Effect of Incubation Media on the Recovery of *Escherichia coli* K12 Heated at 52 °C

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(Received 27 January 1978)

The exposure of exponentially grown *Escherichia coli* K12 to 52 °C for 30 min in Tris/Mg²⁺ buffer resulted in a considerable loss of viability when plated on tryptone agar. When such heated bacteria were held at 37 °C for 2 h in tryptone broth before plating on tryptone agar, there was a significant increase in viability. Thus, heat damage was repaired in tryptone broth but not on tryptone agar. Recovery was greater in tryptone broth than in synthetic medium. In tryptone broth, *recA* or *polA* mutants also recovered but a *lex* mutant did not.

As a result of heating, the sensitivity of bacteria to ultraviolet radiation (u.v.), to mitomycin C and to plating on high salt medium was enhanced. After incubation for 2 h in tryptone broth at 37 °C, the bacteria regained their resistance to u.v. and mitomycin C and tolerance to high salt medium. Recovery of viability required RNA and protein synthesis, whereas recovery of u.v. resistance did not require protein synthesis. Heating for 30 min inhibited the release of acid-soluble material from DNA in all strains of *E. coli* used.

INTRODUCTION

Injury to bacteria caused by exposure to high temperatures and their subsequent recovery has been studied in *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus subtilis*, *Salmonella typhimurium* and *Pseudomonas fluorescens* (Iandolo & Ordal, 1966; Clark, Witter & Ordal, 1968; Bluhm & Ordal, 1969; Rosenthol & Iandolo, 1970; Tomlins & Ordal, 1971; Miller & Ordal, 1972; Gray, Witter & Ordal, 1973). The heat injuries have been characterized as damage to the cell membrane, degradation of ribosomal RNA and alteration in enzyme activity. Heat treatment of *Escherichia coli* at 52 °C apparently induces single-strand breaks in the DNA and post-treatment incubation in phosphate buffer leads to the rejoining of these breaks and an increase in colony-forming ability (Bridges, Ashwood-Smith & Munson, 1969; Mukherjee & Bhattacharjee, 1970; Woodcock & Grigg, 1972). Bridges *et al.* (1969) demonstrated that mutations in certain loci, which render *E. coli* sensitive to radiation, also enhance its sensitivity to heat. The work reported here was undertaken in an attempt to understand the nature of heat lesions in *E. coli* K12, the requirements for recovery and the involvement of known genetic loci. Recovery is defined as an increase in the viability of heated bacteria by incubation in recovery medium before plating on tryptone agar, compared with immediate plating on tryptone agar.

METHODS

Bacteria. These are listed in Table 1.

Media. The minimal glucose/salts medium (MM) contained (per litre): K₂HPO₄·3H₂O, 7 g; KH₂PO₄, 3 g; (NH₄)₂SO₄, 1 g; MgSO₄·7H₂O, 0.1 g; sodium citrate·2H₂O, 0.5 g; MnSO₄ and Fe₂(SO₄)₃, each 0.1 ml of 0.01 M solutions; and thiamin, 1 mg. D-Glucose [50 ml of a 10 % (w/v) solution] was sterilized separately

Table 1. *Escherichia coli* K12 strains used

Strain	Relevant genetic markers	Source
AB1157	<i>thi argB thr leu pro his</i>	P. Howard-Flanders
AB2463	<i>recA13 thi argB thr leu pro his</i>	P. Howard-Flanders
AB2470	<i>recB21 thi argB thr leu pro his</i>	P. Howard-Flanders
AB1886	<i>uvrA6 thi argB thr leu pro his</i>	P. Howard-Flanders
AB2494	<i>lex thi thr leu pro his metB</i>	P. Howard-Flanders
JG112	<i>polA1 thi thyA</i>	J. Gross

and then mixed with the salts. The medium was supplemented with required amino acids at $40 \mu\text{g ml}^{-1}$. The thymine requirement of *E. coli* JG112 was $20 \mu\text{g ml}^{-1}$. Tryptone broth (RM) contained (per litre): tryptone (Oxoid), 10 g; yeast extract paste (Centron Research Laboratories, Pvt. Ltd, India), 5 g; and NaCl, 5 g. Bacteria were always plated on tryptone broth containing 1.5% (w/v) agar (BDH). The high salt medium consisted of tryptone agar containing 2% (w/v) NaCl, on which *E. coli* grows with no detectable loss of viability.

