The Internal Membranes of *Caulobacter crescentus*

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SUMMARY

The intracytoplasmic membranous structures previously observed in various caulobacter species by electron microscopy of thin sections were studied further by electron microscopy of whole organisms of *Caulobacter crescentus* negatively stained with phosphotungstate. The large, complex mesosomes are intrusions of the cell membrane. They appeared, from their central location in dividing cells, to play a role in division. Smaller and less involuted intrusions of the membrane became abundant in oxygen-limited cells, which also had an abnormally high content of haem pigments. These smaller intrusions may therefore be a structural reflexion of an increased content in the membrane of components of the respiratory electron transport system. The membranous organelle which occurred at the site of stalk formation and appeared to be connected with the membranous core of the stalk was not penetrated by phosphotungstate, and therefore had an organization different from that of the mesosome.

INTRODUCTION

A study of the cellular organization of caulobacters belonging to the genera *Caulobacter* and *Asticcacaulis* by electron microscopy of thin sections has shown that these bacteria characteristically contain membranous organelles (Poindexter & Cohen-Bazire, 1964). Large mesosomes were frequent, particularly near the site of cell division, and a membranous structure of variable size occurred at the base of the stalk and was seemingly continuous with the membranes that composed the core of the stalk itself. It was not easy to ascertain the origin and arrangement of these membranous elements from the examination of their profiles in thin sections, and we have accordingly explored another technique for their study.

Zwillenberg (1964) and Bladen, Nylén & Fitzgerald (1964) examined by electron microscopy whole bacteria treated with a solution of potassium phosphotungstate. By virtue of its electron density, phosphotungstate serves as a negative stain. It cannot enter the protoplast; hence, if it is able to penetrate the cell wall, it reveals by its opacity the contours of the cell membrane and its intrusions. Zwillenberg examined a considerable variety of true bacteria, both Gram positive and Gram negative, by this method and found that only four organisms (all Gram positive) possessed walls permeable to phosphotungstate. In the other 8 bacteria he examined, phosphotungstate acted as a negative stain for the whole organism, revealing only the surface structure of the wall. Preliminary trials of this method were conducted with *Caulobacter bacteroides* strain CB 11a, *C. fusiformis* strain CB 27, and *C. crescentus*

strain CB 15, representative of the main structural subgroups in the genus *Caulobacter* (Poindexter, 1964). Only *C. crescentus* proved to possess a wall permeable to phosphotungstate, and it was accordingly used for the experiments reported here.

METHODS

Bacteriological methods. *Caulobacter crescentus* strain CB 15 (Poindexter, 1964) was grown in a liquid medium containing, g./l. of tap water: Bactopeptone 2, Difco yeast extract 1, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2. All cultures were incubated at 30°. Growth was estimated turbidimetrically, by using a Klett-Summerson colorimeter with a red filter (c. 660 m μ).

Bacteria were harvested in three different physiological states for electron-microscopic examination. *Normal bacteria* were derived from fully aerated flask cultures, incubated on a New Brunswick rotary shaker, and harvested during the course of exponential growth. *Oxygen-limited bacteria* were grown in weakly aerated flask cultures, incubated on a slow shaker (80 strokes/min., 2.5 cm. amplitude), and harvested after growth had attained a density sufficient for oxygen to become the limiting nutrient. *Carbon-limited bacteria* were grown in flasks of the standard medium which had been diluted fivefold with 0.002 M-phosphate buffer (pH 6.8) containing, g./l.: NH_4Cl , 0.25 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1. The cultures were fully aerated (incubation on a New Brunswick rotary shaker) and harvested shortly after entry into the stationary phase. Controls showed that the cessation of growth reflected exhaustion of the available carbon and energy sources, and that oxygen was at no point a limiting nutrient.

