Effect of Starvation on Transport, Membrane Potential and Survival of *Staphylococcus epidermidis* under Anaerobic Conditions

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When washed suspensions of *Staphylococcus epidermidis* were starved under anaerobic conditions the viability declined to < 10% within 12 h. Although RNA was slightly degraded during this period the principal substrate for endogenous metabolism was protein and the intracellular amino acid pool. The adenylate energy charge and the ability to transport serine declined markedly within the first 6 h of starvation. With the majority of batches of organism investigated the membrane potential, as measured by the accumulation of Cs⁺ by valinomycin-treated organisms, also decreased significantly during this period. Addition of glucose or serine during starvation reversed these effects to varying extents provided that feeding took place during an early phase (2 h) of starvation. There was no apparent correlation between the magnitude of the membrane potential and viability.

INTRODUCTION

Extensive studies have been carried out on the survival characteristics of the obligate anaerobe *Peptococcus prevotii* (Montague & Dawes, 1974; Reece *et al.*, 1976) and it was concluded that death of the organism was due to depletion of its energy reserves. It was proposed that a critical consequence of energy depletion was the loss of serine transport which was suggested to be energized by a membrane-bound ATPase (Reece *et al.*, 1976). We have extended these studies to the facultative anaerobe *Staphylococcus epidermidis*, an organism which, when grown anaerobically, shows many similarities to *P. prevotii*, including the fermentation of serine. We have previously established that serine is taken up by *S. epidermidis* via a proton-symport system (Horan *et al.*, 1978*a*). This operates in response to a transmembrane proton gradient ($\Delta\bar{\mu}_{H^+}$) generated by a proton-translocating ATPase associated with the plasma membrane. The major component of this driving force under the experimental conditions previously investigated was shown to be the $\Delta\psi$ component; at an external pH of 6.8 no ΔpH component was detected (Horan *et al.*, 1978*a*).

The only previous work on the endogenous metabolism and survival of *S. epidermidis* is that of Strange (1976) who grew the organism aerobically and starved suspensions in aerated saline phosphate buffer. In the present study we have examined the survival characteristics and endogenous metabolism of anaerobically grown *S. epidermidis* starved under anaerobic conditions. Experiments in which the starved organism was fed glucose or serine and the subsequent changes in intracellular ATP and viability were measured demonstrated that the response to these compounds diminished as the starvation period was extended. An attempt has been made to relate this diminution in response to changes in the magnitude of the $\Delta\bar{\mu}_{H^+}$ during starvation, since substrate uptake during recovery from starvation may play a key role in determining viability. A preliminary report of these results has been presented (Horan *et al.*, 1978*b*).

METHODS

Growth of organism. *Staphylococcus epidermidis* was grown and maintained as described by Horan *et al.* (1978a).

Preparation of bacterial suspensions for starvation studies. Cultures (1 l) in the mid-exponential phase of growth were harvested at 37 °C with aseptic precautions under anaerobic conditions by centrifuging at 12000 *g* for 10 min. The organisms were washed once with sterile O₂-free 68 mM-K/NaH₂PO₄ buffer pH 6.8, and suspended in a suitable volume of the same buffer to give a suspension of about 1 mg dry wt organisms ml⁻¹. The temperature to which the organisms were exposed did not fall below 28 °C at any stage during the preparation of anaerobic suspensions. Such suspensions were held at 37 °C in sterile 1 l conical flasks which had facilities for gassing the suspensions and for the aseptic introduction and removal of samples by means of three glass tubes (4 mm diam.) inserted through a rubber bung in the flask neck. Anaerobiosis was achieved by gassing the vessel for 30 min with O₂-free N₂ prior to the introduction of the bacterial suspension.

Determination of viability. The slide culture technique of Postgate *et al.* (1961), as modified for use with anaerobic bacteria by Reece *et al.* (1976), was employed.