Buffers. All dilutions were made in 0.01 M-MgSO₄ solution. Bacteria were heat-treated in Tris/Mg²⁺ buffer (0.01 M, pH 8.0).

Heat treatment. Washed bacteria from an exponentially growing culture (1×10^8 to 5×10^8 viable units ml^{-1}) were suspended in buffer in a test tube and placed in a water bath at 52°C . The temperature in the tube rose to within 0.5°C of the final temperature in 1 min. After heat treatment, samples were diluted and immediately plated to assay colony-forming ability.

Post-treatment incubation. Heated and unheated bacterial suspensions (0.5 ml) were diluted 10-fold into MM or RM and shaken for 2 h at 37°C . At intervals, samples were diluted and plated. Where stated, chloramphenicol (Cam; $100 \mu\text{g ml}^{-1}$) or rifampicin (Rif; $10 \mu\text{g ml}^{-1}$) was added to the recovery medium (these were gifts from May & Baker, India, and Professor G. C. Lancini, respectively). Before plating, the bacteria were washed to remove the drugs. Neither antibiotic reduced the viability of unheated bacteria at the concentrations used.

Exposure to ultraviolet radiation (u.v.) and mitomycin C. Unheated bacteria, heated bacteria and heated bacteria held in recovery medium for 2 h with and without chloramphenicol were compared for u.v. and mitomycin C sensitivity. To measure u.v. sensitivity, bacteria were plated on tryptone agar and the plates were exposed to 254 nm and incubated at 37°C . Irradiation and incubation were performed in complete darkness to avoid photoreactivation. Sensitivity to mitomycin C (Calbiochem) was determined by plating the bacteria on tryptone agar containing the drug at $0.5 \mu\text{g ml}^{-1}$. Mitomycin C at this concentration had no effect on the viability of unheated bacteria.

DNA degradation. Bacteria were radioactively labelled by growing them in minimal medium supplemented with their required growth factors and $5 \mu\text{Ci } [^3\text{H}]\text{thymidine ml}^{-1}$ (sp. act. $6500 \text{ mCi mmol}^{-1}$; Bhabha Atomic Research Centre, India). Labelled bacteria were heated for 30 min at 52°C and incubated with shaking in RM. Samples (0.2 ml) were removed at regular intervals, fixed for 30 min in 1 ml chilled 5% (w/v) trichloroacetic acid and the acid-insoluble fractions were collected on Millipore filters (pore size, $0.45 \mu\text{m}$). The radioactivity was measured in a liquid scintillation counter.

RESULTS

Survival of heated bacteria

The survival of bacteria when plated directly on to tryptone agar immediately after heating is shown in Fig. 1. The mutants *recA*, *lex* and *polA* were more sensitive to heat than the wild type. Mutants *uvrA* and *recB* were as resistant as the parent strain (data not shown). The wild type and the *recA* mutant were more sensitive when grown in MM than when grown in RM. The viability of *polA*, and to a large extent *lex*, was not affected by the growth medium.

Liquid holding recovery in RM and MM

The bacteria were heated for 30 min at 52°C and then shaken for 2 h in MM or RM. Colony-forming ability was assayed at intervals during this period by plating samples on tryptone agar. The viability of unheated bacteria under the same conditions was also assayed (Fig. 2). Recovery was influenced by the composition of both the pre- and post-

Fig. 1. Survival of bacteria heated at 52 °C after growth in (a) MM and (b) RM: ○, wild type; ●, *lex*; △, *recA*; □, *polA*.

treatment media. Recovery is the ratio of the viable numbers of heated and unheated bacteria at 2 h.

The results shown in Fig. 2(a, c) are for bacteria grown in MM and RM, respectively, and shaken in MM after heat treatment. When grown in MM before heat treatment, the wild type and *recA* strains showed recovery during the 2 h incubation, whereas *lex* and *polA* mutants did not (Fig. 2a). When grown in RM before treatment, the colony-forming ability of *recA* and *lex* declined, whereas with the wild type loss of viability occurred for 1 h (Fig. 2c); again, *polA* cells did not recover.

The results shown in Fig. 2(b, d) are for bacteria grown in MM and RM, respectively, and shaken in RM after heat treatment. Except for the *lex* mutant, all the strains recovered significantly. The apparent extent of recovery after 2 h for the wild type, *recA*, *polA* and *lex* strains was 3, 7, 10 and 1, respectively (Fig. 2d). However, the *lex* mutant failed to recover and the increase in viable counts of the *lex* mutant was probably due to the multiplication of survivors since under identical conditions the increase in the number of unheated bacteria was the same.