Electron-microscope examination. The bacteria were harvested by centrifugation and resuspended in distilled water containing 5 g. ammonium acetate and 2 g. sucrose/l. A drop of this suspension was placed on a carbon-stabilized formvar-coated 300-mesh copper grid, and most of the liquid was withdrawn by touching the edge of the drop with a piece of filter paper. A drop of 1% (w/v) potassium phosphotungstate containing 0.2% (w/v) sucrose and adjusted to pH 7.0 was immediately added, and most of the liquid again withdrawn. The air-dried specimens were examined within 24 hr in a Siemens Elmiskop I operating at 80 kV.

Measurements of protein and haem. Suspensions of bacteria in distilled water were treated for 10 min. in the MSE ultrasonic disintegrator. The protein contents of suitably diluted samples were determined by the method of Lowry, Rosebrough, Lewis Farr & Randall (1951). Other samples were used to prepare pyridine haemochromogen by the method of Elliot & Keilin (1963), as modified by Omura & Sato (1964). The spectral difference between the oxidized form and the dithionite-reduced form of the haemochromogen was measured with a Cary 14 recording spectrophotometer, and the total haem content was calculated from the absorption difference between the peak of the α band (557 m μ) and the minimum between the α and β bands, assuming an extinction coefficient of 22 cm.⁻¹ mM⁻¹ (Jones, 1963).

RESULTS

The structure of normal Caulobacter crescentus

Plates 1, 2, 3, figs. 1-5 show typical appearances in phosphotungstate-stained preparations of bacteria growing exponentially in standard medium under conditions

where oxygen is not a limiting nutrient. The cell wall is typically somewhat distorted: irregular, bleb-like extensions (probably derived from the plastic outer layer characteristic of the walls of Gram-negative bacteria) are visible at many points. The contour of the protoplast, sharply revealed by the penetration of phosphotungstate to its outer surface, is smooth and regular. The only visible intracytoplasmic structures are irregularly distributed spherical inclusions that differ intrinsically from the surrounding cytoplasm in their electron opacity. Since the caulobacters characteristically contain deposits of both volutin and poly- β -hydroxybutyric acid (Poindexter, 1964), we tentatively interpret the small, electron-dense inclusions (v) as volutin, and the larger, electron-transparent inclusions (p) as poly- β -hydroxybutyric acid.

The most conspicuous structural elements revealed in these preparations are the large mesosomes (LM), which were deeply penetrated by phosphotungstate so that their number, location, and complex internal structure could be readily observed. Evidently, they represent infoldings of the cytoplasmic membrane. In dividing bacteria, examples of which occur in Pls. 1, 2, 3, figs. 1-4, a single large mesosome occupies the equatorial constriction and extends for some distance into the adjoining cytoplasm on each side of it. The appearance of the mesosome in late division stages (Pl. 2, fig. 3, and Pl. 3, fig. 4) indicates that it was bisected on completion of division, each daughter cell initially containing a single mesosome, derived from this bisection, at the undifferentiated pole (i.e. the pole that bears neither a stalk nor a flagellum).

After the daughter cells have separated, the mesosome apparently undergoes rapid changes in location and structure. This can be inferred from the mesosomal arrangements in Pl. 3, fig. 5, which shows a rosette containing four recently liberated, motile, apical organisms (swarmers). The formation of rosettes in caulobacter results from the adhesion of swarmers at their flagellated poles, which carry hold-fast material; as growth proceeds, each cell in the rosette loses its flagellum and develops a stalk at the previously flagellated pole (Stove & Stanier, 1962). Hence a rosette composed of recently liberated swarmers can be unambiguously recognized, not merely by the shortness of the constituent bacteria, but by the presence of flagella and the absence of stalks. Furthermore, because adhesion always involves the flagellated poles of the participating swarmers, the outer poles of the bacteria in such a rosette can be identified as 'new' poles, formed at the immediately preceding division. Of the four cells in Pl. 3, fig. 5, only the upper one contains a single mesosome at the outer pole, the position that we infer to be characteristic of the immediate post-divisional state. In the cell at the lower right, there is one large, bi-lobed mesosome close to the outer pole, and a second, smaller mesosome not far from the inner pole. In the two other cells, there is a single mesosome close to the flagellated pole. These positional variations might have been brought about either by migration of the post-divisional mesosome, or by its reabsorption and the formation of a new mesosome at another point on the surface of the membrane.

