Nutrient Requirements for the Recovery of Tellurite Resistance by Heat-injured *Streptococcus faecalis*

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Heat-injured *Streptococcus faecalis* recovered tellurite resistance in 4 to 5 h when incubated at 33 °C in a non-growth medium containing $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer pH 7.1, glucose and casein hydrolysate. Only 10 % of the damaged population recovered in the absence of K^+ , or glucose, or casein hydrolysate, or in the presence of valinomycin in medium containing 1 mM- K^+ . The K^+ content of cells increased two- to threefold on heating and a further three- to sixfold during the first 30 min of subsequent incubation. The Na^+ content decreased simultaneously but Mg^{2+} concentrations were not affected. Recovery was not affected by adding 1 mM-EDTA or 2 mM- Mg^{2+} to the medium but the addition of 1 mM-EDTA and 2 mM- Mg^{2+} together reduced recovery to < 20 %. This effect was reversed by adding 2 mM- Ca^{2+} .

INTRODUCTION

Payne & Morley (1976) reported that heat-injured *Streptococcus faecalis* recovered tellurite resistance in heart infusion broth before beginning to grow and divide. However, with media capable of supporting growth, it is not possible to distinguish between changes due to recovery of the damaged cells and those due to multiplication of cells that have already recovered.

Clark *et al.* (1968), investigating the recovery of salt tolerance by heat-injured *Strep. faecalis* R57, reported that recovery was lower in defined media than in trypticase soy broth. However, heat-injured cells of *Staphylococcus aureus* MF31 recovered salt tolerance when incubated in a medium containing glucose, phosphate buffer and a mixture of amino acids (Iandolo & Ordal, 1966). Using the strain s6 of *Staph. aureus*, Hurst *et al.* (1973) found that heat-injured cells recovered in a diluted, dialysed medium that did not support growth of unheated cells, and more recent work has shown that this organism can recover from heat-injury in a medium containing only phosphate buffer, amino acids and Mg^{2+} (Hurst *et al.*, 1975; Hughes & Hurst, 1976).

This paper reports an investigation of the nutrient requirements for the recovery of tellurite resistance by heat-injured *Strep. faecalis*. The cation content of unheated and heated cells, before and after recovery, has also been determined.

METHODS

Heat-injury and recovery procedure. *Streptococcus faecalis* var. *liquefaciens* EB/F/30/39 was grown and maintained as previously described (Payne & Morley, 1976). Unless stated otherwise, cells were heated for 4 min at 60 °C in heart infusion broth by diluting a suspension 50- to 100-fold into 50 ml of pre-heated medium to give about 1×10^8 viable units ml^{-1} (Payne & Morley, 1976). A portion (5 ml) of the heated suspension was harvested by membrane filtration (pore size 0.22 μm) and washed on the membrane with 2×10 ml of 100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ or $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer pH 7.1 before being resuspended in 50 ml of recovery medium and incubated at 33 °C on a rotary shaker. The standard recovery medium, that

Fig. 1

Fig. 2

Fig. 1. Recovery of tellurite resistance by heat-injured *Strep. faecalis* in standard recovery medium. Cells were heated in heart infusion broth for 4 min at 60 °C, harvested by membrane filtration, washed in 100 mM-KH₂PO₄/K₂HPO₄ buffer pH 7.1 and resuspended at about 1×10^7 viable units ml⁻¹ in (a) complete recovery medium (○), (b) recovery medium minus glucose (●) or (c) recovery medium minus casein hydrolysate (□). Cell suspensions were incubated at 33 °C with shaking and samples were removed at intervals for serial dilution and viable count on heart infusion agar with or without 0.04 % potassium tellurite. Recovery is the count in the presence of tellurite expressed as a percentage of the count in the absence of tellurite.

Fig. 2. Requirement for K⁺ during recovery of tellurite resistance by heat-injured *Strep. faecalis*. Cells were heated as in Fig. 1 and allowed to recover at 33 °C in 100 mM-NaH₂PO₄/Na₂HPO₄ buffer pH 7.1 containing 0.5 % glucose and 0.1 % casein hydrolysate with (○) or without (●) 1 mM-KCl. Recovery is expressed as in Fig. 1.

did not support growth, contained 100 mM-KH₂PO₄/K₂HPO₄ buffer pH 7.1, 0.5 % (w/v) glucose and 0.1 % (w/v) casein hydrolysate (Oxoid). The three components were autoclaved (121 °C for 15 min) separately and combined before use. Where required KCl, MgSO₄·7H₂O and EDTA were added as filter-sterilized solutions. Valinomycin (Sigma) was dissolved in ethanol and added to the recovery medium so that the final concentration of valinomycin was 5 µg ml⁻¹ and that of ethanol was < 1 % (v/v).

Viable counts. These were carried out as previously described (Payne & Morley, 1976).