When bacteria grown and treated as in Fig. 2(d) were not shaken but simply held in tryptone broth, the same results were obtained. Thus, holding in tryptone broth for 2 h after heat treatment increased survival compared with immediate plating on tryptone agar. Tryptone broth can therefore be referred to as recovery medium.

U.v. and mitomycin C sensitivity and salt tolerance

The parent strain, AB1157, was heated for 30 min. Half the culture was exposed to u.v., mitomycin C or plated on high salt medium and the colony-forming units were counted. The other half was suspended in recovery medium for 2 h and then exposed to u.v., mitomycin C or high salt medium. Parallel controls of unheated bacteria were also carried out.

Fig. 2. Influence of pre- and post-treatment liquid media on the recovery of bacteria heated at 52 °C for 30 min: ○, wild type; ●, *lex*; △, *recA*; □, *polA*. ---, Increase in the number of unheated bacteria under similar conditions; -.-.-, viable count of heated bacteria in the presence of chloramphenicol.

Table 2. Effect of metabolic inhibitors on the liquid holding recovery of heated AB1157

Bacteria	Incubation medium	Time (min)	Viable units ml ⁻¹ on different plating media:		
			RM	RM + NaCl (2%)	RM + mitomycin C
Unheated AB1157	RM	0	2.0×10^7	2.2×10^7	2.0×10^7
	RM	120	1.4×10^8	ND	ND
	RM + Rif	120	1.2×10^8	ND	ND
	RM + Cam	120	2.0×10^7	ND	ND
Heated AB1157 (30 min, 52 °C)	RM	0	4.3×10^5	3.5×10^4	3.5×10^4
	RM	120	6.5×10^6	1.2×10^6	4.0×10^6
	RM + Rif	120	4.5×10^4	2.2×10^5	3.0×10^3
	RM + Cam	120	1.2×10^6	2.5×10^5	3.3×10^5

ND, not done; Cam, chloramphenicol; Rif, rifampicin.

Heated bacteria were more sensitive to u.v. than unheated bacteria, but regained normal u.v. resistance during the 2 h incubation in recovery medium (Fig. 3). Similarly, heated bacteria were more sensitive to mitomycin C and to plating on high salt medium, but incubation in recovery medium resulted in normal mitomycin C resistance and high salt tolerance (Table 2).

Effect of chloramphenicol and rifampicin on recovery

The presence of chloramphenicol in the recovery medium during 2 h incubation of heated bacteria inhibited the recovery of viability. However, some recovery did occur even in the

Fig. 3

Fig. 4

Fig. 3. U.v. sensitivity of strain AB1157. ○, Bacteria heated at 52 °C for 30 min before exposure to u.v.; ■, unheated bacteria exposed to u.v.; ●, heated bacteria exposed to u.v. after shaking in recovery medium for 2 h; ▲, heated bacteria exposed to u.v. after shaking in recovery medium containing chloramphenicol.

Fig. 4. DNA degradation in heated (—; 52 °C for 30 min) and unheated (---) bacteria during post-treatment incubation in recovery medium: ○, wild type; ●, *lex*; △, *recA*; □, *polA*.

presence of chloramphenicol (Table 2, Fig. 2*d*). Chloramphenicol interfered to a large extent with the recovery of salt tolerance and mitomycin C resistance (Table 2), but had no effect on the recovery of u.v. resistance (Fig. 3).

Rifampicin totally inhibited the repair of heated bacteria in recovery medium. The degree of inhibition of salt tolerance was the same as that by chloramphenicol.

DNA degradation

The initial radioactivity in the acid-insoluble fractions of heated and unheated bacteria was less than 100% and varied from strain to strain, e.g. for unheated wild type, *recA*, *lex* and *polA* bacteria, the values were 90, 65, 80 and 82%, respectively, and the corresponding values for heated bacteria were 95, 70, 80 and 75%. So, for convenience, the initial counts were normalized to 100% and the rest of the results were calculated accordingly. DNA degradation was significantly inhibited in heated bacteria of each strain (Fig. 4).

