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R-Factor-Mediated Nuclease Activity Involved in Thymineless Elimination

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R-factor 1818 (R-1818) had no effect on the efficiency of plating of ligase-deficient phage T4 mutants on strains of *Escherichia coli* containing excess, normal, or defective ligase. However, if the R^+ bacterial strain that overproduced ligase was first starved of thymine, its ability to propagate ligase-deficient phage was reduced by as much as fivefold compared with the burst size on the thymine-starved R^- strain. In contrast, it was found that after ultraviolet irradiation of the host the phage burst size was higher on the R^+ ligase overproducing strain than the R^- derivative. The maximal level of R-factor elimination produced by thymine starvation was inversely related to the ligase level of the host. Ultraviolet irradiation did not cure the R factor from strains containing wild-type levels of ligase, but did cause elimination from strains with excess or defective ligase. The results suggest that R-1818 codes for a nuclease that is induced by thymine starvation and which, possibly in conjunction with host-mediated nucleases, is responsible for its elimination under these conditions.

R-factor 1818 (R-1818) is eliminated from thymine-requiring (*thy*$^-$) mutants of *Escherichia coli* and *Klebsiella aerogenes* during thymine starvation (16). Protein synthesis is necessary for this elimination (17) and, since single-stranded breaks occur in F'*lac* factor deoxyribonucleic acid (DNA) during thymineless death (8), Pinney and Smith (17) have suggested that a nuclease which preferentially degrades R-1818 DNA is induced by thymine starvation. Extracts of thymine-starved *E. coli* contain an endonucleolytic activity not found in unstarved cells (9), and this or a similar nuclease may be involved in the elimination of R-1818. However, since R-7268 and R-TEM are not eliminated by thymine starvation (16), it is conceivable that R-1818 contributes to its own elimination. R-1818 increases the resistance of *Salmonella typhimurium* LT2 (7) and *E. coli* (this paper) to ultraviolet (UV) irradiation, which suggests that the R factor codes for a UV repair enzyme. This enzyme may be the nuclease that is responsible for thymineless elimination of R-1818.

We have consequently investigated whether cells containing R-1818 have a nucleolytic activity not found in R^- cultures. Our approach has been to determine the growth capacity of ligase-deficient bacteriophage T4 mutants in a series of ligase-overproducing and ligase-defective strains of *E. coli* (10) into which the R factor had been introduced. Bacteriophage T4 gene *30* mutants lack phage-induced ligase and will not propagate on wild-type *E. coli*. However, when grown on *E. coli* N1072, a mutant that produces abnormally high levels of bacterial ligase, their burst size is larger than that of normal T4 grown on wild-type strains (10).

Phage T4 gene *30* mutants can also be partially suppressed by secondary phage mutations such as endo II (deficiency of phage endonuclease II) (26) and the *r*II mutation (2, 3, 12). Mutation in the *r*II gene reduces early endonucleolytic attack on injected phage T4 DNA, and this reduction allows phage T4 gene *30* *r*II double mutants to propagate on bacteria with normal ligase levels (13).

Since low levels of endonuclease have been shown to compensate for the ligase requirement of phage T4 ligase-deficient mutants, we proposed that elevated levels of bacterial endonuclease could impair the propagation of these phage mutants. There is no a priori reason why this concept should be specific for the detection of endonuclease activities. An R-factor-mediated exonuclease could produce similar results, provided that sufficient endonuclease is already present in the system to provide a substrate for exonuclease action.

We tested for the presence of R-factor nuclease by determining the burst sizes of a phage T4 gene *30* mutant on R^+ and R^- strains of *E.*

coli N1072. This paper reports that R-1818 does affect the burst size of a phage T4 gene *30* mutant propagated on ligase-overproducing cells of *E. coli* but only after the cells have been starved of thymine or UV irradiated. UV irradiation is shown to cure R-1818 from strains containing abnormally high levels of either normal or defective ligase, but not from wild-type strains. We also present evidence that the maximal level of R-1818 elimination during thymine starvation is affected by the level of bacterial ligase.

MATERIALS AND METHODS

Media. Davis and Mingioli (DM) (6) lipid and solid media were prepared and supplemented as described by Smith (20). DM basal salts solution (termed DM base) was used as diluent for viable counts, washing cultures, and preparing suspensions for UV irradiation. MacConkey agar was Oxoid code CM7, and nutrient agar was Oxoid blood agar base (code CM55). Unless specified in the text, cultures were routinely grown in Oxoid nutrient broth no. 2 (code CM67).

Plaque counts of bacteriophage T4 were made on E.H.A. plates (24) by the soft agar overlay technique. The various indicator strains used are stated in the text.