Preparation of samples for cation determination. In experiments where cation concentrations were to be determined, cells were heated in 50 ml of heart infusion broth at about 1×10^8 viable units ml⁻¹, then harvested by centrifuging (12000 g, 5 min, 10 °C), washed in sterile glass-distilled water, centrifuged (25000 g, 12 min, 10 °C) and resuspended in 1 litre of recovery medium. Such treatments resulted in cell suspensions with a viable count on heart infusion agar of about 5×10^7 viable units ml⁻¹ and a total microscopic count of about 6×10^7 diplococci ml⁻¹ plus 2×10^6 chains of three or more cells. After incubation, cells were harvested by membrane filtration (pore size 0.22 µm) and resuspended in glass-distilled water. Unheated, heated and recovered cells were washed twice in glass-distilled water and finally resuspended in 10 ml of glass-distilled water and stored in plastic bottles at 4 °C. Duplicate dry wt determinations were carried out on 1 ml samples of these washed cell suspensions. Duplicate 3 ml samples were wet-ashed with 1 ml concentrated nitric acid, made up to 10 ml with glass-distilled water and stored in plastic bottles at 4 °C prior to atomic absorption spectroscopy. Samples of recovery medium were similarly treated.

Atomic absorption spectroscopy. Mg²⁺, K⁺ and Na⁺ in ashed samples were determined by atomic absorption spectroscopy using a Varian AA6 spectrophotometer at 285.2, 769.9 and 589.6 nm, respectively. Concentrations were determined from calibration curves obtained with standard solutions and results were corrected for blank digests of glass-distilled water.

Fig. 3

Fig. 4

Fig. 3. Effect of valinomycin and K^+ concentration on recovery of tellurite resistance by heat-injured *Strep. faecalis*. Cells were heated as in Fig. 1 and allowed to recover at 33 °C in NaH_2PO_4/K_2HPO_4 buffer pH 7.1 containing 0.5 % glucose, 0.1 % casein hydrolysate and 5 μg valinomycin ml^{-1} plus either 1 mM-KCl (●) or 100 mM-KCl (○). Recovery is expressed as in Fig. 1.

Fig. 4. Effect of EDTA and Mg^{2+} on recovery of tellurite resistance by heat-injured *Strep. faecalis*. Cells were heated as in Fig. 1 and allowed to recover at 33 °C in (a) 100 mM- KH_2PO_4/K_2HPO_4 buffer pH 7.1 containing 0.5 % glucose and 0.1 % casein hydrolysate (○), (b) medium (a) plus 1 mM-EDTA (●), (c) medium (a) plus 2 mM- $MgSO_4$ (■), (d) medium (a) plus 1 mM-EDTA and 2 mM- $MgSO_4$ (□) or (e) medium (a) plus 1 mM-EDTA, 2 mM- $MgSO_4$ and 2 mM- $CaCl_2$ (△). Recovery is expressed as in Fig. 1.

RESULTS

Effect of medium constituents on recovery of heated cells

When undamaged or heat-injured cells of *Strep. faecalis* were suspended in the standard recovery medium the colony count on heart infusion agar remained constant for at least 30 h. The colony count of heat-injured cells on heart infusion agar containing 0.04 % (w/v) potassium tellurite was about 0.01 % of the count on agar without tellurite on immediate plating but showed an increase within 4 to 5 h which represented recovery of tellurite resistance (Fig. 1). When either glucose or casein hydrolysate was omitted from the recovery medium, less than 10 % of the injured population recovered (Fig. 1). When the potassium phosphate buffer was replaced by sodium phosphate buffer, less than 10 % of the injured population recovered, but addition of 1 mM- K^+ to this medium restored recovery to the normal level (Fig. 2).

Effect of valinomycin on recovery of heated cells

Addition of 5 μg valinomycin ml^{-1} and 1 mM- K^+ to sodium phosphate recovery medium resulted in recovery of less than 10 % of the heat-injured cells (Fig. 3). However, normal recovery was restored when the K^+ concentration was increased 100-fold.

Cation content of unheated, heated and recovered cells

During heat-treatment, the K^+ content of the cells increased two- to three-fold while the Na^+ content decreased slightly (Table 1). After 30 min incubation of heat-injured cells in recovery medium, comparison of colony counts on heart infusion agar with and without tellurite indicated that there was very little recovery but the Na^+ content of the cells had decreased further and the K^+ content had increased a further three- to sixfold. Incubation

Table 1. K^+ , Na^+ and Mg^{2+} contents of unheated, heated and recovered cells of *Strep. faecalis*

Unheated cells, or cells heated for 4 min at 60 °C in heart infusion broth, or cells heat-injured and allowed to recover at 33 °C in 100 mM- KH_2PO_4 / K_2HPO_4 buffer pH 7.1 containing 0.5 % glucose and 0.1 % casein hydrolysate, or unheated cells incubated in similar medium, were washed twice in glass-distilled water. The K^+ , Na^+ and Mg^{2+} contents were determined by atomic absorption spectroscopy of duplicate samples of cells wet-ashed with nitric acid.

