Stabilization of Glucose-starved *Escherichia coli* K12 and *Salmonella typhimurium* LT2 by Peptidase-deficient Mutants

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Escherichia coli K12 and *Salmonella typhimurium* LT2 cells were stabilized during carbon starvation in the presence of peptidase-deficient mutant strains. The rate of loss of viability of the wild-type *S. typhimurium* strain was decreased an average of 2-fold, and the rate for the wild-type *E. coli* strain was decreased about 2·3-fold, when either was starved in the presence of the multiply peptidase-deficient *S. typhimurium* strain TN852; other peptidase-deficient strains exhibited similar stabilizing effects. Starving wild-type *S. typhimurium* LT2 cells utilized peptides excreted by the starving peptidase-deficient cells for protein synthesis, and, to a lesser extent, as respiratory substrates. Provision of free amino acids in steady-state levels to starving *E. coli* K12 cells in a cell recycle apparatus had a stabilizing effect similar to that of mixing with peptidase-deficient cells.

INTRODUCTION

We have been interested in characterizing the survival strategies used by bacterial populations subjected to starvation (Matin *et al.*, 1979; Reeve *et al.*, 1984*a,b*; Zychlinsky & Matin, 1983). Bacteria frequently undergo periods of starvation in their natural environment, making such studies important in the understanding of bacterial ecophysiology. Further, in recent years the value of separating the growth and production phases of bacteria used in industrial fermentation processes has gained increasing recognition. Such a separation allows a more efficient conversion of raw material to product, reducing the amount of metabolic energy expended for growth-related processes. The use of non-dividing cells also obviates the problem of reversion of genetically engineered organisms. Finally, in immobilized cell bioreactors, the limited containment volume makes it necessary to utilize stable non-dividing microbial cells (Inloes, 1982; Inloes *et al.*, 1983).

We have been investigating the role of protein metabolism in the survival of bacteria starved for carbon. Peptidase-deficient mutants of *Escherichia coli* and *Salmonella typhimurium*, which possessed decreased protein-degradation activity, survived starvation poorly (Reeve *et al.*, 1984*a*). Inhibition of the synthesis of functional proteins in wild-type *E. coli* through the use of antibiotics or amino acid analogues, especially in the early phases of starvation, also greatly diminished starvation survival (Reeve *et al.*, 1984*b*). Direct analysis involving two-dimensional gel electrophoresis showed that the onset of carbon starvation led to the induction of about 30 proteins, many of which were absent from the growing bacteria (Groat & Matin, 1986). These results have led us to postulate that bacteria subjected to carbon starvation utilize their amino acid pools, obtained by the degradation of pre-existing proteins, to synthesize a unique set of proteins – the ‘starvation proteins’ – which enhance their resistance to this stress.

We demonstrate here that the stability of glucose-starved wild-type *E. coli* and *S. typhimurium* can be enhanced by starving them in the presence of peptidase-deficient mutants. This effect appears to be due to the provision of amino acids to the starving wild-type organisms in the form

of peptides excreted by the mutants. These amino acids enhance the ability of the wild-type organisms to synthesize protein during starvation.

METHODS

Bacterial strains and growth conditions. *E. coli* K12 and *S. typhimurium* strain LT2 and its derivatives TN852, TN858, TN860 and TN861, together with the compositions of the minimal salts bases used (M9 and 0 salts) have all been described previously (Reeve *et al.*, 1984a). The M9 and 0 salts were supplemented with 0.4% (w/v) glucose as the carbon source; additionally, 0.3 mM-L-leucine was added to media in which *S. typhimurium* mutants were cultivated. Cultures were grown as before (Reeve *et al.*, 1984a).

Carbon starvation. Mid-exponential phase cells were harvested and subjected to carbon starvation as described previously (Reeve *et al.*, 1984a); unless otherwise noted, the starvation medium was 0 salts basal medium minus glucose plus 0.3 mM-leucine. Also unless otherwise noted, mixed culture experiments were initiated by mixing equal volumes of the appropriate strains at the same cell density; a sample of each strain was starved separately as a control.

Viability of cultures was determined by spreading serial dilutions on minimal salts medium agar that contained 0.02% (w/v) glucose as described previously (Reeve *et al.*, 1984a). Because the peptidase-deficient strains had a leucine auxotrophy but the wild-type strains did not, the viable cells of the two strains could be distinguished by plating the culture on minimal salts medium with and without 0.3 mM-leucine.

