Mesosomes in *Bacillus cereus* 569 and the Production of Extra Membranes by Treatment with Actinomycin-D

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SUMMARY

Electron microscopy of thin sections of *Bacillus cereus* 569 fixed under conditions that demonstrate *Bacillus licheniformis* 749c to have only one mesosome per bacterium has shown the former to possess consistently more than one. Treatment of outgrowing *B. cereus* 569 spores with actinomycin-D results in the appearance of extra membrane-like material lying between the plasma membrane and the new wall. Actinomycin-D causes almost immediate cessation of exponential growth in *B. cereus* 569 but extra membrane appears after a lag of approximately half a generation time. Autoradiography of thin sections of exponentially growing bacteria treated with actinomycin-D and labelled during inhibition with tritiated glycerol shows that the grain count is increased over areas of sections where extra membrane is present. Analysis of the lipids extracted shows that some of the labelled glycerol is incorporated during inhibition into material similar to or identical with the normal phospholipids. Virtually no incorporation of labelled amino acid occurred in trichloroacetic acid precipitates during actinomycin-D inhibition. These observations, which suggest that synthesis of new membrane occurs during actinomycin-D inhibition, are discussed in relation to a possible origin from mesosomes.

INTRODUCTION

The existence of intracytoplasmic membrane inclusions (mesosomes) has been confirmed in a large number of Gram-positive organisms (Reusch & Burger, 1973). There is no clear evidence for a common function for these organelles; however, there is circumstantial evidence for their involvement in a number of processes, for example, respiration (Ferrandes, Chaix & Ryter, 1966), nuclear division or synthesis (Ryter & Jacob, 1964; Higgins & Daneo-Moore, 1972) and septation (Imaeda & Ogura, 1963). In part, the lack of firm agreement on any function or set of functions is due to the considerable differences in their structure and number per organism reported from different laboratories. Thus, Highton (1969; 1970*a, b*) showed that *Bacillus licheniformis* 749c and *B. subtilis* 172 contained a single lamellar mesosome. A model for mesosome behaviour in *B. licheniformis* 749c was presented, and it was subsequently shown (Garland & Highton, 1973) that there was only a single lamellar mesosome very early in spore outgrowth. Ryter (1969) obtained different results for *B. subtilis* 168 Ind⁻ where there were several vesicular mesosomes per bacterium. The influence of fixation technique on mesosome structure is well recognized (Ryter, 1969; Burdett & Rogers, 1970), and Highton (1969) demonstrated that breakdown into vesicles, eversion and redistribution of mesosomes occurred in *B. licheniformis* 749c

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if the bacteria were left to stand before fixation. Such observations suggest that variations in mesosome number and structure may represent fixation artifacts, the most likely *in vivo* condition for Gram-positive rods being the presence of a single lamellar mesosome. This paper describes mesosome structure and synthesis in *B. cereus* 569 during exponential growth and spore germination.

METHODS

Organism. *Bacillus cereus* 569 spores were prepared from 12 l cultures in S broth (peptone 10 %, Lab-Lemco (Oxoid) 0.24 %, NaCl 0.2 %) aerated 36 h at 37 °C in a laboratory fermenter ('Micro-ferm', New Brunswick Scientific Co.). After centrifugation and washing in distilled water the spores were purified by centrifugation in 60 % sucrose at 1800 g for 15 min, followed by repeated washing and final suspension in distilled water.

Germination and outgrowth studies. High concentrations of spores (approx. 10^8 /ml) were germinated in 0.01 M-sodium phosphate buffer (pH 7.0) containing 0.01 M-L-alanine and 0.01 M-inosine, and subsequently transferred after centrifugation to L broth (tryptone 1 %, NaCl 1 %, yeast extract 0.5 %) for outgrowth studies. All stages were followed by phase microscopy and assessed on plate counts made after pasteurization at 60 °C for 15 min.

Exponential cultures. Cultures in the exponential phase were derived by inoculating fresh medium with a primary exponential culture. Growth was followed by extinction measurements at 620 nm in 1 cm path-length cells and all cultures were aerated with shaking at 37 °C in flasks with a volume 5 times that of the culture. Media used were L broth, L broth supplemented with 0.05 M-sodium phosphate and 0.002 M-magnesium chloride, and the medium of Pollock (1963) containing trisodium citrate 0.59 %, ammonium sulphate 0.2 %, casein hydrolysate 1 %, glucose 0.2 %, and gelatin 0.1 % in 0.05 M-sodium phosphate and 0.002 M-magnesium chloride.

