell size increased (×2)
Motility	Less detectable H_2S released in TSI[b] agar
Antibiotic spectra	Progressively less quantitative recovery on M9 media
Serological reactions:[a] antigens 4,5,12:i-1,1,2	Increasing incidence of carbon source utilization mutants
Phage type[a]	Increased mutator activity
Qualitative reactions on selective media	Increased reactivation of gamma-irradiated phage
Virulence to mice	Elevated DNA polymerase I activity
	Elevated DNA ligase activity

[a] Confirmed by the Center for Disease Control.
[b] Triple sugar iron agar (Difco).

oresistant cells also contained more titratable total sulfhydryl material, particularly in cold perchloric acid-insoluble form, than the control cells.

With D21R600 cultures, a delayed production of H_2S was observed in the conventional triple sugar iron (TSI) differential agar. The distribution of changes in H_2S release within the populations was examined by the following experiment. TSY plating was used to obtain 100 single colonies, representing each culture, which were subsequently picked onto marked sectors of freshly prepared TSI agar plates and incubated anaerobically. After 2 days at 37 C, H_2S^+ colonies reduced the ferrous sulfate-sodium thiosulfate complex to the black sulfide, whereas H_2S^- colonies remained white. The results showed that on TSI medium the H_2S production pattern changed from 100% positive for the wild type to 100% negative for strain D21R6008, with some heterogeneity evident in the intermediate populations. Further screening of H_2S^+ and H_2S^- colonies from D21R2000 and D21R4000 cultures for radioresistance, however, revealed no direct relationship between H_2S release and irradiation survival abilities.

Distribution of phenotype. The quantitative recoveries of the parent and radioresistant cultures on M9-glucose and TSY agar are shown in Table 4. The more radiation-resistant cultures showed progressively lower recoveries on minimal media. No attempt was made to verify the apparent auxotrophy of these organisms, but the more resistant populations were clearly heterogeneous.

The abilities of 100 single-colony isolates from each resistant culture to use each of the listed substrates as sole source of carbon are shown in Table 5. Loss of ability to grow was limited to the populations displaying the higher levels of radioresistance. This suggests a relationship between the accumulation of cells with an altered phenotype in a population and its cyclic exposure to irradiation. The data provide

TABLE 3. *Average cell composition in exponential cultures of parent and radioresistant strains of S. typhimurium LT2*[a]

Strain	Dry wt[b]	Protein[b]	RNA[b]	DNA[b]	TSH[c]	NPSH[d]	PSH[e]
DB21	3.29×10^{-7}	1.65×10^{-7}	1.50×10^{-7}	1.60×10^{-8}	1.38×10^{-9}	1.08×10^{-9}	3.00×10^{-10}
D21R6008	6.67×10^{-7}	3.40×10^{-7}	2.27×10^{-7}	1.61×10^{-8}	3.96×10^{-9}	1.66×10^{-9}	2.30×10^{-9}

[a] All data are subject to ±10% error based on total count.
[b] Expressed in micrograms per cell.
[c] Total sulfhydryl content (nanoequivalents per cell).
[d] Nonprotein SH material soluble in 0.6 N perchloric acid (nanoequivalents per cell).
[e] SH material insoluble in 0.6 N perchloric acid (nanoequivalents per cell).

further evidence of heterogeneity within the resistant populations and provide no positive correlation between phenotypic changes and enhanced radioresistance.

When 100 pure clones from each of three representative populations were tested for variations in radioresistance, the distributions plotted in Fig. 3 were obtained. Within the sensitivity of the method (frequencies less than 10^{-2} per cell would not be detected), the populations were relatively homogeneous with respect to gamma-irradiation resistance. For example, over 90% of the cells in the DB21R6000 culture shared a 6 D reduction dose between 380 and 410 krad.

Host-cell reactivation of irradiated phage. The ability of strains DB21 and D21R6008 to reactivate irradiated P22c^+ wild-type phage is illustrated in Fig. 4. In the case of UV-irradiated phage (Fig. 4a), both strains exhibited similar host-cell reactivation (hcr) capacities and could be considered to be *hcr*$^+$.

As shown in Fig. 4b, however, the resistant strain D21R6008 was more efficient than strain DB21 in recovering P22c^+ phage which had been irradiated in chilled 2.5% nutrient broth. Phage irradiated in phosphate buffer were more sensitive, and no difference in recovery was observed with the two bacterial hosts. If the DB21C cells are gamma-hcr (+), in relation to the specific damage induced in phage by gamma irradiation in broth, then the D21R6008 cells may be considered to be gamma-hcr (+ +).