Bacterial strains. *E. coli* N1071 (*lac*$^-$ *galE*$^-$ *galK*$^-_{am}$ *trp*$^-_{am}$ *tsy* Su$^-$ [λ*ind*$^-$]), a derivative of strain MB-0 (19), is wild type with respect to DNA ligase production. Mutants derived from strain N1071 by Gellert and Bullock (10), using nitrosoguanidine mutagenesis, were: strain N1072, a DNA ligase overproducer (*lop-8*); strain N1252, a ligase-defective mutant (*lop-8 lig-2*); and strain N1254, a temperature-sensitive derivative that overproduces ligase when grown at 32 C but is ligase defective when grown at 42 C (*lop-8 lig-4*). This series and their λ-cured derivatives were very kindly provided by M. Gellert. When received, the ligase mutants (but not strain N1071) were biotin and thiamine requiring and had an unspecified requirement satisfied by 1% Casamino Acids. After growth on DM medium containing supplements for the known auxotrophic requirements and Casamino Acids, two sizes of colony were observed. The larger colonial type was found to have lost its requirement for Casamino Acids but retained its other auxotrophic mutations. Revertants of this type were isolated for the three *lop-8* mutants (and their λ-cured derivatives) and used throughout this work. Thymine-requiring (*thy*$^-$) mutants of strain N1071 and its derivatives were isolated by trimethoprim selection (23); all required approximately 30 μg of thymine per ml for optimal growth.

R factor. R-1818 (5) is an *fi*$^-$, N-group (4) R-factor and was isolated from a strain first characterized by E. S. Anderson. It confers resistance to ampicillin, streptomycin, sulfonamides, and tetracycline. The donor strain for R-1818 transfer was *E. coli* K-12 58.161/*sp met*$^-$ (R-1818) (15). Transfer was carried out as described by Smith (21), using 10 μg of tetracycline per ml to select for the R factor. R-1818 has been renamed R46 by Meynell and Datta (15).

Bacteriophages. Bacteriophage T4 mutants *am H39X* and *am H39Xr59* from the collection of R. S. Edgar were kindly supplied by M. Gellert. T4 *am H39X* has an amber mutation in the phage T4 ligase gene (gene *30*), and phage T4 *am H39Xr59* carries an additional *rII* mutation. The efficiency of plating (EOP) of these bacteriophages on the amber suppressor strain, *E. coli* CR63, was taken as unity. EOP on other strains were estimated by the method of Gellert and Bullock (10).

Thymine starvation and R-1818 elimination. Exponential cultures were grown in fully supplemented DM medium at 37 C to about 10^8 organisms per ml. They were washed twice with DM base and suspended, at a concentration of approximately 10^7 organisms per ml, in fully supplemented DM medium lacking thymine, prewarmed at 37 C. At various time intervals, samples were diluted and plated on MacConkey agar supplemented with 60 μg of thymine per ml. After overnight incubation, the R-factor content of the resulting clones was analyzed by replica plating as described by Pinney and Smith (16).

UV irradiation. Washed, exponential-phase cultures were suspended in chilled DM base to about 10^8 organisms per ml. They were stirred in glass petri dishes and irradiated with, UV light (254 nm) from a Hanovia low-pressure mercury lamp. The lamp was held at a fixed distance about the sample (18 cm), and the UV dose was varied by exposing the suspensions for different time intervals. After UV irradiation, cells were diluted and plated on nutrient agar. Exposure of irradiated cells to ambient artificial light was minimized to prevent photoreactivation of UV-induced damage (14).

Measurement of average burst size. Thymine-starved or UV-irradiated cultures were mixed with phage T4 mutants at a multiplicity of infection of 0.1. The final mixture, which contained 0.8% sodium chloride for optimal adsorption, was held at 4 C for 15 min to allow adsorption while preventing phage multiplication. A sample was removed and diluted in prewarmed (37 C) nutrient broth (when UV-irradiated cells were used) or fully supplemented DM medium (when thymine-starved cells were used). The remainder of the adsorption mixture, cooled on ice, was shaken with chloroform for 1 min and centrifuged to remove bacterial debris, and the supernatant was assayed for unadsorbed phage. Average burst sizes corrected for unadsorbed phage were determined by standard methods (1).