Electron microscopy. All specimens were prefixed and fixed in osmium-cyanide fixative and embedded in Araldite after uranyl acetate staining and acetone dehydration as described by Highton (1969). Sections cut on a Huxley microtome with a diamond knife were post-stained with lead citrate (*Techniques for Electron Microscopy*, 1965) and viewed on a Siemens Elmiskop 1A operated at 80 kV using plate magnifications of 20000.

Autoradiography of [^3H]glycerol-labelled bacteria. Actinomycin-D was added to a 100 ml exponential culture in L broth which was rapidly transferred after 90 s to a fresh flask containing sufficient [^3H]glycerol to produce a final concentration of 50 $\mu\text{Ci/ml}$ of culture and incubated for 30 min. The culture was immediately prefixed and fixed overnight. It was then washed three times in Michaelis buffer (Ryter & Kellenberger, 1958) to remove native isotope, dehydrated, embedded in Araldite and sectioned as described above. Silver-gold sections were picked up on collodion-coated grids, post-stained with lead citrate and lightly coated with carbon. A film of Ilford L4 emulsion was applied by loop and the grids allowed to develop for 6 to 8 weeks in desiccated light-tight boxes. The autoradiographs were processed in hydroquinone developer and fixed in freshly made thiosulphate fixative. Processed grids were viewed as above.

Graphical methods. Sections were selected for axial cut as judged by clarity of the plasma membrane and traced for measurements. Fifty sections was considered a representative sample.

Uptake of labelled glycerol. For total uptake studies, 100 to 25 ml cultures in L broth and supplemented L broth were inoculated with actinomycin-D. Two minutes after this addition, [^3H]glycerol was added to a final activity of 1 to 5 $\mu\text{Ci/ml}$ and the incubation

continued for 30 min. The cultures were then immediately cooled and 2 % (v/v) of 20 % 'cold' glycerol added to prevent further incorporation. After three washes with 0.05 M-sodium phosphate buffer containing 0.01 M-magnesium chloride, the bacteria were extracted overnight at room temperature with 50 ml chloroform-methanol 2:1. After evaporation and resuspension in 1 to 2 ml chloroform-methanol 2:1, the extract was purified on 6 × 0.5 cm Sephadex columns by the method described by Wuthier (1966). Purified lipid was evaporated down and resuspended in a known volume of chloroform-methanol 2:1, (usually 1 ml) and known volumes (usually 0.1 ml) counted in Dioxan scintillant.

The nature of the radioactivity in the lipid fractions was investigated by silica-gel thin-layer chromatography in chloroform-methanol-water, 65:25:4. The experiments were conducted as described above but with culture activities of 50 μ Ci/ml. After the chromatograms had been run, they were allowed to dry thoroughly at room temperature; the gel was then divided up into 1 cm strips and scraped off the plates into vials containing 5 ml Dioxan for counting. Radioactivity in the residue after lipid extraction was measured after hydrolysis for 4 h in 1 N-HCl at 100 °C, followed by desiccation over sodium hydroxide. The hydrolysed residue was redissolved in a known small volume of water and 0.1 ml amounts counted.

The kinetics of radioactive glycerol uptake was followed by removing known volumes of actinomycin-D-treated labelled cultures at various time intervals and measuring the incorporation into extracted lipid as described above. Control cultures were treated identically but without the addition of actinomycin-D.

Uptake of [14 C]L-leucine. Residual protein synthesis during actinomycin-D treatment was followed by measuring the rate of accumulation of 14 C-labelled L-leucine in trichloroacetic acid (TCA) precipitates. Actinomycin-D and [14 C]leucine (0.28 μ Ci/ml) were added to 50 ml cultures in L broth and supplemented L broth. At various times, 10 ml volumes were removed and pipetted into ice-cooled tubes containing 1 ml 50 % TCA and 0.2 ml of 1 % 'cold' L-leucine carrier. After 1 to 1½ h at 4 °C, the entire contents of each tube were washed onto glass-fibre filters, washed three times with cold 5 % TCA, twice with ether and twice with absolute alcohol, and dried in air. Filters were counted in 5 ml Dioxan scintillant.