The wild-type P22c^+ phage produced turbid plaques on both strains DB21 and D21R6008, a result which indicated that both strains could be lysogenized. The possibility that the increased recovery may be influenced by lysogeny was investigated by repeating the gamma host-cell reactivation experiment with the clear plaque mutant P22c_2. The result (data not shown) confirmed the increased ability of strain D21R6008 to reactivate gamma-irradiated phage. Titers for unadsorbed phage revealed no quantitative differences in irradiated phage adsorption between the bacterial hosts.

Mutator activity. An increase in the frequency of cells resistant to 100 μg of streptomycin/ml, from 10^{-8} to 1.7×10^{-6} (a factor of 170), was found in cultures of strain D21R6008 grown from a pure streptomycin-susceptible clone for approximately 240 generations (Table 6). The frequency for a culture of intermediate resistance, strain D21R3001, increased by a

TABLE 4. *Relative recoveries of the parent and radioresistant cultures of S. typhimurium on TSY and M9-glucose minimal media*

Culture	Viable cells recovered/ml[a] on		Percent recovery on M9-glucose agar
	TSY agar	M9-glucose agar	
DB21	615 ± 37	615 ± 47	100.0
D21R1000	187 ± 19	188 ± 6	100.0
D21R2000	238 ± 16	239 ± 8	100.0
D21R3000	234 ± 16	232 ± 11	99.2
D21R4000	171 ± 5	161 ± 5	94.2
D21R5000	246 ± 13	201 ± 5	81.6
D21R6000	138 ± 9	54 ± 3	39.0

[a] Mean of three determinations ± SD. Values shown are to be multiplied × 10^{-6}.

TABLE 5. *Utilization of carbohydrate substrates as a sole carbon source by single-colony isolates obtained from parent and radioresistant cultures of S. typhimurium*

Carbon substrate	Percentage of isolates incapable of utilizing the carbon source					
	DB21	D21R1000	D21R2000	D21R3000	D21R5000	D21R6000
Arabinose	0	0	2	3	38	43
Cellobiose	0	0	0	0	12	41
Citrate (sodium)	0	0	0	0	0	4
Galactose	0	0	1	0	5	14
Glucose	0	0	0	0	0	0
Glycerol	0	0	0	1	5	11
Lactose	100	100	100	100	100	100
Maltose	0	0	0	3	3	6
Mannitol	0	0	0	2	1	4
Mannose	0	0	0	10	4	6
Ribose	0	0	0	0	0	0
Rhamnose	0	0	0	0	0	0
Succinate (sodium)	0	0	0	0	0	0
Sucrose	100	100	100	100	100	100
Trehalose	0	1	0	7	1	4
Xylose	0	0	0	0	1	2

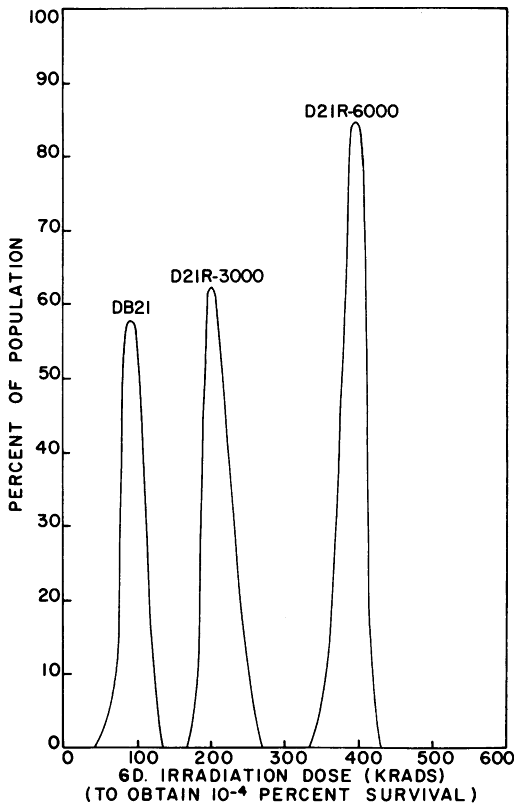

FIG. 3. *Sample containing approximately 10^6 cells from each of 100 single-colony isolates representing each population were placed on marked sectors on the surface of chilled TSY agar plates. Replicate plates were exposed to doses of gamma irradiation varying by increments of 25 krad and then incubated for 48 hr at 37 C. The dose needed to inactivate 10^6 cells (6 D dose) of each isolate was estimated within the limits ± 25 krad. Curves represent histogram data with points not shown.*

factor of 7, whereas no significant mutator activity was observed for cultures of low resistance or for the parent culture.