The membranous organelle which lies at the base of the stalk and is seemingly continuous with the membranous core of the stalk (see Pl. 6, figs. 10, 11) frequently has a structure indistinguishable from that of the mesosome in thin sections; occasionally, its component membranes are more regularly arranged, more or less parallel to the long axis of the bacteria (Poindexter & Cohen-Bazire, 1964). In phosphotungstate-treated bacteria, neither the organelle itself nor the membranous core

of the stalk is appreciably penetrated by phosphotungstate. This can be seen with particular clarity in Pl. 1, fig. 2, where the protoplast has retracted slightly from the wall at the stalked pole, so that its surface is sharply outlined by negative contrast. As is also particularly evident in this figure, the core of the stalk shows up by negative contrast with the enclosing, phosphotungstate-permeated wall of the stalk. These observations indicate that the system of membranes occupying the stalked pole of the bacteria and extending into the core of the stalk do not (in contrast to the mesosome) represent a simple invagination of the cell membrane.

Effects of oxygen starvation on membrane structure

Earlier unpublished electron-microscopic studies of thin sections suggested that membranous intrusions are particularly abundant in caulobacter which have been grown in low concentrations of oxygen. Since these bacteria are strict aerobes, oxygen is an essential nutrient, so that the observed abundance of membranes in such cells could be a non-specific consequence of the slowing down or cessation of division. However, no such proliferation of membranes could be detected in sections of cells that had entered the stationary phase as a result of the depletion of the carbon and energy source. Negative staining with phosphotungstate completely confirmed the earlier observations on thin sections. Bacteria which have entered the stationary phase as a result of carbon depletion are essentially indistinguishable in both size and mesosomal structure from normal bacteria harvested during the exponential phase of growth; but bacteria from oxygen-limited cultures have a completely different aspect, illustrated in Pls. 4, 5, figs. 6-9.

Oxygen limitation evidently deranges cell growth and division. Most of the bacteria are greatly elongated (up to eight times as long as normal), and bear unusually short stalks; swimmers are very rarely observed. These elongated bacteria contain large numbers of membranous intrusions which are freely penetrated by phosphotungstate, and which vary greatly in size, shape and internal complexity. Some show the characteristic internal complexity of mesosomes; others seem to be simple sac-like structures. As many as 80 may occur in a single bacterium. Division figures are relatively rare in oxygen-limited bacteria; but when they do occur (see Pl. 5, fig. 8) the plane of constriction is characteristically occupied by a single large mesosome, entirely analogous in position and structure to the mesosome of a dividing normal bacterium. It should be specially noted that the development of the large mesosome in a dividing oxygen-limited bacterium is not accompanied by the disappearance of the smaller membranous intrusions, which are clearly evident at the other points on the surface of the protoplasts of dividing bacteria. Division in oxygen-limited bacteria is often highly asymmetric, as seen in the upper organism of Pl. 5, fig. 8; and in such cases the large mesosome still occupies the plane of constriction.

There is much evidence that in aerobic bacteria the cell membrane is the site of the respiratory electron transport system (see Hughes, 1962, for a review). Lenhoff, Nicholas & Kaplan (1956) have demonstrated that in *Pseudomonas fluorescens* oxygen limitation leads to a substantial increase in the cellular content of cytochrome *c*. These facts suggested a possible explanation for the abundance of membranous intrusions characteristic of oxygen-limited caulobacters. If, as in *P. fluorescens*, oxygen limitation increases the differential rate of synthesis of com-

ponents of the respiratory electron transport system, intrusion of the cytoplasmic membrane may be necessary to accommodate the increased cellular content of these membrane-bound enzymes. An analogous situation has been shown in *Rhodospirillum rubrum* and other non-sulphur purple bacteria, where the content of membrane-bound photosynthetic pigments, which can be greatly varied in response to environmental factors, is correlated with the extent of the internal membrane system (Cohen-Bazire & Kunisawa, 1968; Cohen-Bazire, 1968).