RESULTS

Effect of R-1818 on the propagation of phage T4 am gene 30 mutants. To assess whether the possession of R-1818 affected the ability of phage T4 *am H39X* and T4 *am H39Xr59* to propagate on *E. coli* N1071 and its ligase mutants, the EOP of these phages were determined on R$^+$ and R$^-$ strains. The results (Table 1) for the R$^-$ strains were very similar to

TABLE 1. *Efficiencies of plating of T4 gene 30 mutants on ligase mutants of E. coli with and without R-1818*

E. coli strain[a]	Ligase genes	R-1818	EOP	
			T4 *am H39X*	T4 *am H39Xr59*
N1071	Wild type	−	5.0×10^{-5}	1.0
N1071	Wild type	+	5.0×10^{-5}	1.0
N1072[b]	*lop-8*	−	0.80	0.75
N1072[b]	*lop-8*	+	0.75	0.70
N1252[b]	*lop-8 lig-2*	−	1.4×10^{-6}	9.0×10^{-5}
N1252[b]	*lop-8 lig-2*	+	1.2×10^{-6}	8.0×10^{-5}
N1254[b]	*lop-8 lig-4* 32C	−	0.75	0.90
N1254[b]	*lop-8 lig-4* 32C	+	0.75	0.90
N1254[b]	*lop-8 lig-4* 42C	−	9.0×10^{-5}	2.0×10^{-3}
N1254[b]	*lop-8 lig-4* 42C	+	8.5×10^{-5}	2.0×10^{-3}

[a] T4 *am H39Xr59* was assayed on strains cured of λ, whereas T4 *am H39X* was assayed on strains lysogenic for λ(*ind*⁻).

[b] These strains no longer require a Casamino Acid supplement (10). EOP were determined by using log-phase cells on E.H.A. plates at 37 C unless specified.

those published by Gellert and Bullock (10) and indicate that the revertants of the unspecified growth requirement(s), selected for use in this work, had still retained their described ligase properties. The presence of R-1818 had no significant effect on the EOP of either phage mutant in any bacterial strain tested (Table 1).

We conclude that under normal conditions R-1818 does not contribute significantly to the effective levels of ligase or nuclease in phage T4-infected bacteria. However, it is possible that the R factor mediates ligases or nucleases that may be induced by conditions of thymine starvation or UV irradiation.

Effect of R-1818 on phage propagation after thymine starvation or UV irradiation of the host. Attempts were made to investigate the effects of thymine starvation and UV irradiation on the EOP of the two phage T4 gene *30* mutants on the R⁺ *E coli* N1071 series, but since both of these treatments are bactericidal it was impossible to obtain a confluent lawn of the indicator strain in which to observe lysis.

The effect of R-1818 on the burst size of phage T4 *am H39X* in *E. coli* N1072 *lop-8 thy*⁻ after periods of thymine starvation was therefore determined. This strain was chosen because it gives a large burst size of phage T4 *am H39X* under normal conditions (10), which facilitates the detection of any reduction. The results (Fig. 1) show that the burst size of phage T4 *am H39X* was not affected by thymine starvation of the R⁻ host for periods of up to 2 h, but during this time the burst size in the R⁺ strain was markedly reduced. When the period of thymine starvation was extended beyond the 2 h, reductions in burst sizes occurred in both hosts.

The effect of R-1818 on the burst size of phage T4 *am H39X* in strain N1072 that had previously been UV irradiated was determined next. It was found, contrary to the results obtained with thymine starvation, that the presence of the R factor increased the burst size of the phage relative to that obtained in R⁻ cells (Fig. 2). These results may suggest that R-1818 produces enzyme(s) which affect phage T4 *am*

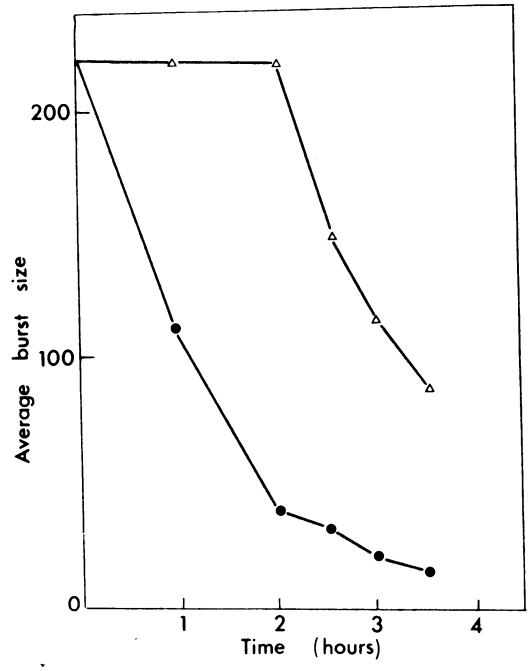

FIG. 1. *Effect of R-1818 on the ability of Escherichia coli N1072 lop-8 thy⁻ to support phage T4 am H39X propagation after periods of thymine starvation. Exponential-phase cultures were deprived of thymine for various times, after which samples were removed for phage propagation. Symbols: △, N1072 lop-8 thy⁻; ●, N1072 lop-8 thy⁻ (R-1818).*