DNA polymerase activity. Triphosphate incorporation into acid-insoluble polymeric material by extracts of strain DB21 and the two radioresistant isolates D21R1001 and D21R6008 is shown in Fig. 5. In the conditions of the assay, where triphosphate substrate concentrations were initially in excess, the parameter of deoxythymidine triphosphate ^3H counts per minute incorporated in the first minute was used to compare the relative DNA polymerase I activities of the bacterial extracts. Results for both exponential- and stationary-phase cultures of the three strains at 22 C and also at 15 C (to facilitate differentiation of initial rates) are summarized in Table 7.

The extracts from radioresistant cells contained more assayable DNA polymerase activity than extracts of parent cells, to the extent of a maximal sevenfold increase in the case of stationary-phase D21R6008 cultures measured on the basis of activity per cell. Expressed in terms of specific activity (counts per minute incorporated per milligram of protein per minute), the polymerase activity was elevated equally in both strains D21R1001 and D21R6008 to a level between two and three times that of the wild-type control.

It should be noted that the data shown for the parent culture DB21 in Fig. 5 suggest that the wild-type DNA polymerase I activity level in *S. typhimurium* LT2, assayable by the DeLucia and Cairns (9) procedure, is considerably less than that reported by these authors for *E. coli* strain W3110T$^-$.

Polynucleotide ligase activity. The results of the assay for polynucleotide-joining enzyme activity in extracts of strains DB21, D21R1001, and D21R6008 are listed in Table 7. A twofold increase in ligase activity, expressed on a per cell basis, was observed for strain D21R6008 compared to the parent, but no corresponding increase in specific activity was evident. The results also show that measurable ligase activity may be detected in 2 hr at 0 C.

DISCUSSION

The tabulated values of the LD_{90} and the 3 D doses listed and defined in Table 1 serve as a basis for comparison with resistance increases reported by other authors. Radioresistant *E. coli* (12, 31, 45) and *S. typhimurium* (19), irradiated as stationary-phase cultures in their growth media, increased their resistance by factors equivalent only to that between strains DB21 and the D21R1000 in the present study. The subsequent increases obtained for strains D21R200 through D21R6000 considerably exceed those levels.

The survival characteristics under more defined conditions (Fig. 1 and 2) point to the concomitant stepwise development of resistance to both gamma and UV irradiation. This observation adds to the already abundant evidence of shared relationships in response to the two types of irradiation. A lack of total identity between the effects of UV and gamma irradiation (5), however, is also reflected in Fig. 1 and 2. The increments in UV resistance, relative to the strain DB21 control, are much less dramatic than the corresponding increases in gamma resistance for the same populations, and changes in asymptotic slope are less evident. The LD_{90} values, however, which include the

