Production of Single Strand Breaks in the DNA of *Streptococcus faecalis* After Mild Heating

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INTRODUCTION

Although moist heat is known to damage many components of the bacterial cell, the cause of thermal death remains unknown (for reviews, see Allwood & Russell, 1970; Tomlins & Ordal, 1976; Welker, 1976).

Single strand breaks in DNA as a result of exposure to moist heat have been observed in *Escherichia coli* (Bridges *et al.*, 1969; Sedgwick & Bridges, 1972; Woodcock & Grigg, 1972; Pauling & Beck, 1975; Grecz & Bhatarakamol, 1977) and *Salmonella typhimurium* (Gomez & Sinskey, 1973, 1975). These breaks are not considered to be the direct effect of heat on the DNA, since their formation is dependent on the recovery conditions (Gomez & Sinskey, 1973), the strain used and physiological state of the bacteria (Sedgwick & Bridges, 1972), and could not be demonstrated by heating the DNA *in vitro* (Woodcock & Grigg, 1972). Rather, the damage has been attributed to heat-activated nucleases (Sedgwick & Bridges, 1972; Gomez & Sinskey, 1973), the breaks being greatly reduced in an endonuclease-defective strain or if enzyme inhibitors are present during heating (Grecz & Bhatarakamol, 1977).

The role of this damage in cell death is unclear. While Woodcock & Grigg (1972) reported that breakage and repair of the DNA in *E. coli* were accompanied by corresponding changes in viability, Bridges *et al.* (1969) were unable to show any repair of the breaks in their strains during recovery. However, Pauling & Beck (1975) found that a DNA ligase-defective mutant of *E. coli* was much more heat-sensitive than the wild-type strain and suggested that this sensitivity was due to an inability of the mutant to repair the thermally induced breaks. In *S. typhimurium* the damage was only detectable after many bacteria had lost their viability (Gomez & Sinskey, 1973), yet the lesions have to be repaired before the injured cells can resume growth on complex media (Gomez & Sinskey, 1975), and hydroxyurea, which depletes the bacteria of DNA precursors, interferes with recovery (Gomez *et al.*, 1976).

Clearly, DNA damage must be sought in other species, particularly Gram-positive bacteria, before its general significance in thermal death can be assessed. We have looked for evidence of DNA scissions in two thymine-requiring mutants of *Streptococcus faecalis* and we report here our preliminary findings that single strand breaks are produced when these bacteria are heated at 60 °C.

METHODS

Strains and growth conditions. Thymine-requiring mutants of *Streptococcus faecalis* 775 and EB/F/30/82 were grown at 37 °C in SF35 synthetic medium (Andrew, 1973) supplemented with 5 µg thymine ml⁻¹. For labelling experiments the medium contained 2.5 µCi [methyl-¹⁴C]thymine ml⁻¹ (specific activity 55 mCi mmol⁻¹) or 100 µCi [methyl-³H]thymine ml⁻¹ (specific activity 5 Ci mmol⁻¹) (both from The Radiochemical Centre, Amersham), together with 3 µg thymine ml⁻¹.

Growth [colony-forming units (c.f.u.) ml⁻¹] was monitored by a model Z_B Coulter counter (Coulter Electronics, Harpenden, Herts.) set to count all particles greater than 1 µm diameter using a 30 µm orifice tube and 2 % (w/v) saline as the electrolyte.

Heat treatment. Overnight cultures grown in the ³H-labelled medium were used to inoculate fresh batches of the same medium and incubation at 37 °C was continued until the cultures reached the mid-exponential growth phase (about 5 × 10⁸ c.f.u. ml⁻¹). Samples (1 ml) of these cultures were then transferred to flasks containing 9 ml of thymine-supplemented SF35 medium, pre-heated to 60 °C, in a Heto thermistor-controlled water bath (V. A. Howe, London), accurate to ± 0.1 °C. The flasks were agitated by a reciprocating shaker attachment operating at 60 strokes min⁻¹. ¹⁴C-Labelled bacteria, treated similarly in medium at 37 °C, served as unheated controls. Survivors were estimated by periodically withdrawing samples from the flasks, diluting them in sodium phosphate buffer (pH 6.8) at 20 °C, and pour-plating samples of appropriate dilutions in triplicate with 10 ml of thymine-supplemented SF35 medium solidified with 1.2 % (w/v) Oxoid agar no. 3. Colonies were counted after 3 d incubation at 37 °C.