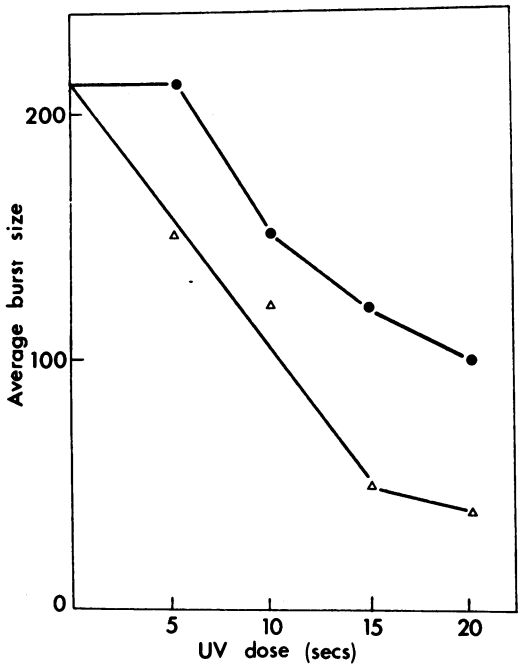

FIG. 2. *Effect of R-1818 on the ability of Escherichia coli N1072 lop-8 to support phage T4 am H39X propagation after UV irradiation of the host cells. Washed, exponential-phase cultures were UV irradiated for various times, after which samples were removed for phage propagation. Symbols: △, N1072 lop-8; ●, N1072 lop-8 (R-1818).*

H39X propagation when the host bacteria have been starved of thymine or UV irradiated.

Elimination of R-1818 from E. coli N1071 and its ligase mutants. The phage burst size experiments suggest that the R factor may mediate nuclease activity, and we have suggested (17) that nuclease action may be implicated in thymineless elimination of R-1818. Consequently, we investigated the elimination of R-1818 from hosts with different ligase activities. The results (Table 2) show that R-1818 was eliminated from *E. coli* N1071 *thy*⁻ and its ligase mutants during thymine starvation. Moreover, the host ligase level affected the degree of elimination. In strain N1072 *lop-8 thy*⁻ (R-1818), i.e., the ligase overproducer, the maximal R-factor elimination rate obtained was reduced, whereas in strain N1252 *lop-8 lig-2 thy*⁻ (R-1818), i.e., the ligase-defective strain, it was increased. These results were reproducible and the differences were statistically significant ($P < 0.02$). Thus, the maximal rate of R-factor elimination during thymine starvation was inversely related to the level of bacterial ligase. The results (Table 2) also show that R-1818 conferred resistance to thymineless death.

R-1818 additionally confers protection against UV irradiation on *Salmonella typhimurium* (7). Nucleases are involved in both excision and post-replication repair of UV damage (18, 22), and it is possible that R-1818 protects its host by mediating nuclease(s) that assist in DNA repair. It may be that these nuclease(s) are the same as those suggested to cause thymineless elimination of the R factor. If this were the case, then UV irradiation should induce R-1818 elimination. The effect of UV irradiation on R-1818 was therefore investigated. The results (Table 3) show that R-1818 also protects *E. coli* N1071 from UV irradiation. However, the R factor was not eliminated from this host by UV irradiation. In contrast, a maximum of 10% R-1818 loss was found from strain N1072 *lop-8* (R-1818) and a maximum of 15% loss occurred from strain N1252 *lop-8 lig-2* (R-1818) after UV irradiation.

DISCUSSION

R-1818 is eliminated from *E. coli* K-12 *thy*⁻ during thymine starvation (16) and protects bacteria from UV irradiation and thymineless death. These characteristics could depend on R-factor-coded nucleases. This paper reports two genetic methods of testing this concept: (i) the effect of the R factor on the propagation of a ligase-deficient bacteriophage that is known to be sensitive to changes in endonuclease levels (13, 26), and (ii) the elimination of the R factor from bacterial strains with altered ligase levels.

Under normal growth conditions, R-1818 did not influence the plating efficiency of any phage T4 ligase mutant on any bacterial strain tested and, therefore, normally does not contribute to the effective levels of ligase or nuclease in phage T4-infected cells. It was not possible to test the ability of phages to plaque on thymine-starved or UV-irradiated indicator bacteria because the latter are killed by these treatments so, as an alternative, phage burst sizes were measured under these conditions.