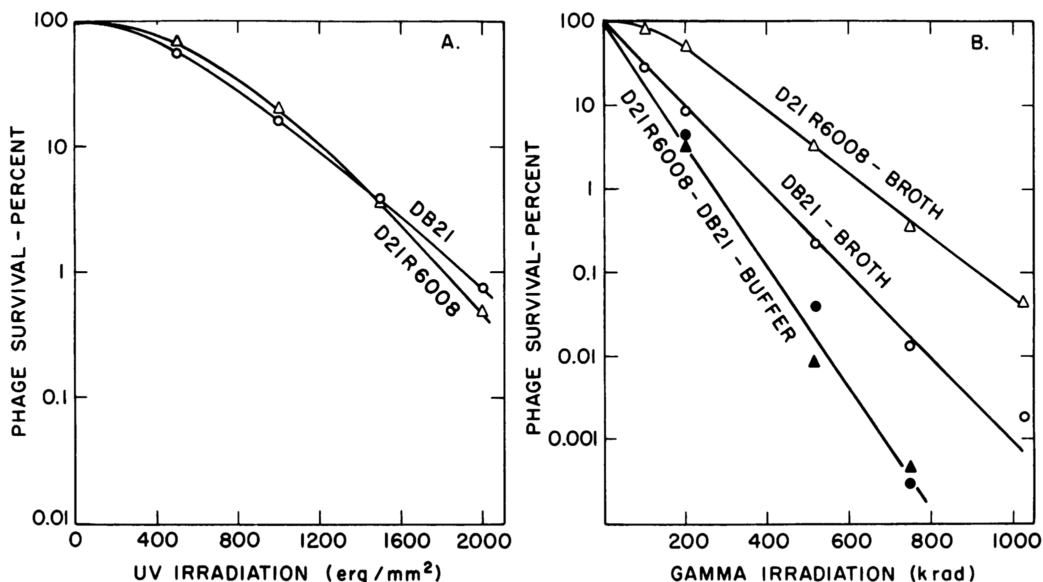

FIG. 4. *Host-cell reactivation of irradiated P22 wild-type phage by parent and radioresistant strains of S. typhimurium LT2. (A) UV irradiation. P22c$^+$ phage were UV-irradiated (incident dose rate, 18 ergs/mm^2 per sec) on the surface of prepoured TSY agar plates. Indicator organisms were added in 2.5-ml quantities of overlay agar. The counts on each indicator are expressed as percentage survival of untreated phage on the respective host: (Δ) on D21R6008; (O) on DB21. (B) Gamma-irradiation. P22c$^+$ phage were suspended at 10^{10} PFU/ml in 0.067 M phosphate buffer or 2.5% nutrient broth (w/v) and exposed to ^{60}Co gamma rays at 0 C (dose rate, 5.17 krad/min). Survivors were estimated by the overlay plate method with DB21 and D21R6008 as indicators. The P22 counts on each indicator are expressed as percentage survival of untreated phage on the respective host.*

TABLE 6. *Mutator activity in parent and radioresistant strains of S. typhimurium LT2*

TSY broth culture	*str-r* frequency/cell		
	Initial	After 90 generations	After 240 generations
DB21	2×10^{-7} (1.0)[a]	1.7×10^{-7} (0.9)	3×10^{-7} (1.6)
D21R1001	1×10^{-6} (1.0)	1.2×10^{-6} (1.2)	1.6×10^{-6} (1.6)
D21R3001	8×10^{-7} (1.0)	1.6×10^{-6} (2.0)	5.8×10^{-6} (7.3)
D21R6008	1×10^{-8} (1.0)	3.6×10^{-8} (3.6)	1.7×10^{-6} (170.0)

[a] Figures in parentheses refer to relative increase determined as: *str* frequency per cell after *n* generations/initial *str* frequency per cell.

contribution of the shoulder regions, increase at a faster rate than D_{10} values for both forms of irradiation.

The geometric parameters of shoulder and slope in a survival curve have been attributed to dual repair systems in the case of X ray-induced damage in *M. radiodurans* (30), and Haynes (16) used a similar concept to account for shoulders in the UV-survival curves of *E. coli* b/r. It is conceivable that the cyclic development of radioresistance obtained in the present study represents a selection of strains enhanced in their ability to repair irradiation-induced damage. Thus, if the widely held thesis that the principal target is DNA (16, 33) is accepted, the excessive increase in gamma versus UV resistance could then be attributed to a selection of processes either unique to the direct rejoining of interrupted strands (7, 26) or pertaining only to the late stages of excision repair (18).

The alternative possibility of a high target multiplicity is less attractive. Calculated extrapolation numbers are unacceptably high, and a re-treatment of the survival data according to the methods of Miller (27), in which log survival is plotted against the square of dose, reveals an average ploidy of unity even