Scintillant. This contained 130 g naphthalene, 8 g 2,5-diphenyloxazole and 0.3 g dimethyl 1,4-bis-(2-(4-methyl-5-phenyloxazolyl)) benzene (Koch-Light Laboratories, Colnbrook, Buckinghamshire) per litre of Dioxan. A Beckman scintillation counter was used. All results are given as background-subtracted and corrected for quenching on the basis of quench correction curves constructed with the same isotope.

Chemicals. Actinomycin-D was used at a final concentration of 2 μ g/ml in all experiments and was a gift from Merk, Sharp and Dohme Ltd, Hoddesdon, Hertfordshire.

Radioactive materials. Glycerol 2- 3 H], specific activity 100 mCi/mm, and L-leucine 14 C, specific activity 270 mCi/mm, were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. When used at a high concentration the glycerol was evaporated down in a gentle current of air to remove carrier alcohol.

RESULTS

Mesosomes in exponential cultures

The mass doubling times were 17 min in L broth and supplemented L broth and 32 min in Pollock's medium (Fig. 1). Samples were taken at points A to E and Table 1 shows an analysis of mesosome distributions in the various samples. Fig. 2 illustrates a typical *Bacillus cereus* 569 exponential-phase organism from L broth at an extinction (*E*) 0.35

Fig. 1. Growth of *Bacillus cereus* 569. Extinction was measured at 620 nm in 10 mm path-length cells and samples for electron microscopy were taken at points A to E. The mesosome distributions are given in Table 1. ×, L broth with phosphate and magnesium; ●, L broth; ○, Pollock's medium.

Table 1. Mesosome distribution in *Bacillus cereus* 569 cells

Samples were taken at points A to E on the growth-curves given in Fig. 1.

Medium	Corresponding point on Fig. 1	No. of sections	Average mesosome number/section	Septations c. mesosome(s) associated* (%)	Lamellar mesosomes (%)
L broth	A	79	2.1	46	79
L broth	B	80	1.9	47	97
L broth	C	79	1.3	90	3
Supplemented L broth	D	78	1.9	45	95
Pollock's medium	E	79	1.4	30	95

* Mesosomes were classified as septum-associated if they were within 0.5 μ m of the septal plane.

(point A on Fig. 1); no essential differences were observed in samples from the alternative media and in all samples the average mesosome content per bacterium was greater than one. Mesosomes were separated by apparently random distances and were almost entirely lamellar (Fig. 2), with the exception of cultures sampled during early stationary phase in L broth (point C, Fig. 1) where mesosomes were almost all vesicular and the plasma membrane darker staining and more wavy in outline (Fig. 3 and Table 1). No evidence was obtained for mesosome division, but the proportion of septa associated with mesosomes in random axial sections of septating bacteria (Table 1) makes it probable that septations

Fig. 2. Exponential-phase *Bacillus cereus* 569 grown in L broth. More than one mesosome is present and they consist of largely concentric lamellar membranes.

Fig. 3. Bacterium from an early stationary-phase culture in L broth. Mesosomes are vesicular and both they and the plasma membrane are darkly stained.

Fig. 4

Fig. 5

Fig. 4. Dormant spore of *Bacillus cereus* 569. The core is structureless and surrounded by a structureless cortex (Cx.), laminated coat (Ct.) and exosporium (Ex.).

Fig. 5. Spore germinated 15 min in alanine-inosine-phosphate buffer. Indistinct structures are visible in the core and the cortex is thinner with a darker-staining region closely applied to the plasma membrane.

Fig. 6

Fig. 7

Fig. 6. Inhibition by actinomycin-D of exponential-phase *Bacillus cereus* 569 growing in L broth. Actinomycin-D ($2 \mu\text{g}/\text{ml}$ of culture) was added at the time indicated by the arrow.

Fig. 7. Growth of *Bacillus cereus* 569 in L broth after exposure to actinomycin-D. Bacteria were washed 3 times and resuspended in fresh medium to the same extinction after inhibition. Actinomycin-D was added at the time indicated by the arrow. $\times$, 10 min exposure; $\bullet$, 20 min exposure. The curves are staggered due to the time which elapsed between the addition of actinomycin-D and the commencement of re-incubation.

normally occur in association with a mesosome. Serial sections also showed that two apparently separate mesosomes occurring at a septal site were not connected.