When thymine-starved bacteria were infected with phage T4 *am H39X*, its burst size was decreased by the presence of R-1818, possibly indicating the presence of an R-factor-mediated nucleolytic activity that was being induced by these conditions.

Elimination of R-1818 during thymine starvation occurred in all strains tested, irrespective of ligase genotype, and the maximal rate of elimination was inversely proportional to the level of host ligase. This could be a reflection of antagonism between ligase and the nucleases that may be induced by thymine starvation.

When UV-irradiated bacteria were infected

TABLE 2. *Resistance to thymineless death mediated by R-1818 and elimination of the R-factor by thymine starvation*[a]

Duration of thymine starvation (min)	Wild-type ligase N1071 *thy*⁻ R⁻	Wild-type ligase N1071 *thy*⁻ (R-1818)		Ligase-overproducer N1072 *thy*⁻ (R-1818)		Ligase-defective N1252 *thy*⁻ (R-1818)	
	Initial count (%)	Initial count (%)	R⁻ clones (%)	Initial count (%)	R clones (%)	Initial count (%)	R⁻ clones (%)
0	100	100	0.4	100	0.12	100	0.7
60	94	100	0.9	54	0.60	29	1.2
120	19	61	13.0	0.90	3.0	0.30	3.5
180	1.0	6.2	18.0	0.10	8.5	0.07	14.5
210	0.25	1.0	17.6	0.06	7.7	0.02	28.0
240	0.05	0.15	18.5	0.06	9.2	0.01	26.0

[a] Cultures were grown to log phase at 37 C, washed, and resuspended in DM medium without thymine to give a viable count of 10^7 cells per ml, and incubation was continued at 37 C. Samples were removed at intervals and plated on MacConkey agar containing 60 μg of thymine per ml. After overnight incubation, suitable plates were replica-plated onto fully supplemented DM medium containing 10 μg of tetracycline per ml, and onto fully supplemented DM without tetracycline. These plates were incubated overnight and scored for R⁻ colonies.

TABLE 3. *UV protection mediated by R-1818 and elimination of the R factor from the E. coli N1071 ligase series by UV irradiation*[a]

UV dose (s)	Wild-type ligase N1071 R⁻	Wild-type ligase N1071 (R-1818)		Ligase-overproducer N1072 (R-1818)		Ligase-defective N1252 (R-1818)	
	Initial count (%)	Initial count (%)	R⁻ clones (%)	Initial count (%)	R⁻ clones (%)	Initial count (%)	R⁻ clones (%)
0	100	100	0.60	100	0.12	100	0.69
5	41	71	0.14	20	1.8	0.62	3.4
10	11	38	0.30	2.2	4.7	0.05	6.0
15	0.8	10	0.25	0.18	8.5	0.003	8.0
20	0.04	1.0	0.17	0.03	10.0	0.0001	15.0
30	0.001	0.004	0.30				

[a] Cultures were grown to log phase at 37 C, washed, and resuspended in DM basal medium to give a viable count of approximately 10^8 cells/ml. This suspension was irradiated at 254 nm for different time intervals. After irradiation, samples were plated on nutrient agar and incubated overnight at 37 C. Suitable plates were replica plated to determine R-factor content.

with phage T4 *am H39X*, its burst size was increased by the presence of R-1818. This result is opposite to that found with bacteria subjected to thymine starvation and may reflect the UV-repair activity of the R factor acting on the DNA of the UV-irradiated host. Cells containing fewer unrepaired DNA lesions should be better fitted to propagate the phage.

Since both excision and post-replication repair of UV irradiation-induced damage involve nuclease action (18, 22), the enhanced UV resistance conferred by R-1818 could be mediated by an R-factor nuclease. Indeed, Marsh and Smith (14) have suggested that UV-protecting R factors aid post-replication repair (18). If thymineless elimination of R-1818 were due to the same nuclease that confers UV protection, then UV irradiation might also be expected to induce elimination. However, the R factor was not eliminated from *E. coli* N1071, which has a wild-type ligase level. On the other hand, R-1818 was eliminated by UV irradiation from the ligase-overproducing and the ligase-defective strains. Both these *lop-8* strains have an additional mutation that increases UV sensitivity (11), and this latter mutation may be responsible for UV-induced R-factor loss. However, the elimination of R-1818 from these mutants could result from the effects of abnormal ligase levels on the excision repair of UV-induced damage.

In conclusion, these findings are compatible with a nuclease activity mediated by R-1818 that is induced by thymine starvation and may result in elimination of the R factor. Bacteria with wild-type ligase levels are protected from UV irradiation by R-1818. This may also be due to an R-factor-mediated nuclease activity.

However, UV irradiation does not eliminate the R factor from these bacteria.

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