Table 1. *Variation of the specific haem content of Caulobacter crescentus in response to changes in the conditions of growth*

Conditions of growth...	Haem content, μ moles/mg. cellular protein		
	Aerobic exponential	Oxygen- limited	Carbon- limited
Expt. 1	0.182	0.415	0.25
Expt. 2	0.187	0.5	—
Expt. 3	0.20	0.53	—

In order to obtain some indication of the cellular content of respiratory enzymes in *Caulobacter crescentus*, we determined the haem content, measured as pyridine haemochromogen. As shown in Table 1, oxygen-limited bacteria contained more than twice as much haem on a protein basis as normal. Carbon-limited bacteria had a slightly greater haem content than normal, but the value was far below that characteristic of oxygen-limited organisms. These findings are accordingly consistent with the functional interpretation of the abundance of membranous intrusions in oxygen-limited caulobacters that has been outlined above.

DISCUSSION

Caulobacter crescentus contains at least two kinds of membranous organelles, distinguishable both by their location in the organism and by their structure. One type of organelle is situated at the site of stalk formation, and is probably connected with the membranous core of the stalk. This membrane system is not penetrable by phosphotungstate, and cannot therefore be interpreted as a direct intrusion of the cytoplasmic membrane. We propose to term it the *stalk organelle*.

The other type of organelle, for which we adopt the term 'mesosome', is a complex intrusion of the cytoplasmic membrane, readily penetrable by phosphotungstate. In exponentially growing populations, there are typically only one or two mesosomes in the interdivisional bacteria, and only one in dividing bacteria, invariably associated with the site of division. In oxygen-limited populations there are many more mesosomes in each bacterium, and they are more varied in size and structure. Since oxygen depletion leads to a substantial increase in the cellular haem content, we suggest that this proliferation of membranous intrusions reflects an increased content of membrane-bound respiratory enzymes, induced by low oxygen concentration.

In Gram-positive bacteria there is much evidence that mesosomes may play a role in the processes of nuclear and cell division (FitzJames, 1965), and their frequent association with transverse septa has led several workers to suggest that they may function in septum formation (van Iterson, 1961; Glauert, Brieger & Allen,

1961; Imaeda & Ogura, 1963). In 1963 Jacob, Brenner & Cuzin proposed that the bacterial chromosome may be replicated at an attachment site on the cell membrane, and that physical separation of the daughter chromosomes may result from interstitial growth of the membrane between their attachment points. The first direct evidence in support of this hypothesis was produced by Ryter & Jacob (1964), who demonstrated a constant topological association and probable physical attachment between the nucleoplasm and the mesosome in *Bacillus subtilis*. The invariable location of the mesosome at the site of cell division in *Caulobacter crescentus* is certainly compatible with the notion that it has important functions in division, and provides the first indication of a mesosomal role in the division of Gram-negative bacteria.

When oxygen-limited organisms of *Caulobacter crescentus* divide, the small intrusions characteristically formed as a result of oxygen limitation remain dispersed on the surface of the protoplast, even though a single large mesosome occupies the site of division. The increase in haem content which accompanies this cytological modification provides evidence that mesosomes can function as areas of concentrated respiratory activity; this is in accord with the cytochemical observations of van Iterson & Leene (1964) in *Bacillus subtilis*. Further, one of us (J.S.P.) has found that phosphate limitation similarly results in the proliferation of small, peripheral mesosomes. In light of the studies on oxygen limitation in these strictly aerobic bacteria, this suggests that restrictions of energy metabolism are responsible for formation of the small mesosomes, and that their formation may be controlled by a high-energy phosphate compound within the cell.