Radiochemical techniques. The membrane potential and rates of serine and 2-aminoisobutyrate uptake were measured using the techniques described by Horan *et al.* (1978a). The concentration of serine was 0.3 mM, 1 μ Ci μ mol⁻¹ (37 kBq μ mol⁻¹). For measurement of the $\Delta\psi$ component of the $\Delta\bar{\mu}_H^+$, the organisms, starved in 68 mM-sodium/potassium phosphate buffer, were washed and resuspended in 68 mM-sodium phosphate prior to addition of valinomycin and ¹³⁷CsCl.

Extraction of intracellular materials. Adenine nucleotides were extracted using H₂SO₄ as described by Montague & Dawes (1974). The acid-soluble intracellular pool was extracted with cold perchloric acid according to the method of Herbert *et al.* (1971). RNA was extracted from the bacterial pellet remaining after cold perchloric acid extraction by resuspension in 0.5 M-perchloric acid (4 ml) and heating in a water bath at 70 °C for 15 min with occasional shaking. The extract was then centrifuged at 12000 *g* and 30 °C for 10 min. The supernatant fluid was retained and the hot perchloric acid extraction was repeated with the bacterial pellet. The two supernatant fluids were then pooled and made up to 10 ml with 0.5 M-perchloric acid.

Analyses. ATP in bacterial extracts was measured by the firefly luminescence method using a high gain photomultiplier tube as described by Dawes & Large (1970). ADP and AMP in extracts were determined as ATP after enzymic conversion of each to ATP (Johnson *et al.*, 1970). The pentose content of the acid-soluble intracellular pool and of the suspending fluid was determined with the orcinol reagent of Herbert *et al.* (1971). A spectrum of the coloured complex obtained on reaction with the orcinol reagent, over the range 400 to 800 nm, indicated that the orcinol-positive material consisted of pentoses. The RNA content of the hot-acid extract was determined by the method of Herbert *et al.* (1971). Total amino acids in the suspending fluid or the intracellular pool were measured by the method of Yemm & Cocking (1955) using alanine as a standard. For quantitative analysis, samples containing about 150 nmol amino acid were concentrated to dryness by rotary evaporation under reduced pressure. The residue was taken up in 750 μ l water containing 36.5 nmol norleucine as an internal standard. A sample (500 μ l) of this mixture was applied to a Locarte Mark IV bench analyser. The values presented were derived from a comparison of peak areas with those of standards containing 25 nmol of each of 25 amino acids. The protein content of 1 ml portions of bacterial suspension was estimated by boiling with 1 M-NaOH (4 ml) in stoppered tubes for 10 min and total protein was then determined by the Lowry method. Bovine serum albumin was used as a standard.

RESULTS AND DISCUSSION

Substrates of endogenous metabolism

The products of endogenous metabolism in *S. epidermidis* under anaerobic conditions are acetate, formate, lactate, CO₂ and ammonia (Sivakanesan & Dawes, 1980). In an attempt to discover the endogenous substrates utilized by this organism, the intracellular concentrations of protein and of RNA were measured during starvation. RNA was the only component to change significantly, declining from 15.5 to 7.5% of the dry weight over 12 h starvation at 37 °C; the protein content decreased slightly from 19 to 16% of the dry weight over the same period. The extent of RNA degradation was lower than that observed for aerobic starvation by Strange (1976). There was a concomitant rise in *A*₂₆₀-absorbing material in the suspending fluid and, assuming an average molar absorption coefficient of 13.1×10^3 l mol⁻¹ cm⁻¹ for the bases of RNA, the value for *A*₂₆₀ accounted for the bases produced by RNA degradation. The remaining RNA breakdown product was ribose, detected as orcinol-positive material in

Table 1. *Effect of anaerobic starvation on the amino acid composition of the intracellular pool of S. epidermidis*

Staphylococcus epidermidis (3.2 mg dry wt organisms ml⁻¹) was starved and amino acids were analysed as described in Methods.