Alkaline sucrose gradient analysis. A modification of the alkaline sucrose gradient technique (McGrath & Williams, 1966; Kato & Kondo, 1970) was used to detect single strand breaks in the DNA. Samples (1 ml) of the heated bacterial suspension were centrifuged for 2 min in a Quickfit & Quartz microcentrifuge, and the pellets were resuspended in 0.1 ml of the supernatant. Equal volumes (50 µl each) of the heated and unheated bacterial suspensions were then added together to 0.2 ml of a solution of 1 % (w/v) sodium dodecyl sulphate, 0.25 M-NaOH, 0.005 M-EDTA, 0.05 M-NaCl and 0.005 M-Tris which had been layered on top of a 4.7 ml 5 to 20 % linear sucrose density gradient containing 0.2 M-NaOH and 0.001 M-EDTA. The gradients were left at room temperature for 30 min to allow the bacteria to lyse, and then centrifuged in an SW-50L rotor in a Beckman L2-65B ultracentrifuge for 75 min at 42000 rev. min⁻¹ at 20 °C. Approximately 30 fractions of equal volume (165 µl) were collected from the bottom of the centrifuge tube into the wells of a microhaemagglutination tray. Samples (100 µl) of all fractions were then applied to 24 mm diam. Whatman no. 1 filter paper discs and the discs were dried under infrared lamps. Acid-insoluble material was precipitated by washing the discs twice in ice-cold 5 % (w/v) trichloroacetic acid for 15 min and once in acetone. The discs were dried again, placed in vials containing 10 ml of a solution of 0.3 % (w/v) 2,5-diphenyloxazole and 0.03 % (w/v) 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene in toluene, and assayed for radioactivity in a model 2002 Packard Tricarb scintillation counter.

The number of breaks (*n*) per single strand genome was calculated using a modification of the equation of Town *et al.* (1971) to allow for the asymmetrical distribution of the DNA profiles:

$$n = \frac{M_w}{M_n} \left[\left(\frac{S'_1}{S''_1} \right) \frac{1}{\alpha} - 1 \right]$$

where *M_w* is the molecular weight of 'native' single strand DNA, *M_n* is the weight-average molecular weight of 'undamaged' DNA on the gradient, *S'₁* and *S''₁* are the distances (in fractions) beyond which one-half the mass of damaged and control molecules, respectively, sediment (Veatch & Okada, 1969) and α is a constant with a value of 0.4 (Studier, 1965).

M_w was calculated from buoyant density measurements of Schildkraut *et al.* (1962) in comparison with the molecular weight of single strand *E. coli* DNA (1.25 × 10⁹). *M_n* was calculated from its sedimentation coefficient (Studier, 1965) using a value for α of 0.4 and a value for *k* of 0.0528. The ratio *M_w*/*M_n* was approximately 7, which is of the same order as the values of McGrath & Williams (1966) and Town *et al.* (1971). The fast sedimenting material at the bottom of the gradients and the degradative products at the extreme top of the gradients were excluded from the calculations.

RESULTS AND DISCUSSION

The modification of the alkaline sucrose gradient technique for examining *E. coli* DNA was found to be suitable for use with these streptococci: satisfactory profiles of DNA were obtained, the position of the peak in the unheated control cells being essentially the same in all experiments.

When the cells were heated there was a progressive decrease in the molecular weight of the DNA in both strains. Figure 1 shows the results for strain 775 T⁻.

A striking feature of the results is that damage was detectable after much shorter heating periods and at lower levels of killing than reported for *E. coli* (Bridges *et al.*, 1969; Woodcock & Grigg, 1972) or *S. typhimurium* (Gomez & Sinskey, 1973, 1975). Shifts in the peaks were seen after only 90 s of heating, when little or no loss of viability had occurred. This may

Fig. 1. Alkaline sucrose gradient sedimentation of DNA from heated ($\circ$, ^3H -labelled) and unheated ($\bullet$, ^{14}C -labelled) *Streptococcus faecalis* 775 T $^-$. (a) No heat treatment (100 % viability); (b) heated for 5 min at 60 °C (90 % viability); (c) heated for 13 min at 60 °C (30 % viability); (d) heated for 31 min at 60 °C (0.06 % viability).

indicate that this early damage is repairable when the cells have been plated, while the more extensive damage occurring later is not. Recently, a similar situation was described in *E. coli* (Ahmad *et al.*, 1978).

The loss of acid-insoluble material during the heat treatment observed in *E. coli* (Sedgwick & Bridges, 1972; Woodcock & Grigg, 1972) was not seen here, suggesting that no degradation of DNA by exonucleases occurred in these streptococci.

The relationship between the strand scissions and cell death is indicated in Fig. 2, where a plot of the number of breaks against percentage loss of viability gives a straight line. It is interesting to note that EB/F/30/82 T $^-$, which is the slightly more heat-resistant strain, requires a larger number of breaks than 775 T $^-$ to produce the same level of kill.

In conclusion, these results show that the production of single strand DNA breaks by mild heat occurs not only in Gram-negative bacteria but also in Gram-positive bacteria. Moreover, a direct relationship exists between the number of breaks and loss of viability in these streptococci, suggesting that genetic damage may well be responsible for their inactivation at 60 °C. We are presently investigating whether repair of the breaks is necessary before the cells can recover from thermal injury.

Fig. 2. Relationship between the number of single strand breaks in the DNA of *Streptococcus faecalis* 775 T⁻ (a) and EB/F/30/82 T⁻ (b) and the loss of viability after heating at 60 °C.

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