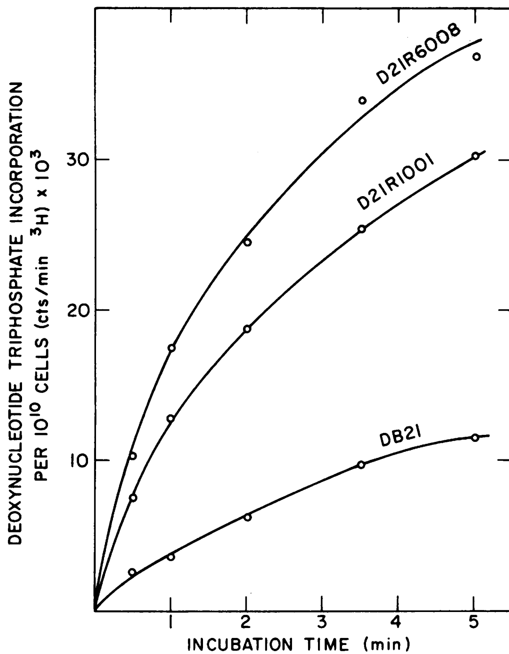

FIG. 5. *DNA polymerase activity in cell extracts of parent and radioresistant strains of S. typhimurium LT2. Strain DB21 and D21R6008 cells were grown to 5 × 10^8 cells/ml in EM9-glucose medium, chilled, centrifuged, and suspended in 0.1 M Tris(hydroxymethyl)aminomethane-0.01 $MgSO_4$ (pH 7.4) at a concentration of 10^10 cells/ml (cell counts determined in a Petroff-Hausser chamber). The suspensions were disrupted by sonic oscillation at 0 C, and centrifuged at 1,000 × g for 10 min. Samples (0.9 ml) of supernatant fluid were held at 22 C for 5 min with 20 μg of sonically treated calf thymus DNA per ml added, followed by 0.3 ml of triphosphate solution (final concentrations, 100 nmoles of deoxyguanosine, deoxyadenosine, and deoxycytidine triphosphates per ml, and 6 nmoles (11.4 μCi/ml) or ^3H-thymidine triphosphate per ml. Samples of 0.2 ml were taken at intervals, transferred to 5 ml of 5% trichloroacetic acid-1% sodium pyrophosphate, and held in ice for 2 hr. These samples were then washed on 24-mm, type HA filters (Millipore Corp.) with 5% trichloroacetic acid and with 5% acetic acid, dried, and counted in a Beckman cpm 100 scintillation counting system.*

in the more resistant cultures. Furthermore, no multinucleate, filamentous structures (20, 34) were observed, and the cellular content of DNA was quantitatively similar between strains DB21 and D21R6008 (Table 3).

The radioresistant cultures generally retained the qualitative identification characteristics of the wild-type parent, including serological and phage-typing characteristics. The maintenance of lysogeny in the DB20 series of

TABLE 7. *DNA polymerase I and polynucleotide ligase activities in radioresistant strains of S. typhimurium relative to the parent strain*

Determination	Growth phase[a]	Enzyme activity[b]		
		DB21	D21R1001	D21R6008
DNA polymerase I activity[c]				
Activity/10^10 cells				
15 C	Exp.	2,200 (1)	4,900 (2.2)	6,700 (3.0)
15 C	Stat.	950 (1)	3,620 (3.8)	6,800 (7.2)
22 C	Exp.	3,500 (1)	12,700 (3.7)	17,200 (4.9)
Activity/mg of protein[d]				
15 C	Exp.	1,146 (1)	2,168 (1.9)	2,058 (1.8)
15 C	Stat.	805 (1)		2,490 (3.1)
22 C	Exp.	1,823 (1)	5,619 (3.1)	5,470 (3.0)
Ligase activity[e]				
Units/cell				
30 C	Exp.	2.63 (1)	4.15 (1.6)	5.18 (2.0)
0 C	Exp.	0.51 (1)	0.83 (1.6)	1.15 (2.2)
Units/mg of protein[d]				
30 C	Exp.	1.88 (1)	1.78 (0.9)	1.56 (0.8)
0 C	Exp.	0.36 (1)	0.35 (1)	0.35 (1)

[a] Exp., exponential; stat., stationary.

[b] Relative activities are given in parentheses.

[c] Activity expressed as deoxynucleotide triphosphate ^3H counts per minute incorporated into trichloroacetic acid-insoluble polymeric material in the first minute of incubation of the assay mixture at the indicated temperature.

[d] Protein in extracts of sonically disrupted cells estimated by the Lowry method and recovery always more than 97.7% of total 5 N NaOH-soluble protein present in intact cells.

[e] One unit converts 100 nmoles of double-stranded deoxyadenylate and deoxythymidylate [d(A-T)$_n$] to an exonuclease III-resistant form in 30 min under assay conditions at 15 C or in 2 hr at 0 C.

resistant cultures and the continued susceptibility of the DB21-based organisms to both lysis and lysogenization (as evidenced by turbid plaques in the host-cell reactivation experiments) provided further confirmation that the isolates are true derivatives of *S. typhimurium* and not contaminants.

Phenotypic considerations relating to identification clearly provide only limited information concerning a potentially mixed population. The presence of a few survivors bearing wild-type characteristics could be sufficient to give a positive result. Failure to recognize the genetic heterogeneity of irradiation-cycled microbial cultures may lead to premature assumptions of correlation between phenotypic traits and radioresistance. In the case of the D21R6000 resistant culture, for example, the heterogeneity with respect to ability to grow on minimal media, utilization of carbon source, and release of H_2S is not reflected in its distribution of elevated radioresistance (Fig. 3). The implication emerges, therefore, that many of the phenotypic changes reported in radioresistant cultures are the result of ancillary mutations and are not directly responsible for the elevated resistance.