Amino acid	Intracellular amino acid concn [nmol (mg dry wt organisms) ⁻¹]				
	Time from start of harvesting (h):				
	0.75	2	5.75	7.25	24
Glutamate	40.0	6.9	2.6	2.1	0
Aspartate	33.6	31.5	10.2	5.1	0
Glycine + Cysteine	4.6	0.6	0.6	0	0
Alanine	4.3	1.2	0.9	0	0
Lysine	12.2	6.6	0.6	0.5	0
Threonine	7.5	0	0	0	0
Serine	7.0	0	0	0	0
Proline	4.7	0	0	0	0
Valine	1.7	0.4	0	0	0
Leucine	2.8	0	0	0	0
Isoleucine	8.0	2.7	0.5	0	0
Tyrosine	1.8	0	0	0	0
Phenylalanine	2.9	0	0	0	0
Histidine	3.5	0	0	0	0
Total	134.6	49.9	15.4	7.7	0
NH ₄ ⁺	29.0	29.3	43.0	44.0	44.2

Table 2. *Effect of anaerobic starvation on the release of amino acids and ammonia by S. epidermidis*

Staphylococcus epidermidis (3.2 mg dry wt organisms ml⁻¹) was starved and amino acids released into the supernatant fraction were analysed as described in Methods.

Amino acid	Amount released [nmol (mg dry wt organisms) ⁻¹]					
	Time from start of harvesting (h):					
	1.25	3	5	7	8.25	24
Glutamate	7.1	13.7	28.1	27.3	26.9	28.3
Aspartate	1.1	9.2	9.4	9.4	9.0	8.4
Alanine	6.1	7.9	16.8	19.1	16.4	16.8
Lysine	2.3	13.6	12.0	14.4	15.7	16.6
Threonine	0	0	3.0	3.5	6.0	6.0
Histidine	5.5	8.8	13.8	17.2	18.0	16.8
Total	22.1	53.2	83.1	90.9	92.0	92.9
Ammonia	11.0	17.0	22.0	26.0	28.0	38.0

the suspending buffer. Using conventional Warburg manometry it was found that the organism did not ferment ribose as judged by acid production and gas evolution, although ribose was rapidly accumulated by *S. epidermidis* (data not shown). In the first 12 h of starvation there was a release of orcinol-positive material into the suspending fluid and an increase of orcinol-positive material in the acid-soluble intracellular pool sufficient to account for the ribose released in RNA degradation. Thus, since the products of RNA degradation can be quantitatively recovered, this process apparently provides no endogenous substrates for *S. epidermidis* under anaerobic conditions.

During the first 5 h of starvation the intracellular amino acid pool declined rapidly from a value of about 8 µg (mg dry wt)⁻¹ and after 11 h was negligible, whereas the amino acid content of the suspending fluid steadily increased approaching values of 12 µg (mg dry wt)⁻¹ after

Fig. 1. Effect of anaerobic starvation on viability, the adenylate energy charge and rate of serine uptake in suspensions of *S. epidermidis*. Viability (○), adenylate energy charge (●) and the rate of serine uptake (□) were measured as described in Methods.

Table 3. *Effect of anaerobic starvation on adenine nucleotide concentrations in S. epidermidis*

Washed bacterial suspensions were starved under anaerobic conditions as described in Methods.

Time from start of harvesting (h)	Adenine nucleotide concn [nmol (mg dry wt organisms) ⁻¹]						Adenylate energy charge
	Intracellular			Extracellular			
	ATP	ADP	AMP	ATP	ADP	AMP	
1	14.4	5.9	2.5	0	0	0	0.76
2	5.2	5.0	8.7	0	0	0	0.41
3.3	3.8	4.4	11.1	0	0	1.8	0.31
5	2.0	3.5	18.4	0	0.1	3.2	0.16
6.5	0.8	2.5	13.4	0	1.0	6.7	0.12

