# THE INVOLVEMENT OF PROTEASES IN THE MORPHOGENESIS OF *ROD* MUTANTS OF *BACILLUS SUBTILIS*

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## 1. Introduction

Morphological mutants of a variety of species of bacteria are known that exist either as cocci or rods according to the conditions of growth (reviewed [1]). There are a number of distinct genetic groups of such mutants of *Bacillus subtilis* [2] with different physiological and biochemical phenotypes [3–6]. One type, *rodA*, grows as rods at 30°C and is distinguished by derepression of peptidoglycan synthesis, inhibition of teichoic acid synthesis and loss of autolysins when grown at 45°C as cocci. Another, *rodB*, shows none of these characteristic changes but unlike *rodA* strains is affected in its morphology and growth rate by the concentration in the growth medium of $Mg^{2+}$ and certain anions such as the halides, as well as by temperature [6]. For example it changes from rods to cocci if $Br^-$ is removed from media, or if the growth temperature is raised from 20°C to 35°C in the absence of halides. Here we show that the change in morphology of both mutants is grossly affected by protease inhibitors which also inhibit an alteration in both mutants of the molecular weight of membrane proteins.

## 2. Materials and methods

### 2.1. *Micro-organisms*

The *rod* mutants of *B. subtilis* studied were 200B (*rodA*) and 104 (*rodB*), both were in the genetic background of strain 172 which is referred to as the wild-type. The strains were kept as freeze-dried cultures for long-term storage. Over shorter periods of a few months the strains were subcultured on LA agar at weakly intervals.

### 2.2. *Media and growth conditions*

The experimental medium was the TRM buffer–salts–glucose solution in [5] suitably supplemented by amino acids to satisfy the auxotrophic requirements of the strains, $MgSO_4$, and halides as required. It was dispensed into side-arm flasks of 5 times the volume of the fluid medium. The initial inoculum was taken from agar plate cultures into 5 ml of either CHY [7] or TRM [5] medium. These cultures were incubated for 5–6 h at 35°C, a series of dilutions of them was then prepared and inoculated into TRM medium which according to the experiment was either used as such or supplemented by either 15 mM KCl or 15 mM KBr. The following morning the cultures that had reached the early stage of exponential growth were diluted with the same medium to give an $A_{430}$ of 0.08. The organisms in these cultures had thus passed through $\geq$10 generations of growth before the conditions were changed so that the opposite morphology would be expected. All cultures were shaken to provide aeration.

### 2.3. *Measurement of bacterial diameter*

A wire loopful of the culture was placed on a microscope slide and allowed to dry at room temperature. A drop of 0.01% gentian violet was then added and a cover-slip pressed down on it. Measurements of the smaller axis of the cells were made with a split-image eye-piece attached to a phase contrast microscope giving an overall magnification of 900. One eye-piece unit was equivalent to 0.0227 μm. The diameters of 20 randomly selected bacteria were measured, the coefficients of variation for the groups was ~10%. Where differences between averages were $<$2 or 3 standard deviations larger populations of $\leq$300 bacteria were measured and the significance

of the differences between averages subjected to Student's *t*-test. No difference was accepted as significant unless the probability of identity was <0.1%. The method was checked in a single experiment in which electron microscope pictures of negatively-stained whole bacteria were measured. Two groups of 60–70 cells were compared. The coccal form has about twice the diameter of the rod-shaped form.

2.4. *Preparation and examination of membranes*

Cultures (100 ml) were cooled to 4°C and subjected to shear for 45 s in an atomiser (MSE) to remove flagella. They were then centrifuged and the bacteria lysed by treatment for 20 min at 37°C with egg-white lysozyme (250 μg/ml) while suspended in 25 mM Tris–HCl (pH 7.2) containing 10 mM $MgSO_4$ and 5 μg DNase/ml. The membranes were deposited by centrifuging and washed once with 25 mM Tris–HCl (pH 7.2). They were finally resuspended in the same buffer and a sample taken for assay of protein. The solutions were adjusted to contain the same amount of membrane (equiv. 3 mg protein/ml) along with 0.002% bromophenol blue, 1% (w/v) sodium dodecyl sulphate, 10% (w/v) glycerol and 1 mM dithiothreitol. The suspension was heated at 100°C for 1 min and the preparation examined by slab polyacrylamide gel electrophoresis. The gels were adjusted to give 13% cross-linkage and post-stained with Coomassie blue. Markers of bovine serum albumin, ovalbumin and trypsin inhibitor were always run alongside the samples.