	Time in recovery medium (h)	Expt	Cation content [μg (mg dry wt) $^{-1}$]		
			K^+	Na^+	Mg^{2+}
Unheated cells	0	A	2.21	9.80	2.65
		B	3.79	11.53	2.62
		C	2.04	9.97	2.50
		D	3.33	11.13	2.53
		E	1.73	9.56	2.64
	0.5	A	26.55	4.60	1.96
	6.0	A	33.16	2.94	2.16
Heated cells	0	B	9.26	10.46	3.60
		C	7.63	6.88	3.25
		E	3.46	9.30	2.83
	0.5	B	31.52	5.80	2.98
		E	21.28	4.81	2.34
	6.0	C	20.35	5.22	2.32
		D	28.30	2.29	2.99

for up to 6 h resulted in no significant further change in the K^+ and Na^+ contents of the cells (Table 1). Unheated cells incubated under the same conditions showed similar changes in K^+ and Na^+ contents. The level of Mg^{2+} in the samples showed little change.

Effect of EDTA and Mg^{2+} on recovery

Heat-injured cells of *Strep. faecalis* may not require an exogenous source of Mg^{2+} in order to recover tellurite resistance since no marked changes in Mg^{2+} content were detected during either heat treatment or recovery. (Mg^{2+} was present in the recovery medium as an impurity at 3.3 μM .) Addition of 1 mM-EDTA or 2 mM extra Mg^{2+} had little effect on recovery although the Mg^{2+} did reduce the initial rate of recovery without affecting the final level (Fig. 4). However, addition of 1 mM-EDTA and 2 mM- Mg^{2+} together allowed less than 20 % of the injured population to recover. This effect could be reversed by adding 2 mM- Ca^{2+} (Fig. 4).

DISCUSSION

The results presented in this paper show that heat-injured cells of *Strep. faecalis* recovered tellurite resistance in a medium that did not support growth. Most previous studies on the recovery from heat-injury of non-sporing bacteria have been with cells incubated in media permitting growth or in the presence of penicillin to inhibit growth (for review, see Tomlins & Ordal, 1976).

In this study, a requirement for K^+ , glucose and a source of amino acids during recovery has been demonstrated. Payne & Morley (1976) reported that heat-injured cells failed to recover normally in the presence of chloramphenicol, suggesting that ability to synthesize protein was a prerequisite for recovery. The dependence of recovery on a supply of amino acids is consistent with this and such a requirement may partly explain the involvement of K^+ . In *Escherichia coli*, the process most sensitive to K^+ deprivation is protein synthesis

(Lubin & Ennis, 1964), while in *Strep. faecalis*, K^+ -depleted cells are incapable of incorporating [^{14}C]leucine into protein (Harold & Baarda, 1968). Glucose most likely provided the energy for the injured cells to accumulate K^+ and amino acids: the generation of a proton-motive force is required for the active transport of certain metabolites (for reviews, see Hamilton, 1975; Simoni & Postma, 1975) and the relationship between glycolysis, the membrane-bound ATPase and the generation of this potential in *Strep. faecalis* has been adequately demonstrated (Harold & Baarda, 1969; Harold *et al.*, 1970*a, b*; Harold & Papineau, 1972).

Valinomycin inhibits the growth of *Strep. faecalis* by interfering with the selective accumulation of K^+ (Harold & Baarda, 1967), this inhibition being overcome by adding excess K^+ . In the present study, valinomycin inhibited recovery but, again, addition of excess K^+ reversed the effect, indicating that during recovery the damaged cells need to accumulate K^+ . Glycolysing cells have been shown to accumulate K^+ (Zarlengo & Schultz, 1966; Harold & Baarda, 1967, 1968), and the present results agree with these earlier reports and show that, during recovery, heated cells require and accumulate K^+ , suggesting that at least some of the metabolic processes characteristic of undamaged, non-growing cells are essential for recovery of heat-injured cells. These results differ from those obtained with heated *Staph. aureus* s6 (Hurst *et al.*, 1973) in which K^+ and Na^+ are not required for recovery from thermal injury (Hughes & Hurst, 1976).

Although no Mg^{2+} was added to the recovery medium, the amount present as an impurity may have satisfied a similar Mg^{2+} requirement to that observed with *Staph. aureus* s6 (Hurst *et al.*, 1975; Hughes & Hurst, 1976). However, with *Staph. aureus*, a Mg^{2+} requirement could be demonstrated by adding 1 mM-EDTA, but no such requirement was observed with heat-injured *Strep. faecalis*. Even in the presence of 10 mM-EDTA with no added Mg^{2+} , recovery was only slightly affected (results not shown). The observation that added Mg^{2+} failed to stimulate recovery supports this conclusion and seems reasonable since the cells did not lose Mg^{2+} during heating. The effect on recovery of EDTA plus Mg^{2+} and its reversal by Ca^{2+} suggest an effect of EDTA- Mg chelate on heated cells since, in the presence of Ca^{2+} and Mg^{2+} , EDTA binds Ca^{2+} preferentially (see Martell & Calvin, 1953).

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