AN IMMUNOFLUORESCENT STUDY OF CELL WALL REPLICATION IN CERTAIN BACTERIA AND A BUDDING YEAST

by

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ABSTRACT

Cell wall replication in Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Escherichia coli, Streptococcus faecalis and Saccharomyces cerevisiae was studied by differential labelling* of living cells with fluorescent and non-fluorescent antibody.

Cells labelled with fluorescent antibody were incubated in broth after the free fluorescent antibody was removed or its further uptake blocked by the addition of non-fluorescent antibody. Examination of smears at consecutive intervals of 10 minutes showed that the new cell wall was non-fluorescent while the old wall remained as discrete fluorescent areas.

Growth of new cell wall in Bacillus cereus was initiated near the poles. In the old wall, additional new wall segments gradually developed to form an alternating pattern of new and old wall segments. Further growth elongated the new wall and pushed the old segments apart. Separation of daughter cells appeared to involve splitting

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*The term differential labelling is in current usage. It is of course recognized that the labelling is uniform and the differentiation occurs during the subsequent growth.

of the transverse septa laid down at or near the old wall segments.

Growth of new cell wall in Bacillus megaterium was initiated either at one of the poles or at the central area of the cell. Multiple segments of new and old wall appeared along the cell length. Further elongation was followed by the formation of transverse septa and separation of daughter cells incorporating either old or new wall segments.

Cell wall replication in Bacillus subtilis was studied by differential labelling with fluorescent polypeptide and polysaccharide antibodies. The new segments were replicated at multiple sites along the cell length forming an irregular pattern. Unlike the discrete areas of new cell wall seen in B. cereus and B. megaterium there was no clear cut separation of old and new segments.

In Escherichia coli the cross wall appeared as non-fluorescent or fluorescent bar depending on whether the free fluorescent antibody was removed before or after the formation of the cross wall. The initial step in cell division was the formation of a cross wall at the cell equator, followed by the appearance of new cell wall on either side of the cross wall. The process was repeated in sequence at subsequent

sites in the polar, the subcentral, and the subpolar areas. Constriction occurred at random so that the divided parent cells were composed of several daughter cells.

A polar type of unidirectional cell wall growth and elongation was also observed in Escherichia coli. It was initiated by the synthesis of a ring of new cell wall material around the polar tip. A second ring was then formed at the subpolar area during the rapid enlargement of the first ring in a single direction.

Evidence shows that cell wall synthesis is independent of cell division and that in Escherichia coli, it is initiated at multiple but specific sites within the cell.

Contrary to the E. coli synthesis of cell wall at multiple sites, Streptococcus faecalis replicated new cell wall at only one site per coccus. The new cell wall segment was initiated and enlarged at the coccal equator, and was followed by the formation of a cross wall, centripetal growth and constriction to separate the daughter cells.

The bud formation of Saccharomyces cerevisiae was initiated as a small bulge in the cell wall. This bulge from the mother cell enlarged and gradually developed into a small bud. Further increase in size of the bud was accompanied by the formation of a cross wall and constriction at the base

area to separate the bud from the mother yeast. It appears that the cell wall of the bud was newly synthesized first at the base area, and then added to the actively growing new bud in a direction away from the base. After separation of the mother and daughter yeasts, the birth and bud scars were clearly visible on the fluorescent cell wall.

The effect of chloramphenicol on cell wall replication of B. cereus, B. megaterium, Bacillus subtilis and E. coli studied by differential fluorescein-labelling and found to be similar to that observed in untreated cells. These antibiotic-treated cells formed long filaments and replicated new cell wall at multiple sites along the cell length; their daughter cells contained both old and new wall segments. In the long cells of B. cereus and E. coli, the initiation of the first new wall segment occurred slowly but subsequent new segments appeared very rapidly at multiple sites simultaneously along the cell length.

Experimental evidence clearly shows that growth and elongation of the microbial cells examined in this investigation do not occur by diffuse intercalation of new cell wall into the old.

