PULLULOMYXA BOTRYTIS N. SP.

A. C. THAYSEN

Microbiological Section, Chemical Research Laboratory of the Department of Scientific and Industrial Research, Teddington, England

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A brief note was published some years ago by W. T. Morgan of the Lister Institute, London, and the writer (1932) describing the isolation from decayed woody tissues of a micro-organism which decomposes the specific polysaccharides of *Shigella dysenteriae*, Shiga; *Shigella dysenteriae*, Flexner Y; *Pneumococcus* Type II; and the tubercle bacillus.

At the time a study of the relationship of this organism was not proceeded with as it was felt that its morphological peculiarities deserved closer attention than it was then possible to devote to them. The organism was provisionally referred to as a *Myxococcus*, a designation which was based solely on its microscopic appearance. Strained *in vivo* with a dilute solution of methylene blue it resembles the coccoid stage of many myxococci as depicted by Krzemieniewscy (1928), and incidentally of *Spirochaeta cytophaga* as described by Hutchinson and Clayton (1919), an organism which Kremienewska (1930) states approaches the myxococci in its characters.

Figure 1 shows the polysaccharide-decomposing organism stained *in vivo* with dilute methylene blue. Its apparently thick-walled coccoid cells contain deeply staining plasmatic inclusions which, in many cases, appear to adhere to one side of the cell wall. The cells measure between 2.5 and 3 μ in diameter; a few may exceed 3 μ and some, not including those attached to larger cells, may be no more than 2 μ in diameter. In the production of smaller coccoid cells by normally sized spheres the organism differs strikingly from the coccoid stage of the myxococci. These attached smaller coccoid cells can be seen to be

the daughter cells of the normally sized spheres, arising from the latter by a process of budding. There is therefore only one cell form in the life cycle of the polysaccharide-splitting organism.

Among the myxococci, on the other hand, the coccoid stage represents the resting stage in the life cycle; on germination it gives rise to rod-shaped cells. These rods are the active or reproductive stage of the life cycle. Only when reproduction ceases does a coccoid stage again appear among the myxococci

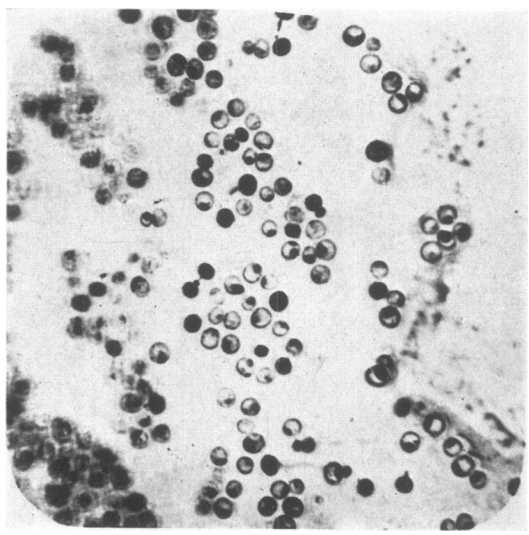

FIG. 1. THREE-DAY-OLD CULTURE OF PULLULOMYXA BOTRYTIS, STAINED IN VIVO WITH METHYLENE BLUE. × 2550 APPROX.

and this through the contraction of rodshaped cells into spheres, never as a result of the budding of coccoids.

It is true that in other groups of myxobacteria the two stages in the life cycle are morphologically more uniform than is the case among the myxococci. But in these cases the cell form is invariably rod shaped, both during the resting stage and the reproductive stage. No case has so far been described in which an organism belonging to the *Myxobacteriales* has shown a complete absence of rod-formed cells and a mode of reproduction reminiscent of budding.

It is for this reason that the writer has felt compelled to refrain from incorporating the organism here described among the *Myxobacteriales.*

The observation that reproduction in the polysaccharide-splitting organism takes place by budding naturally led to a comparison of this process as it occurs here with the budding process among the yeasts in order to see whether the new organism could be incorporated among the latter. But here again the writer was unable to find convincing evidence for doing so. In

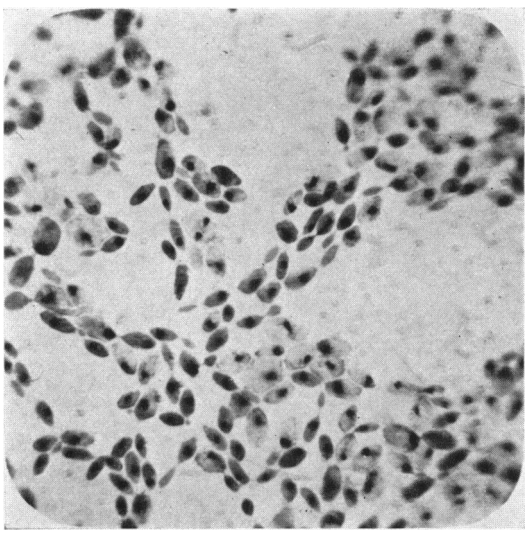

FIG. 2. TWENTY-FOUR-HOUR CULTURE OF SACCHAROMYCES ELIPSOIDEUS, STAINED WITH FEULGEN'S STAIN. × 950

fact the observations which he made led him to conclude that very considerable differences exist between the process of budding in the two cases, notably in the behaviour of the thymonucleic substances.