The carbohydrate utilization patterns of strain DB21 and its derivative cultures (Table 5) show an increasing accumulation of mutants. The preponderance of mutations affecting arabinose utilization may be of some significance. A single *araB* locus has been reported for *S. typhimurium* (36) which lies in a position similar to that of the equivalent locus in *E. coli* (41). The *araE* locus, however, controlling the L-arabinose permease in *E. coli*, is close to the *recB* and *recC* loci involved in the repair of irradiation damage (43). The homology of the two genetic maps (36) leads to the reasonable speculation that an *araE* gene may also be adjacent to the *rec* loci which have already been located in this region of the *S. typhimurium* genome by Eisenstark (36). A causal relationship between arabinose utilization and radioresistance is not implied, but the observation could be consistent with a hypothesis that the increases in resistance stem from mutations, probably of deletion type, in the vicinity of genes controlling DNA repair enzyme systems.

The high reversion frequency of strain D21R6008 to streptomycin resistance may indicate the presence of a mutator activity such as that described for *S. typhimurium* by Miyake (28). A mutator locus *mutS* and a thymidylate synthetase locus *thyA* are both located close to the *rec* loci of *E. coli* (41), and in *S. typhimurium thyA* is allocated to the same map position as an unidentified *rec* locus (36). A possible correlation is again suggested between radioresistance and mutations in the vicinity of DNA repair loci.

The host-cell reactivation studies provide further support for the involvement of DNA repair processes in hyper-radioresistance. The method essentially examined the ability of the bacterial hosts to repair lesions in phage DNA induced in the absence of interference from any host-intracellular radioprotective factors. Possible differences in irradiated phage adsorption were also not involved, as has been reported previously for P22 infection of *S. typhimurium* (40) and for coliphage systems (13).

Strain D21R6008 reactivated UV-irradiated phage with an efficiency similar to that of the DB21 parent and similar to that reported previously for *S. typhimurium* LT2 (38, 46). This observation agrees with the report (31) that gamma-induced radioresistant mutants of *E. coli* K-12 do not display an elevated host-cell reactivation response to UV-irradiated phage. In the case of the gamma host-cell reactivation experiments, however, the marked enhancement in the recovery of P22 phage, after gamma irradiation in concentrated nutrient broth, is particularly significant. Under these conditions, irradiation-induced damage in phage DNA has been shown in T7 coliphage to be primarily pyrimidine base damage (13) and in T1 coliphage irradiated in 0.1 M histidine (2) to be primarily single-strand breaks. It seems reasonable to postulate, therefore, that strain D21R6008 has acquired an enhanced ability to repair this type of DNA damage. On the other hand, phage irradiated in buffer or exposed to UV light were not hyper-reactivated. This perhaps reflects that in the first case the damage probably involved lethal double-stranded lesions (13) and in the second case the sensitivity of the method was insufficient to detect the rather less pronounced differences in the relative hosts' UV survival mechanisms.

The detailed enzymology of DNA repair, reviewed, for example, by Howard-Flanders (17), is not completely understood, but clearly DNA polymerase I and polynucleotide-joining enzyme activities are considered important participants. Mutants of *E. coli* which are deficient in DNA polymerase I activity are UV sensitive (15), X-ray sensitive (23), and incapable of completing DNA excision repair (4, 21) or rapid repair of X-ray-induced DNA strand breaks (42). A DNA ligase mutant *E. coli* strain TAU-bar ts-7 has been found to be X-ray-sensitive (8). Studies in vitro have shown that polymerase activity is necessary before

polynucleotide ligase can rejoin fragments of X-irradiated DNA (22). Against this background, therefore, the evidence of the present study, that radioresistant cells contain up to four or five times more DNA-polymerase I activity and doubled ligase activity compared to the parent strain (Table 7), provides circumstantial support for the postulate that enhanced DNA-repair mechanisms contribute to the observed hyper-resistance. The relative abilities of these strains to restore irradiation-induced strand breakage in their DNA is currently being investigated.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants UI-00087 from the National Institute of Urban and Industrial Health and FD-00165 from the Food and Drug Administration.

We thank David Botstein for many stimulating discussions.

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