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INTRODUCTION

The present-day concepts of microbial cell wall replication are widely divergent. Bisset (1951, 1953, 1955, 1956) postulated that growth of the cell envelope of unicellular bacteria occurred from a growing point at one pole, and that growth of septate bacteria occurred from the points of junction of cell wall and cross-wall. On the other hand, Stocker (1956) maintained that cell growth and elongation occurred diffusely by the intercalation of the cell wall along its length. Williams (1959), using cell inclusions as markers in phase contrast observations of Spirillum anulus, also concluded that cell growth and elongation occurred diffusely by intercalation of the old and new cell wall.

In a cytological study of bacterial cell wall replication it is essential to relate cell growth with the formation of cross-wall involved in the process of cell division. Swann (1957) believed that cell growth and cell division, although dependent upon one another, were controlled by different mechanisms. Lark and Lark (1960), observing cell wall synthesis by means of short-term incorporation of

radioactive alanine and methionine, reported that synthesis occurred continuously throughout the cell division cycle; moreover, cells became elongated during the interval between successive divisions. Clark, Webb, and Chance (1956) noted that the cells of Corynebacterium pseudophtheriticum examined by phase contrast microscopy enlarged slightly prior to division and became less dense. Prescott (1955) investigating the relation between cell growth and division observed that there was no growth during cell division, but there was a sharp transition to a period of maximum growth at the end of division. The growth of single cells of Schizosaccharomyces pombe was studied by Mitchison (1958), who showed that the dry weight of the cell increased linearly between divisions and that the rate was doubled at some points during the later stages of division, as the one parent cell split into two daughter cells. Henrici (1928) had earlier observed that bacterial cells increased in size before division and became smaller during the later stages of growth.

The differential labelling* of cell wall material with fluorescent and non-fluorescent antibody has recently provided

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a unique technique for the study of bacterial cell wall replication in a living system. Fluorescent photomicrographs of Streptococcus pyogenes, published by Cole and Hahn (1962), have shown that cell wall growth in this organism initiates and extends, from a minimum of two sites per coccus, both centripetally and peripherally from the coccal equator. Cole (1964) used the same technique to follow the cell growth of Salmonella typhosa, and as discrete and microscopically resolvable fluorescent areas of old cell wall were not observed, he concluded that cell wall replication occurred by diffuse intercalation of new cell wall in this species.

It is apparent that diverse patterns of cell wall replication may occur among different bacterial species. The present work was undertaken to obtain information about the manner and location of cell wall replication, and the relation between cell wall growth and the formation of cross wall in Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Escherichia coli, Streptococcus faecalis, and Saccharomyces cerevisiae by means of differential fluorescent and non-fluorescent labellings.

HISTORICAL

A. Bacterial cell growth was defined by Hartsell (1959) as a combination of cell morphology, spatial organization, and the patterns of synthesis. Webb (1949, 1951), studying the effect of magnesium on cell division, and Nickerson and Sherman (1952), elucidating the metabolic aspects of bacterial growth, provided evidence that cell division was not an obligatory requirement for cellular growth.

In the study of cell growth, Migula (1894) reported the observation of a transverse plasma boundary in the dividing cells of Bacillus oxalaticus. Subsequent investigation by this author (1897) showed that the cell, prior to division, became elongated while a cell-sap vacuole appeared in the middle of the cell. The cytoplasm, like a dense peripheral ring, closed inward to form a plasma disc which separated the vacuole into two. The membrane then grew centripetally toward the centre and split the plasma disc, the latter subsequently increased in thickness until constriction occurred on both sides to separate the daughter cells. Schaudinn (1902) noted that the first evidence of cell division in Bacillus butschli was the appearance of a highly refractile granule in what later became the plane of

division. This granule, always detected in the long axis of the cell, broadened into a disc perpendicular to the long axis of the cell. The disc, increased in size as well as thickness until it reached the lateral membrane, split to make a complete separation of daughter cells.