Determination of peptide cross-feeding. *S. typhimurium* LT2 and TN852 were grown for two generations. One-half of the TN852 culture was labelled during the second generation by adding $0.4 \mu\text{Ci ml}^{-1}$ of a ^{14}C -labelled amino acid mixture (50 mCi mmol^{-1} ; $14.8 \text{ MBq mmol}^{-1}$; ICN). The three cultures (TN852, ^{14}C -labelled TN852 and LT2) were then harvested, washed twice and resuspended in starvation medium. The cultures were starved for 2 h, during which time considerable radioactivity was excreted by the labelled TN852 culture. The three starved cultures were harvested and the unlabelled LT2 and TN852 cells were suspended in the labelled supernatant obtained from the labelled TN852 culture. The cultures were dispensed into serum vials fitted with cap and bucket assemblies, and incorporation of radioactivity into cellular protein and respiratory CO_2 was determined as described previously (Reeve *et al.*, 1984b).

RESULTS

Stabilization of glucose-starved S. typhimurium LT2 and E. coli by TN852

S. typhimurium LT2 was starved for glucose in the presence or absence of TN852, which lacks four peptidase activities, and its viability was monitored. The wild-type bacteria lost viability much less rapidly when starved in a TN852:LT2 cell ratio of 1:1 than when they were starved alone (Fig. 1). In five separate experiments, the mean half-life of the LT2 culture was 2.9 ± 0.3 d, while starvation in the presence of the mutant TN852 increased this half-life to 5.7 ± 0.5 d, an average 2-fold increase in half-life. When the TN852:LT2 ratio was increased to 9:1 in one experiment, the half-life of the wild-type bacteria was increased to 12 d (data not shown). Mixing LT2 cells with other peptidase-deficient mutants (TN858, TN860 or TN861) produced similar results, i.e. the glucose-starved LT2 organisms were greatly stabilized by the presence of the individual mutant (data not shown). For unknown reasons, TN852 cells lost viability more rapidly during starvation in mixed culture than in pure culture (Fig. 1). As a consequence, the initially mixed starving culture became essentially a pure culture of LT2 within about 30 h (Fig. 1). Yet, LT2 continued to show a greater half-life in such cultures for at least another 70 h (Fig. 1). Thus, the benefit derived by the wild-type from the presence of the mutant was a long-term one, and transcended the elimination of the viable cells of the latter.

To test whether the stabilizing effect was limited to *S. typhimurium* LT2 cells, we repeated the mixing experiments using *E. coli* as the wild-type strain. The results were similar to those with *S. typhimurium*: the presence of TN852 cells in the starvation medium significantly enhanced the stability of the wild-type *E. coli* during carbon starvation (Fig. 2). In two experiments, the half-life of the wild-type culture was increased 2.3- and 2.4-fold by mixing in a 1:1 ratio with TN852 cells.

Cross-feeding of the wild-type by the mutants

We have presented evidence that starving bacteria need to synthesize new proteins to enhance their resistance to this stress (Reeve *et al.*, 1984a, b). Further, it has been shown (Reeve *et al.*,

Fig. 1

Fig. 2

Fig. 1. Viability of *S. typhimurium* LT2 (○, ●) and TN852 (△, ▲) starved in pure (filled symbols) or mixed (1:1 cell ratio) cultures (open symbols). The two strains were grown to mid-exponential phase, harvested, washed and resuspended in growth medium minus glucose. Viability during starvation was determined by plating on 0 salts medium plus or minus leucine.

Fig. 2. Viability of *E. coli* K12 starved singly (●), in the presence of equal numbers of *S. typhimurium* TN852 cells (○), or in the presence of the 20 amino acids added at a rate of $0.6 \mu\text{g}$ of each amino acid $(\text{ml culture})^{-1} \text{h}^{-1}$ (△). The last experiment was done with a cell recycle apparatus as discussed in the text.

1984a; Yen *et al.*, 1980) that peptidase-deficient mutants of *S. typhimurium* excrete peptides during starvation. Possibly, the presence of the mutants stabilized the wild-type organisms by the provision of peptides which the latter took up, degraded to amino acids, and utilized in new protein synthesis during starvation. To test this possibility a TN852 culture whose protein had been labelled during growth with ^{14}C -labelled amino acids was starved for 2 h. In the light of previous data (Reeve *et al.*, 1984a), the mutant could be expected to excrete [^{14}C]peptides into the starvation medium during this time, and indeed considerable radioactivity ($12000 \text{ d.p.m. ml}^{-1}$) was found in the supernatant. Unlabelled *S. typhimurium* LT2 and TN852 cells, which had also been starved for 2 h, were each resuspended in the supernatant from the labelled TN852 culture, and incorporation of radioactivity from the supernatant into protein and respiratory CO_2 was measured as described in Methods. As expected, TN852 did not assimilate the radioactivity or convert it to CO_2 . However, LT2 incorporated about 17% of the label into protein during a 30 min incubation, and at the same time about 8% of the label appeared as CO_2 . These results are consistent with the supposition that the presence of the mutant promoted protein synthesis in the starving wild-type organisms.