10 h starvation. Quantitative analysis of the intracellular pool (Table 1) showed a rapid disappearance of amino acids, over 60% being lost within 2 h. Six of these appeared in the suspending fluid (Table 2), and the final extracellular amounts of alanine, lysine and histidine exceeded their initial intracellular values, confirming that some protein degradation occurred during the starvation period. As more glutamate, aspartate and serine disappeared from the intracellular pool than appeared in the extracellular pool it was probable that these were the principal amino acids used as endogenous substrates. Ammonia increased both in the intracellular pool and in the suspending fluid (Tables 1 and 2) and presumably was derived from deamination of amino acids since purine and pyrimidine degradation did not occur under these conditions. Strange (1976) did not determine ammonia release under aerobic conditions. These results are compatible with the products of endogenous metabolism as measured in Warburg studies (Sivakanesan & Dawes, 1980).

Viability and energy charge

The viability of buffered suspensions of *S. epidermidis* declined rapidly to a low value within a period of 12 h starvation (Fig. 1); the death rate was higher than under the aerobic conditions studied by Strange (1976). This loss of viability was reproducible with different batches of organism and was not affected by the bacterial density during starvation over the range 0.35 to 16 mg dry wt organisms ml⁻¹. Such reproducibility over a range of bacterial densities suggests that cryptic growth was not a major factor under these conditions. If cultures were harvested while in the late-exponential phase of growth the organisms had a high

Fig. 2

Fig. 3

Fig. 2. Effect of feeding with serine (a) or glucose (b) on the intracellular ATP concentration of anaerobically starved suspensions of *S. epidermidis*. Samples (20 ml) were removed from suspensions of the organism after different periods of starvation (○) and incubated with 10 mM-serine or 5 mM-glucose (●). Two independent batches of organism were used in the experiments illustrated. Intracellular ATP was measured as described in Methods.

Fig. 3. Effect of addition of glucose on the intracellular ATP concentration, membrane potential, serine transport and viability of anaerobically starved *S. epidermidis*. A suspension of the organism was divided and to one half was added 10 mM-glucose (●) at the time indicated by the arrow. The remaining suspension served as the control (○). (a) Viability, (b) serine transport, (c) intracellular ATP concentration and (d) membrane potential (expressed as a valinomycin-dependent Cs⁺ accumulation ratio) were measured as described in Methods.

ATP content, whereas if cultures were harvested as they entered the stationary phase then the decline in intracellular [ATP] and energy charge that occurred during starvation was already initiated. These differences in [ATP] had no effect on the loss of viability. The adenylate energy charge dropped rapidly during the initial period of starvation; in the experiment illustrated in Fig. 1 it fell from a value of 0.76 to 0.33 within 3 h of initiating starvation. This was largely due to a rapid decline in [ATP] with a concomitant rise in [AMP] (Table 3). After 4 h starvation the energy charge stabilized at a value between 0.1 and 0.2 due to a lowering of intracellular [AMP], the AMP appearing in the suspending fluid (Table 3).

Effect of glucose or serine during starvation

Feeding experiments were performed with glucose or serine to assess their effect on maintaining viability, the adenylate energy charge and the intracellular [ATP]. The ability to produce ATP from glucose or serine declined rapidly after 3 h starvation, and after 8 h the organism failed to produce ATP in response to such feeding (Fig. 2). The ability of glucose feeding to maintain the viability of *S. epidermidis* was also investigated. If glucose was added after 2 h starvation, viability was initially maintained and the intracellular [ATP] increased (Fig. 3). If

Fig. 4. Variation of the membrane potential and viability of anaerobically starved suspensions of *S. epidermidis*. The results of three independent experiments are illustrated. Viability (●, ■, ▲) and membrane potential (expressed as a valinomycin-dependent Cs^+ accumulation ratio) (○, □, △) were measured as described in Methods. Open and closed symbols refer to the same experiment.

feeding was delayed until 4 h after the start of starvation, no effect on viability was observed although there was an increase in the intracellular [ATP] (data not shown).