DISCUSSION

Bridges *et al.* (1969) found that 'reckless' and 'cautious' recombination-deficient strains of *E. coli* B were sensitive to mild heating whereas the *exr* (*lex*) mutation had little or no effect except when combined with a *lon* or *hcr* mutation. The heat sensitivity of *polA*⁺ and *polA* strains was identical (Sedgwick & Bridges, 1972) and the authors explained this as being due to the lack of endonucleolytic incision activity. Part of our data on the apparent involvement of known loci on heat sensitivity do not agree with these results. We found that besides the *recA* strain, *polA* and *lex* strains were also sensitive to heat whereas the 'cautious' strain *recB* was as resistant as the wild type. The 'cautious' recombination-deficient strain used by Bridges *et al.* (1969) was later shown to have a *resA* mutation (Kato & Kondo,

Fig. 5. Scheme for heat lesions and their mode of recovery.

1970), which is an allele of *polA*. This might explain why the 'cautious' recombination-deficient strain of Bridges *et al.* (1969) was sensitive to heat killing.

Survival was enhanced when the heated bacteria were held in tryptone broth as compared to direct plating on tryptone agar. The effect of 'liquid holding' on the recovery of u.v.-irradiated *E. coli* in MM is well documented (Roberts & Aldous, 1949; Ganesan & Smith, 1968). Contrary to the results with u.v., the liquid holding recovery of heated bacteria was poor in MM compared with recovery in RM.

Recovery of viability was considerably inhibited by rifampicin and chloramphenicol, suggesting a possible role for RNA and protein synthesis in recovery. *Salmonella typhimurium* also required both for recovery from thermal injury (Tomlins & Ordal, 1971), although other bacteria do not require protein synthesis (Clark *et al.*, 1968; Miller & Ordal, 1972; Gray *et al.*, 1973).

DNA degradation was inhibited by heat treatment, probably due to inhibition of DNAases. Chapmann & Pollard (1969) reported inhibition of DNA degradation when *E. coli* was heated at temperatures greater than 45 °C prior to γ -irradiation. Heated bacteria were more sensitive to u.v. and to plating on high salt medium, and during liquid holding normal u.v. resistance and salt tolerance were regained. These data suggest inactivation of radiation repair enzymes and damage to the cell membrane by heat and their subsequent recovery. The recovery of normal u.v. resistance in the presence of chloramphenicol (Fig. 3) suggests that *de novo* synthesis of repair enzymes was not required and under favourable conditions, provided by tryptone broth, renaturation of the repair enzymes might be involved. Chloramphenicol inhibited the recovery of mitomycin C resistance and salt tolerance (Table 2) which suggests that repair of the cell membrane requires *de novo* protein synthesis. Thus,

the failure to achieve normal mitomycin C resistance in the presence of chloramphenicol could be due to increased permeability of mitomycin C. The recovery of normal u.v. resistance in the presence of chloramphenicol (Fig. 3) but inhibition of recovery of viable bacteria in recovery medium (Fig. 2*d*) suggest that the two phenomena may be independent of each other.

The repair of certain heat lesions occurred during liquid holding and not on agar plates of the same medium composition. We suggest that lesions in protein and membrane are repaired in the liquid medium but inhibited on agar plates. A similar result was reported by Roberts & Aldous (1949) who tested the inhibitory effect of agar on recovery of u.v.-irradiated *E. coli*. Recovery was obtained in liquid medium but was inhibited on agar medium and they attributed this effect to a typical diffusion which was not possible on the solid medium.

We propose the following explanation for the heat lesions and their mode of recovery in *E. coli* (Fig. 5). Short periods of heat treatment (low dose) cause local opening of DNA at various points (Inman, 1966). These specific regions are attacked by DNAase(s) (Goldmark & Linn, 1970; Sedgwick & Bridges, 1972) resulting in nicked DNA. A few breaks are repaired by DNA polymerase I and ligase (Town, Smith & Kaplan, 1971, 1972; Srivastava, 1974), while the remaining breaks require functional *lex* and *recA* genes. Thus, at low doses only the lesions in DNA are important and are repairable (Woodcock & Grigg, 1972), but after longer periods of heating, damage to RNA and protein becomes effective. This involves inactivation of repair enzymes and DNAase(s), and damage to the cell membrane. Recovery at higher doses would eventually require the synthesis of membrane and activation and/or *de novo* synthesis of enzymes. Post-treatment incubation in tryptone broth, but not on tryptone agar, brings about resumption of enzyme activities and cell membrane recovery, as shown by the increase in u.v. and mitomycin C resistance and high salt tolerance.

We are grateful to Dr Nitya Nand for facilities to carry out this work. One of us (M.A.) is indebted to C.S.I.R., New Delhi, for the Junior Research Fellowship, C.D.R.I. communication no. 2207.

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