Spore germination and the effect of actinomycin D

Fig. 4 and 5 show an ungerminated spore and one after 15 min suspension in the germination buffer at which time 95 % of spores had germinated and were heat-sensitive. Germination appeared typical as demonstrated by the appearance of intracellular structure (ribosomes, DNA, plasma membrane) and a thinning of the cortex. After 60 min incubation in L broth mesosomes were identifiable, often more than 1 per bacterium, and emergence was commencing as indicated by the separation of the cortex into two layers with localized dissolution of the spore coats. Cultures incubated similarly but with the addition of actinomycin-D throughout the 60 min outgrowth period showed very little progress from the germinated state. Addition of actinomycin-D during the first 30 min of outgrowth followed by washing and resuspension in fresh medium without actinomycin-D for a further 30 min incubation appeared simply to delay outgrowth. However, when actinomycin-D was added during the last 30 min of outgrowth, development was still delayed but approximately a third of the sections showed collections of membranous material lying between the plasma membrane and new wall (Fig. 8). In some cases this had an apparent origin from, or connection with, a mesosome (Fig. 9).

Production of extra membranes in exponential cultures

Addition of actinomycin-D at $2 \mu\text{g}/\text{ml}$ abruptly halted growth (Fig. 6). The growth rate was markedly reduced or completely arrested on transfer back to fresh medium after

Fig. 8. Germinated spore grown for 60 min in L broth with the addition of actinomycin-D during the last 30 min of outgrowth. Extra membranes (arrowed) lie between the plasma membrane and new wall.

Fig. 9. Extra membranes possibly arising from, or attached to, a mesosomal site. Growth conditions were as in Fig. 6.

Fig. 10. Extra membranes in exponential-phase *Bacillus cereus* 569 after 30 min exposure to actinomycin-D in L broth.

Fig. 11. Sample of autoradiographed culture used in the grain analysis. The random background count on control grids was less than 1 grain/plate.

washing the culture free of actinomycin-D, the degree of inhibition depending on the duration of exposure to actinomycin-D (Fig. 7). In both L broth and supplemented L broth 30 min exposure to actinomycin-D during the exponential phase resulted in the appearance of extra membranes (Fig. 10) often associated with vesicles and in several layers. An analysis of 50 randomly chosen longitudinal sections and a small number of bacteria sectioned serially indicated that there was no preferential site from which extra membranes originated. Mesosomes were also present although reduced in size. Samples taken at various intervals after the addition of actinomycin-D to L broth cultures showed that, after a lag of approximately 15 min, the frequency of extra membranes steadily increased to about 35 % of sections at 30 min, after which lysis prevented further meaningful analysis (see Fig. 6). In supplemented L broth, the frequency was reduced to about 10 % at 30 min. This was unchanged after subjecting actinomycin-D treated bacteria to a 'regrowth' period.

Autoradiography of labelled exponential cultures

In sections autoradiographed after cultures had been labelled with tritiated glycerol during actinomycin-D inhibition, the grain size was large in relation to that of the structures examined; also, interference could possibly originate from wall glycerol polymers, and the only effective controls were sections in the same sample which lacked extra membrane. A rigorous analysis was therefore considered unjustified. Supporting evidence (but not conclusive proof) for new membrane synthesis would be provided if it could be shown that the grain count was increased in sections where extra membrane occurred. As cytoplasm and extra membranes usually lay very close to the wall, distinction between possible wall- and membrane-derived grains was frequently not possible. Grains were therefore classed as wall/membrane- (i.e. combined), mesosome-, or nucleus-associated if any part of the grain was within 50 nm of the respective structure. (This corresponds to 'probability circles' of 280 nm average diameter.) Wall/membrane grains were 92 % of all grains counted.

Fig. 12. Histograms of grain distribution over areas in sample exposed to [^3H]glycerol during actinomycin-D inhibition. A; extra membrane absent (212 areas); B, extra membrane present (65 areas). For details of method of analysis see text.

Grain counts were made on areas from whole-field reproductions (Fig. 11). Fig. 12 shows the distribution of 320 grains associated with wall/membranes (including mesosomes) over 277 areas. An area was defined as any section or incomplete section that contained a section of wall and/or membrane. A plane of wall was excluded whether extra membrane was present or not. Both transverse and longitudinal sections were included and a correlation was made with the frequency of extra membranes in complete transverse and longitudinal sections in a separate control. This established that the inclusion of incomplete sections did not materially distort the distribution of extra membranes. The average grain count over areas not showing extra membranes was 0.87/area; that for areas with extra membrane was 2.1/area. The number of grains recorded as wall/membrane associated in areas containing extra membrane and in excess of the average recorded in control areas formed 25 % of the total wall/membrane grains counted.