If stabilization of the wild-type in mixed culture is indeed due to the extra provision of amino acids from peptides excreted by the mutant, then starvation of the wild-type in an amino acid mixture ought to have a similar stabilizing effect to that produced by the presence of the mutant. To simulate constant provision of amino acids during starvation, as probably occurs in the presence of the mutant, a cell recycle apparatus, described previously (Reeve *et al.*, 1984b), was used. Starvation medium (M9 salts minus glucose) was continuously removed from the culture suspension by filtration at the rate of about one culture volume every 100 min, and replaced with fresh starvation medium. The fresh medium was supplemented with the 20 amino acids at $0.95 \mu\text{g ml}^{-1}$ each for the first 28 h of starvation, thus supplying the culture (250 ml) with $0.6 \mu\text{g}$ of each amino acid $\text{h}^{-1} (\text{ml culture})^{-1}$ during this period. The concentration at which the amino acids were used was chosen to match the amount of extra amino acids expected to be available to

the wild-type in mixed culture based upon the known capacity of TN852 for protein degradation, i.e. about $1\% \text{ h}^{-1}$ (Yen *et al.*, 1980; unpublished results). Similarly, the duration of the supplementation was intended to simulate the mixed culture with respect to the length of time for which the mutant remained significantly viable and served as a source of peptides (Fig. 1; see Discussion). The direct provision of amino acids during starvation stabilized the wild-type *E. coli* to the same extent as the presence of the mutant (Fig. 2). It is noteworthy that previous results (Reeve *et al.*, 1984*b*) have established that the carbon starvation survival pattern of *E. coli* under the cell recycle conditions closely resembles that found in shake flasks, i.e. that cell recycling itself has no effect on the survival pattern.

DISCUSSION

It is known that peptidase-deficient mutants excrete peptides during starvation (Reeve *et al.*, 1984*a*; Yen *et al.*, 1980), and that wild-type bacteria can make use of exogenous peptides (Payne, 1980). The results of the labelling experiments presented in this study showed that peptides excreted by *S. typhimurium* TN852 cells during starvation were utilized by the starving wild-type, presumably after being degraded to amino acids. Thus, it is logical to conclude that the enhanced resistance to starvation stress exhibited by the wild-type cells in the presence of the peptidase-deficient mutants was due to the availability of extra amino acids during starvation in mixed cultures. This conclusion is strengthened by the finding that direct provision of amino acids to starving wild-type cells also enhanced their survival.

The extra amino acids made available to the wild-type could have enhanced survival solely by serving as a source of energy, and the data indeed show that the amino acids were respired. However, it is likely that their utilization in protein synthesis also played a crucial role. The data demonstrate that in strain mixing experiments *S. typhimurium* LT2 continued to show improved survival for up to 3 d after the elimination of TN852 from the culture (Fig. 1). It is unlikely that extra peptides continue to remain available to LT2 in mixed culture after the elimination of TN852, given the high rate of peptide utilization by starving LT2 and the fact that excretion of peptides by peptidase mutants is confined mainly to the first 24 h of starvation (Reeve *et al.*, 1984*a*; unpublished results). Thus, the continued enhanced survival of LT2 in mixed culture after the elimination of viable TN852 was evidently due to peptide utilization by LT2 in about the first 30 h of starvation. If the role of amino acids had been confined solely to provision of energy, such a long-term effect would be unlikely. Rather, it appears that the provision of extra amino acids in early starvation facilitated synthesis of protein required in long-term survival. Our other published and unpublished data are consistent with this interpretation. We have found that protein synthesis in bacteria starved for carbon is limited by the availability of amino acids, and that new protein synthesis during the first few hours after the onset of starvation is necessary for improved half-life later in starvation (Reeve *et al.*, 1984*a, b*). Also, we have recently isolated a mutant deficient in a gene that is expressed at the onset of carbon starvation; this mutant survives such starvation poorly (E. Zychlinsky, A. Bockman & A. Martin, unpublished).

One objective of our work on starving bacteria has been to define conditions conducive to their stability. The method reported here can conceivably have application in enhancing the longevity and productivity of non-growing cells in the production phase of immobilized cell bioreactors (Inloes, 1982; Inloes *et al.*, 1983).

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