The diminished response to nutrient feeding observed in the early stages of starvation was not due to a decrease in the amount of enzymes of glucose or serine metabolism since extracts prepared from suspensions starved for 5 h demonstrated no marked decline in these activities (Sivakanesan, 1979; M. Midgley & E. A. Dawes, unpublished observations).

Effect of starvation on transport

Experiments were conducted to ascertain whether the diminished response to feeding was due to a decline in the transport of substrates into the organism. Thus, the uptake of serine and of 2-aminoisobutyrate, a non-metabolized amino acid previously shown to enter via the serine transport system in this organism (Horan *et al.*, 1978*a*), was measured in bacterial suspensions that had been starved (Fig. 1). Only the results obtained with serine uptake are presented but for both substrates there was a 50% loss in the initial rate of transport within 4 h of starvation; after 12 h, transport had fallen almost to zero in three experiments and to < 20% of the initial value in a fourth experiment. Thus the decline in ATP production on feeding serine could be explained by the diminished ability of the starved organism to transport serine.

The $\Delta\psi$ component of the $\Delta\bar{\mu}_{\text{H}^+}$, previously shown to be a major driving force for serine and 2-aminoisobutyrate transport, was also measured during starvation (Figs 3*d*, 4). We have been particularly concerned with the relationship between $\Delta\psi$ and viability since it might be expected that, for recovery from starvation and subsequent growth, the magnitude of the $\Delta\bar{\mu}_{\text{H}^+}$ would be a critical factor. However, in a series of ten experiments, varying patterns of behaviour of starved suspensions were observed. In all cases the loss in viability was reasonably reproducible but this was not matched by a similar response in the $\Delta\psi$ (Fig. 4). The $\Delta\psi$ is expressed in Figs 3 and 4 as a valinomycin-dependent Cs^+ accumulation ratio since the logarithmic relationship between the accumulation ratio and $\Delta\psi$ masks significant changes. The two extremes in response found are illustrated together with a more typical response shown in the majority of experiments (Fig. 4). The accumulation ratios measured at the earliest time in the experiments illustrated corresponded to 104, 89 and 65 mV (inside

negative). The generation of $\Delta\psi$ during starvation was stimulated when glucose was added (Fig. 3d) and the decline in viability was less rapid but survival was not prolonged beyond 12 h.

The death of starved *S. epidermidis* was accompanied by a diminished ability to transport serine and, presumably, other substrates when attempting to recover from starvation in the medium for viability measurements. Since the $\Delta\bar{\mu}_{H^+}$ probably provides a major driving force for the uptake of many nutrients (Hamilton, 1975), it is of interest that no clear-cut relationship between viability and the magnitude of this driving force has emerged from our studies on this organism. The fact that loss of transport activity and the fall in the energy charge somewhat precedes the fall in viability may indicate that these parameters have to fall to below certain threshold values before the organism is unable to recover from the stress induced by starvation.

In a majority of experiments (seven out of ten) there were also significant changes in the $\Delta\psi$ during the early phase of starvation. Contemporary studies on energy-coupling mechanisms for active transport in bacteria have been mainly concerned with assessing the relationship between the magnitude of the driving force and the accumulation ratio achieved by a transport system (Booth *et al.*, 1979) although kinetic effects have also been investigated (Mitchell *et al.*, 1979). Thus, the initial rate of glutamate transport into *Staphylococcus aureus* was inhibited in a pH-dependent manner when the $\Delta\psi$ component of $\Delta\bar{\mu}_{H^+}$ was experimentally lowered in value (Mitchell *et al.*, 1979). In organisms recovering from starvation, and metabolizing substrates as they enter, the rate of transport rather than the extent may well be more important for eventual recovery. In some cases, such as K^+ uptake, it is probable that the accumulation ratio achieved by the operation of the transport system is, however, of prime importance. This is borne out by the observation of Harold & Brunt (1977) using *Streptococcus faecalis*. Provided that the growth medium contained a high concentration of K^+ , and was rich in other nutrients, then the $\Delta\bar{\mu}_{H^+}$ could be abolished (by the addition of suitable ionophores) without hindering growth. The requirement for $\Delta\bar{\mu}_{H^+}$ was manifest when the concentration of amino acids or K^+ in the growth medium was deliberately lowered. Whether these observations can be extended to organisms recovering from starvation remains to be determined. When *S. epidermidis* was starved under anaerobic conditions the fall in the value of $\Delta\psi$ during the early period of starvation, observed in the majority of experiments, appears to be the reason for the diminished rate of substrate uptake (when subsequently exposed to substrate) and hence diminished ATP generation. The mechanism of glucose uptake in this organism is not yet characterized, but attempts to detect the involvement of a phosphotransferase system have so far been negative (N. J. Horan, unpublished observations).