Incorporation of [^3H]glycerol and [^{14}C]L-leucine during actinomycin-D treatment

The addition of [^3H]glycerol had no effect either on the normal growth rate or on actinomycin-D inhibition at the concentrations used. However, electron microscope experiments showed that extra membrane was inhibited by alcohol carrier at concentrations above 5 % alcohol, hence it was removed by evaporation when using high levels of isotope. Counts of wash supernatant fluid showed a decrease of at least 100-fold in isotope activity at each wash. Significant activity was present in lipid extract after 30 min exposure to actinomycin-D and label. Table 2 shows the distribution of [^3H]glycerol label in lipid and residue fractions of actinomycin-D treated cultures. Activity in lipids continued to increase for at least 40 min after the addition of actinomycin-D but at a much lower rate than for control cultures (Fig. 13). Although the incorporation of [^{14}C]L-leucine into 5 % TCA precipitates from control cultures increased exponentially in both L broth and supplemented L broth, there was no such increase in the precipitates from similar but actinomycin-D treated cultures. There was however an initial rapid uptake of both [^3H]glycerol and [^{14}C]L-leucine

Table 2. *The effect of actinomycin-D on [^3H]glycerol incorporation into lipid*

Medium	Control cultures		Cultures treated with actinomycin-D (extinctions constant)		
	Lipid counts	Change in extinction	Lipid counts	Residue counts	Counts in lipid (%)
L broth	280 000	0.42-1.62	62 000	250 000	20
Supplemented L broth	390 000	0.47-1.55	40 000	46 000	46

Counts are given as d.p.m. per total residue or extract; activities for actinomycin-D cultures are corrected to an E 0.6. Isotope ($500\ \mu\text{Ci}$) was added to each 100 ml, and the cultures incubated for 30 min at 37°C .

Fig. 13. Radioactivity isolated from cultures labelled with tracers during inhibition by actinomycin-D in L broth (●) and supplemented L broth (×). Actinomycin-D was added 90 s before the addition of label at time 0. Control culture and -.-.- actinomycin-D treated culture labelled with $5\ \mu\text{Ci}$ [^3H]glycerol/ml. Points show activity extracted from 100 ml culture by chloroform-methanol. — Control and ---- actinomycin-D inhibited cultures labelled with $0.28\ \mu\text{Ci}$ [^{14}C]L-leucine/ml. Points show activity precipitated by 5% TCA from 10 ml culture and deposited onto glass-fibre discs.

before the steady state was reached. A possible explanation for this is a rapid exchange between the isotopes and labile or exchangeable material in metabolic pools or as 'nascent' material (J. Davies, personal communication).

Labelled lipid extracts were subjected to thin-layer chromatography and the distribution of radioactivity measured. The major peaks were clearly separable from a [^{14}C]glycerol marker and appeared to be identical in both control and actinomycin-D inhibited cultures. Chromatographic analysis of lipids extracted from batch cultures showed that these were mainly phosphatidyl ethanolamine, lyso-phosphatidyl ethanolamine, diphosphatidyl glycerol, phosphatidyl glycerol and neutral lipid.

The effect of actinomycin-D on Bacillus licheniformis 749c

Actinomycin-D caused rapid but incomplete cessation of growth in *B. licheniformis* 749c. Extra membranes were not observed when inhibited bacteria were sectioned.

DISCUSSION

This study has shown that under fixation conditions demonstrating single lamellar mesosomes in *Bacillus licheniformis* and *Bacillus subtilis* (Highton 1969; 1970b), *Bacillus cereus* 569 has several mesosomes per bacterium from the commencement of spore outgrowth and throughout exponential growth, nearly all of which are lamellar irrespective of the growth medium apart from those found in organisms in the stationary phase. It appears likely that there is a close association of at least one of these mesosomes with septation and association becomes very high in the stationary phase (Table 1). The absence of evidence for mesosome division and the apparently random separations of mesosomes from each other precludes the type of model for mesosome behaviour proposed by Highton (1969) in *B. licheniformis* 749c.