2.5. *Protease inhibitors*

The following were used: L-1-tosylamide-2-phenylethyl chloromethyl ketone HCl (TPCK); *N*-α-*p*-tosyl-L-lysine chloromethyl ketone HCl (TLCK); phenyl-methyl-sulphonyl fluoride (PMSF); diphenylcarbamyl chloride (DPCC); hydrocinnamic acid (HC); 6 amino-hexanoic acid; antipain; pepstatin; and trypsin inibitor. Of these TPCK, PMSF, DPCC and HC all affected the change in morphology of one or both mutants at low concentrations. The remainder were without affect even at high concentrations (20–200 μg/ml). All the inhibitors were to varying degrees growth inhibitory for the mutant strains irrespective of their effects on morphological change. They were very much more toxic to the wild-type strain.

## 3. Results and discussion

TPCK is a chymotrypsin inhibitor attacking a histidine residue in the active centre of the enzyme [8]. Fig.1 shows its effect upon the change in diameter of mutant 200B when the growth temperature is altered from 45°C to 25°C. When the morphology was changed in the reverse direction, from rods to the fatter cocci, TPCK (14.2 μm) had no similar effect on the rate or extent of the change (fig.2). TLCK a trypsin inhibitor [8] had no effect at 54 μM on the morphological changes of strain 200B in either direction. TPCK had only a small but statistically significant affect on mutant 104. PMSF (5 mM), on the other hand acting on 104 had the effect shown in fig.3 on the reduction in diameter of the cocci changing to rods. Similar results were obtained irrespective of whether the shape change was effected by the addition of 15 mM KBr to the growth medium maintained at a constant temperature of 37°C or by changing the temperature from 37°–20°C in the absence of the halide.

Transverse sections of mutant 104 incubated with 15 mM PMSF under the former conditions for two

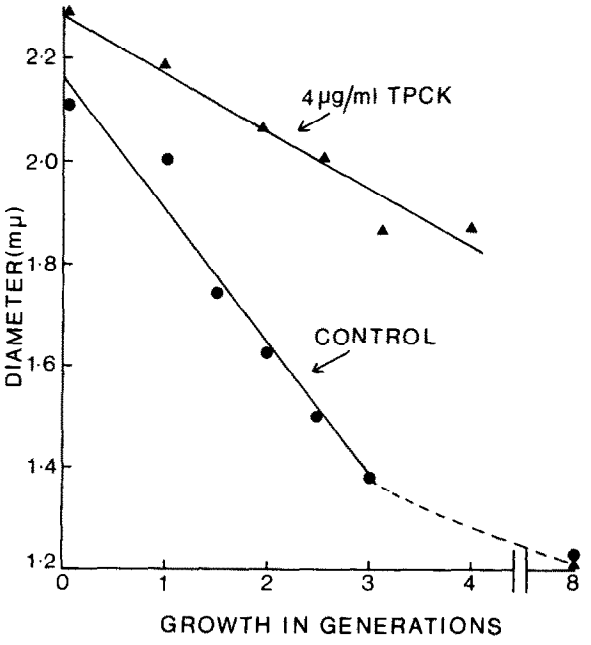


Fig.1. The effect of TPCK upon the reduction in the diameter (shorter axis) of the cells of mutant 200B (*rodA*) that takes place when the culture is transferred from a growth temperature of 45°C to one of 25°C.

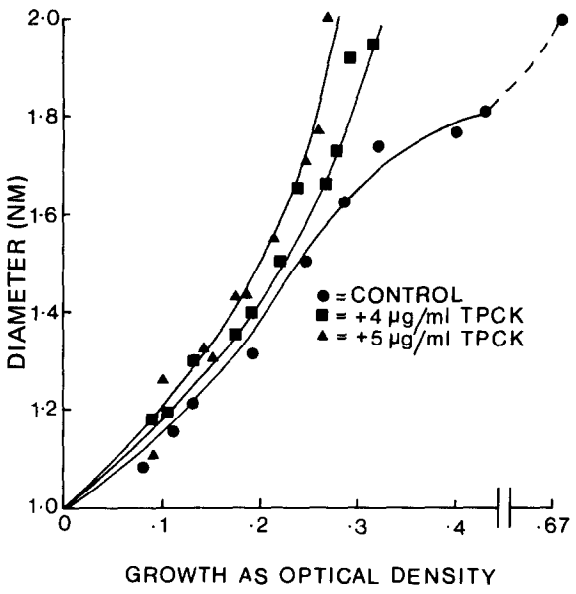


Fig.2. The lack of effect of TPCK on the increase in diameter (short axis) of cells of 200B (*rodA*) that takes place when cultures are transferred from a growth temperature of 25°C to one of 42°C.