The accumulating observations on bacterial membrane systems suggest that the term 'mesosome' may in fact encompass organelles that have different functions. Some mesosomes, derived by intrusion of the cell membrane, may have specific roles in cell division, while others of the same derivation serve to extend the surface of the membrane in order to accommodate a large complement of respiratory enzymes. The stalk organelle, which is superficially similar to mesosomes in structure, provides evidence that membrane organelles may have a third function, i.e. participation in differentiation of subcellular structures. The significance of the apparent independence of this organelle from the cell membrane is not clear.

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EXPLANATION OF PLATES

All figures are electron micrographs of *Caulobacter crescentus* strain CB 15. Plates 1–5, negative-stained with phosphotungstate. Plate 6, thin sections of osmium-fixed caulobacters, embedded in Vestopal and post-stained with lead hydroxide.

Abbreviations used in figures: S, stalk; c, core of stalk; cb, cross-band in wall of stalk; f, flagellum; H, holdfast; v, volutin granule; p, poly- β -hydroxybutyric acid granule; M, mesosome; LM, large mesosome; W, cell wall; mo, membranous organelle.

PLATE 1

Dividing organisms from a culture in exponential growth.

Fig. 1. Note the large mesosome at the equator of the organism, symmetrically distributed between the two future daughter cells. The holdfast material at the tip of the stalk is clearly shown. Magnification, $\times 36,000$.

Fig. 2. The large mesosome is somewhat unequally distributed between the two future daughter cells. At the stalked pole the continuity between the protoplast (containing the membranous organelle at the base of the stalk) and the core of the stalk itself is particularly clear. Magnification, $\times 40,000$.

PLATE 2

Fig. 3. Caulobacters from a culture in the course of exponential growth. The two stalked organisms are adherent to a common holdfast. The upper organism, in a late stage of division, has a single large mesosome at the site of constriction. The lower organism, in the interdivisional state, contains a large mesosome near the apical pole, and a much smaller one near the basal, stalked pole. Cross-bands are visible in the stalks of both organisms. Magnification, $\times 36,000$.

PLATE 3

Rosettes of caulobacters from a culture in the course of exponential growth.

Fig. 4. A mature rosette, composed of three stalked bacteria at different stages of division. In each organism there is one large mesosome, located in the area of division. Magnification, $\times 39,000$.

Fig. 5. A newly formed rosette, composed of four swarmer, adherent by the flagellated poles. Note the differences in the number and position of the mesosomes. Magnification, $\times 30,000$.

PLATE 4

Caulobacters from oxygen-limited cultures. Note the cellular elongation, the short stalks, and the numerous small mesosomes.

Fig. 6. Electron-transparent material appears to have exuded from the mesosomes and to have accumulated between the surface of the protoplast and the cell wall. Magnification, $\times 24,000$.

Fig. 7. The diversity of mesosomal size and shape is particularly striking in this preparation. Two cross-bands in the wall of the stalk of the right-hand organism are shown very clearly. Magnification, $\times 20,000$.

PLATE 5

Fig. 8. Rosette composed of three caulobacters from an oxygen-limited culture. Two organisms are dividing, and contain characteristic large mesosomes at the sites of division, as well as smaller mesosomes elsewhere. In the bacterium at left the division is highly asymmetric. Magnification, $\times 24,000$.

Fig. 9. A group of stalked bacteria from an oxygen-limited culture, showing the diversity in size and form of the mesosomes, and the shortness of the stalks. Magnification, $\times 27,000$.

PLATE 6

Figs. 10 and 11. Thin sections of caulobacters from an oxygen-limited culture. The membranous organelle at the base of the stalk, which cannot be seen in whole organisms stained with phosphotungstate, is evident in two bacteria. Profiles of mesosomes are also abundant. Magnification, $\times 84,000$.