Cell division in a number of spore-forming aerobes including Bacillus radicosus, B. mycoides, B. megaterium, B. subtilis, B. limosus, B. alvei and B. asterosporus was described by Guilliermond (1908) as two granules sending slender extensions from the side of the membrane toward the centre to form a biconcave disc. This newly formed partition increased gradually in thickness at the median area, and then split into two bands by the formation, in its middle, of a hyaline zone. The division occurred frequently in an oblique plane. A similar finding was reported in cells of Proteus vulgaris by Knaysi (1929). This author (1930) observed the inward growth of the 'ectoplasm' of B. subtilis to form a band through which a sharply defined colourless line of division appeared. He pointed out that young cells continued to divide and to grow rapidly to maintain the size of the daughter cells, but old cells did not increase in size fast enough to keep up with the cell division, resulting in smaller

daughter cells. Bisset (1939) presented his view of cell division in Bacillus mycoides as the sudden appearance of an extremely fine line across the cell, and the development of a slight curvature of the dividing line to produce a concavity on one side and a corresponding convexity on the other. The concave side expanded to change into a convex. The final stage involved the formation of an indentation at the end of the dividing line to separate the cells.

The formation of a transverse plasma membrane in Escherichia coli was reported by Robinow (1942, 1944). Subsequent investigations with light microscopy by Robinow (1945) and by Knaysi (1941, 1949, 1951) suggested that the initial stage of cell division involved the inward annular growth of the cytoplasmic membrane to form a transverse septum; this was subsequently split by the centripetal growth of the cell wall. Chapman and Hillier (1953), on the other hand, using electron microscopy of ultra-thin sections to study the cellular division in Bacillus cereus, demonstrated that the inward growth of the cell wall initiated the partition of mother and daughter cells, and that the process of cell division involved the secretion of cell wall material as a ring on the inner surface of the wall to form an annular disc

which closed in the manner of an iris diaphragm. The transverse wall laid down in this manner gradually became thickened, and split eventually into two layers, separating the mother and daughter cells. Dawson and Stern (1954), recording the stages of bacterial cell division by electron microscopy of the isolated cell walls of Streptococcus faecalis and Staphylococcus aureus, described the initiation and centripetal growth of a transverse septum in the cell wall, followed by the splitting of the septum into two layers and constriction of the cell wall through the line of the double septum to make the final separation. Additional experimental evidence from Chapman (1959a,b) indicated that the cytoplasmic membrane in an unidentified organism formed a laminar septum on which cell-wall material was deposited; this deposition was followed by constriction to separate the daughter cells. Murray, Francombe and Mayall (1959) also presented experimental evidence to show that the major area of cell wall synthesis was at the site of septum formation. Conti and Gettner (1962), after reporting that the centripetal growth of the cell wall in Escherichia coli was responsible for the partition of the cell during division, concluded that the cell septum could be formed either by the annular centripetal growth of the cytoplasmic membrane or by the cell wall

with a closely apposed cytoplasmic membrane.

Knaysi (1951) believed that the formation of transverse cell wall was an independent function of each daughter cell while Bisset (1948, 1950) suggested that bacteria divided by the production of a septum from the cell membrane. Chapman and Hillier (1953), however, considered the peripheral bodies which were observed to line up at the site of the annular ring prior to cell division to be responsible for the secretion of cell wall material as the ring moved in towards the centre. Chapman (1959a) further proposed that cell wall material might be synthesized by both the cytoplasm as well as the cytoplasmic membrane.

The formation of a cell plate in a number of bacterial cells prior to division was demonstrated through the use of crystal violet nuclear staining by Chance (1953a,b). Clark and Webb (1954) later observed that the cell plate of protein origin gradually matured into a cross-septum coated with cell wall material, the formation of which caused the separation of the nucleus into two fragments. Further studies on the centrifugal division by cell plates in actively growing cells of Corynebacterium pseudodiphthericum and Micrococcus pyogenes var. aureus by Clark, Webb and Chance (1957) showed that the

cell plate was first formed as a thin disc near the cell equator. Subsequent growth and elongation of the cell extended the cell plate to the outer cell wall. As a result, new cell wall material began to develop at the point of juncture of the disc and the outer cell wall. The centripetal growth of this heavy ring of wall material partitioned the mother and daughter cells by the formation of a double wall. During the last stage of cell division, the proteinaceous cell plate simply served as an interface on which wall material was deposited.