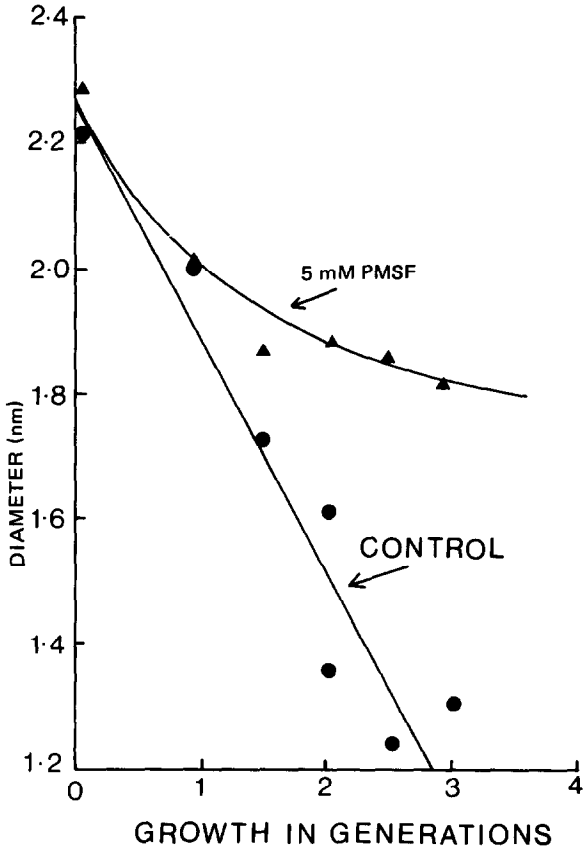


Fig.3. The effect of PMSF on the reduction in diameter (short axis) of the cells of 104 (*rodB*) when 15 mM sodium bromide is added to cultures in the TRM basal medium. Incubation was at 37°C.

generations showed little change from the original cocci when examined by electron microscopy. In the absence of the inhibitor the mutant has assumed a rod-shaped morphology similar to that of the wild-type. PMSF had very little effect on the morphological change of 200B. Thus whereas TPCK greatly inhibits aspects of the coccus to rod change in a *rodA* mutant but not a *rodB* one, PMSF affects the *rodB* mutant but not *rodA*. TPCK was too toxic to the wild-type strain at 5.7 μM to test its affect on morphology, PMSF alone had no effect, but a combination of PMSF and DPCC slightly but significantly increased its diameter.

The membrane proteins of the rod and coccal forms of both mutant strains showed a characteristic difference. The cocci had a protein of $M_r$ ~33 000 which in the rod forms was replaced by one with $M_r$ ~30 000. No similar changes occurred in the membrane proteins of the wild-type when grown under the same conditions. Addition of 3 μg TPCK/ml to cultures of 200B or of 5 mM PMSF to those of 104 under conditions giving a coccal to rod change in morphology by the control cultures led to considerable inhibition of this change in molecular weight. For example, after two generations of growth by 200B, the 33 000 $M_r$ band had almost disappeared whereas in the presence of TPCK the 30 000 and 33 000 $M_r$ bands stained about equally. There was a similar but less distinct change with proteins of $M_r$ ~80 000. This change also appeared to be inhibited by TPCK.

It has been suggested that proteases are involved in the export of enzymes such as penicillinase [9] α-amylase [10] and lipase [11] by bacilli and in the passage of alkaline phosphatase to the outer membrane of *Escherichia coli* [12]. Protease inhibitors including TLCK inhibit the differentiation of *Dictyostelium discoideum* [13]. This paper shows their involvement in the coccus to rod morphogenesis of *rod* mutants of bacilli which may suggest that enzyme processing is important for the growth and division of bacteria.

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