REFERENCES

- BOOTH, I. R., MITCHELL, W. J. & HAMILTON, W. A. (1979). Quantitative analysis of proton-linked transport systems. The lactose permease of *Escherichia coli*. *Biochemical Journal* **182**, 687–696.
- DAWES, E. A. & LARGE, P. J. (1970). Effect of starvation on the viability and cellular constituents of *Zymomonas anaerobia* and *Zymomonas mobilis*. *Journal of General Microbiology* **60**, 31–42.
- HAMILTON, W. A. (1975). Energy coupling in microbial transport. *Advances in Microbial Physiology* **12**, 1–53.
- HAROLD, F. M. & BRUNT, J. V. (1977). Circulation of H^+ and K^+ across the plasma membrane is not obligatory for bacterial growth. *Science* **197**, 372–373.
- HERBERT, D., PHIPPS, P. J. & STRANGE, R. E. (1971). Chemical analysis of microbial cells. *Methods in Microbiology* **5B**, 209–344.
- HORAN, N. J., MIDGLEY, M. & DAWES, E. A. (1978a). Anaerobic transport of serine and 2-aminoisobutyric acid by *Staphylococcus epidermidis*. *Journal of General Microbiology* **109**, 119–126.
- HORAN, N. J., MIDGLEY, M. & DAWES, E. A. (1978b). Effect of starvation on transport, membrane potential and survival of *Staphylococcus epidermidis*. *Society for General Microbiology Quarterly* **6**, 34.
- JOHNSON, R. A., HARDMAN, J. G., BROADUS, A. E. & SUTHERLAND, E. W. (1970). Analysis of adenosine 3',5'-monophosphate with luciferase luminescence. *Analytical Biochemistry* **35**, 91–97.

- MITCHELL, W. J., BOOTH, I. R. & HAMILTON, W. A. (1979). Quantitative analysis of proton-linked transport systems. Glutamate transport in *Staphylococcus aureus*. *Biochemical Journal* **184**, 441–449.
- MONTAGUE, M. D. & DAWES, E. A. (1974). The survival of *Peptococcus prévotii* in relation to the adenylate energy charge. *Journal of General Microbiology* **80**, 291–299.
- POSTGATE, J. R., CRUMPTON, J. E. & HUNTER, J. R. (1961). The measurement of bacterial viabilities by slide culture. *Journal of General Microbiology* **24**, 15–24.
- REECE, P., TOTH, D. & DAWES, E. A. (1976). Fermentation of purines and their effect on the adenylate energy charge and viability of starved *Peptococcus prévotii*. *Journal of General Microbiology* **97**, 63–71.
- SIVAKANESAN, R. (1979). Ph.D. thesis, University of Hull, U.K.
- SIVAKANESAN, R. & DAWES, E. A. (1980). Anaerobic glucose and serine metabolism in *Staphylococcus epidermidis*. *Journal of General Microbiology* **118**, 143–157.
- STRANGE, R. E. (1976). *Microbial Response to Mild Stress*, pp. 24–25. Shildon: Meadowfield Press.
- YEMM, E. W. & COCKING, E. C. (1955). The determination of amino-acids with ninhydrin. *Analyst* **80**, 209–213.