Division by simple constriction of the cell was observed by Vahle (1909) in Myxococcus ruber, by Dobell (1911) in Bacillus flexilis, by Guilliermond (1908) and Swellengrebel (1909) in Spirillum volutans. Ellis (1902, 1922) described cell division as a simple partition and separation of the parent cell into two completely rounded off portions connected by a thick bridge of mucilaginous material shaped like a biconcave lens.

Cell division by cytoplasmic retraction was reported by Knaysi (1929a,b) in cells of Mycobacterium tuberculosis as a clear zone formed by the drawing back of the cytoplasmic substance into two dense masses on either side. The clear

zone was then separated by membranes to complete the division process. Similar findings were also reported by Ellis (1902, 1922). On the basis of dark-field observations, Knaysi (1941) showed that separation of cytoplasm inside the dividing cell preceded the deposition of cell-wall material.

The differences in the mode of cell division between morphologically smooth and rough forms of bacteria were emphasized by Bisset (1948). According to this author, the process of cell division occurred in two main stages: the formation of a transverse septum which was continuous with the cell membrane, and the secretion of the new cell wall by this membrane. In rough bacilli, the membranous septum was formed long before cell division occurred. The new wall material was secreted internally in one piece to cover the whole membranous septum at the first sign of cell division. During separation of the daughter bacilli, a slight degree of constriction was visible at the septum area. In smooth forms, the membranous septum was formed immediately before cell division. The existing cell wall together with the new wall material secreted by the membranous septum constricted to form the ends of the new daughter cells. On the basis of observations from subsequent studies, the same author (1950,

1951) put forth the concept that the main growth of the cell wall of Gram-negative bacteria was initiated from one pole of the cell, and that the formation of the secretory septum after cell elongation was accompanied by a second growing point at the point of cell division. A "germ-tube" type of growth at both ends of the cell was also reported by Gilmour (1961) in Bacterionema matruchotii, and by Adler and Hardigree (1964) in Escherichia coli. Jeynes (1957) conducted an investigation on the growth and properties of protoplasts suggested that the process of growth and cell division was controlled by a growing point consisting of lipoprotein and Feulgen-positive material.

Cell division in Corynebacterium diphtheriae was reported by Hewitt (1951). According to this worker, the cross-septa divided the cell into multiple compartments, and cell division occurred at the cross-septum with no indication of any previous constriction of the cell at this point. Bisset (1949) related the multicellular characteristics of Corynebacteria and Mycobacteria to the mode of cell division of these organisms. He considered that reproduction occurred either by multiple cell divisions or by fragmentation into single cells which subdivided without separation, and then

grew into multicellular bacilli.

A different approach to the study of cell division was reported by Bisset (1951) using flagella as cell-wall markers. Based on the observation that one daughter cell was usually flagellated while the other was not, this author suggested that the main growth of the cell envelopes in unicellular bacteria was from a growing point at one pole and from the junction of cell wall and cross wall in septate bacteria. The phenomenon was further investigated by Bisset and Pease (1957) using two strains of bacteria (Salmonella typhimurium and Proteus vulgaris) which were capable of producing flagella at 36° C but not at 44° C. The cells were first grown at 36° and then transferred to 44° for observations on cell division. These authors demonstrated that the flagella were retained by the parent cells after several divisions, and that the daughter cells had no flagella. Stocker (1956), and Quadling and Stocker (1956) however reached different conclusions about the same organism. According to these workers, the flagella were distributed among the daughter cells after several divisions instead of being retained by the mother cell. They proposed that growth of the cell wall was diffuse and not localized at definite growing points. Other experimental evidence to