The production of extra membranes appears to be the normal response of *Bacillus cereus* 569 but not *Bacillus licheniformis* 749c to treatment with actinomycin-D. Extra membrane could be due to new synthesis in *B. cereus* 569 or to some form of membrane redistribution without new synthesis. In favour of the latter are that mesosomes are easily everted (Highton, 1969) and on treatment with actinomycin-D mesosomes appeared to be reduced in size and possibly in number. An estimate can be made however of the amounts of membrane material in mesosomes of normal *B. cereus* 569. Thus, taking a typical bacterium $1.7\text{ }\mu\text{m}$ long and $0.65\text{ }\mu\text{m}$ in diameter and assuming it has hemispherical ends, the total surface area of plasma membrane is about $4.5\text{ }\mu\text{m}^2$ of which about $0.6\text{ }\mu\text{m}^2$ covers each hemispherical end. A mesosome $0.2\text{ }\mu\text{m}$ in diameter containing 5 concentric lamellae spaced about $0.2\text{ }\mu\text{m}$ apart would have a total membranous area of just over $0.2\text{ }\mu\text{m}^2$, and three such mesosomes would have a total membranous area of about $0.7\text{ }\mu\text{m}^2$. There is thus enough total mesosome membrane to produce one extra membrane round one end of the bacterium as a hemispherical sheet. Such a calculation is of course liable to fairly gross error depending on the bacterial size and mesosome number. However, in a significant number of bacteria it was clear that the amount of extra membrane exclusive of any mesosomes present was greatly in excess of what might reasonably be expected if all the mesosomes had been reorganized into extra membrane. Additional evidence is afforded by the following data. First, extra membranes were produced only after a time lag of about 15 min, which is more compatible with new synthesis than gradual reorganization. Secondly, as the only difference seen in the populations examined by autoradiography was the presence or absence of extra membranes in the same sample, about 60 % of the grains counted in sections containing extra membranes could have originated from the extra

membranes and constituted 25 % of all grains counted. These figures correspond well with the distribution of tritiated glycerol recorded in the chemical studies (20 % counts in lipid, Table 2). Thirdly, labelled glycerol was still incorporated into lipid during actinomycin-D inhibition (Fig. 13 and Table 2) although there was virtually no growth or protein synthesis and radioactivity in lipid extracts from actinomycin-D inhibited cultures was indistinguishable by thin-layer chromatography from that from control cultures. Such observations suggest that during actinomycin-D inhibition of *B. cereus* 569, membranes continue to be produced. The nature of the extra membranes is uncertain and in the absence of protein synthesis may be phospholipid 'myelin forms' or possibly lipoteichoic acids (Wicken, Gibbens & Knox, 1973). Alternatively, they may be similar to normal membranes, the proteins being supplied from non-specific endogenous proteins (Mindich, 1970) or from a very low level of continued membrane-protein synthesis not detectable by the method used. Attempts were made to isolate labelled membranes without success, partly due to contamination by label in what was presumably teichoic acid in the wall and partly due to the resistance of the cultures to lysozyme.

The production of extra membrane material is well recognized in Gram-negative bacteria, especially *Escherichia coli*, for example in temperature-sensitive strains grown at the restrictive temperature (Wiegand, Holt, Shiveley, Decker & Greenawalt, 1973), normal strains infected with mutant phage (Onishi, 1971) or grown under conditions of magnesium deprivation (Fill & Branton, 1969); it is also found in other organisms such as *Vibrio marinus* (Felter, Kennedy, Colwell & Chapman, 1970) and *Acetobacter suboxydans* (Batzing & Claus, 1973) where their production is related to the growth phase. However, there appears to be no precedent for the appearance of extra membranes in Gram-positive organisms. Mach (1964) reported the production of extra membranes in *Streptomyces olivaceus* after treatment with mitomycin C, but their origin could not be clearly correlated with mesosomes found in other strains (Hopwood & Glauert, 1960). No definitive correlation is evident between the mesosomes and extra membranes reported in this study of *Bacillus cereus* 569. The effect of actinomycin-D is primarily to inhibit RNA synthesis and the almost immediate cessation of growth suggests that bacterial but not membrane growth may be tightly coupled to some very short-lived mRNA. Data from the spore study also indicates that the mRNA or enzymes involved in the production of extra membrane material are not operative until some time after the commencement of outgrowth, when true mesosomes first become visible (Garland, 1971). It is thus tempting to postulate that production of the extra membranes in *B. cereus* 569 is related in some way to mesosomal function.

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