Among the yeasts, reproduction by budding proceeds, as shown in figure 2, by the formation of a daughter cell which invariably attains a considerable size before part of the thymonucleic substances of the mother cell proceeds towards it, and eventually enters it through the narrow connection between the two cells.

The process of transfer of thymonucleic substances from the mother cell to the daughter cell is comparatively simple. The original spherical form of this material will stretch to produce a shorter or longer thread depending on the distance it is removed from the connecting passage between mother and daughter cell. Part of this thread will squeeze through the passage and form a sphere in the daughter cell, whilst the remainder, still in the mother cell, will again contract and form a sphere. The partition of the thymonucleic substances in the yeast cell thus corresponds to an amitotic nuclear division. The staining method adopted to

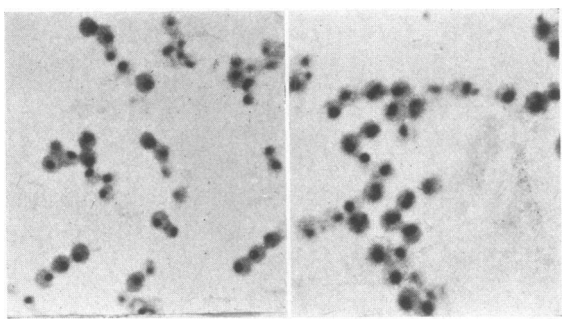

FIG. 3 FIG. 4

FIG. 3. SEVENTY-DAY-OLD CULTURE OF PULLULOMYXA BOTRYTIS, STAINED WITH FEULGEN'S STAIN. × 2550 APPROX.
FIG. 4. FORTY-EIGHT-HOUR-OLD CULTURE OF PULLULOMYXA BOTRYTIS, STAINED WITH FEULGEN'S STAIN. × 4000 APPROX.

ascertain the behaviour of the thymonucleic substances was based on that of Feulgen (1926).

The behaviour of the thymonucleic substances during the cell division of all the other yeast types which the writer has been able to examine, including a species of the small anascosporogene yeast *Pityrosporum rhinoserosum*, was exactly the same as that described above for a typical *Saccharomyces ellipsoideus*.

In the polysaccharide-splitting organism here discussed, the behaviour of the thymonucleic substances during budding is more complex. In the resting cell this material forms a well defined sphere usually in the centre of the cell, as shown in figure 3, illustrating a 70-day-old culture stained with Feulgen's stain. At this age the cells are smaller than those of a young and active

culture. When growth commences, and before any extraneous signs of budding are noticeable, the sphere of thymonucleic substances elongates to form an equatorial band in the cell. Eventually this band may stretch further and become U shaped. In other cases the band of thymonucleic substances is represented by two parallel placed rods. These early stages can be observed in figures 4, 5, 6 and 7.

It has not been possible to ascertain with certainty the details of the further subdivision of the thymonucleic substances as revealed by Feulgen's stain. A closer scrutiny of figures 4 and 5 will show cells with two and three separate globules of these substances, indicating perhaps a further subdivision of the two above-described parallel rods, or possibly of the original U shaped

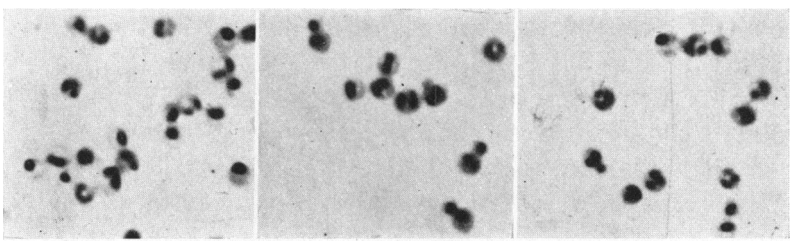

FIG. 5 FIG. 6 FIG. 7

FIGS. 5, 6 AND 7. FIVE-DAY-OLD CULTURES OF PULLULOMYXA BOTRYTIS, STAINED WITH FEULGEN'S STAIN. × 4000 APPROX.

band. In some cases a subdivision of the substances into 4 separate units has been observed, and it cannot be excluded that the stage of three or of two separate globules may represent a subsequent fusion of a previously larger number of units. Throughout the period of subdivision of the thymonucleic substances the cell has remained globular without excrescences. However, when the stage of the existence of two or three definite globules of thymonucleic substances has been reached the cell can be seen to acquire a slightly egg-shaped form with one granule of thymonucleic substances eventually occupying the pointed end of the cell. Here the bud finally arises.

The mode of separation of the daughter cell from the mother cell in the polysaccharide-splitting organism also differs from that

of the yeasts, or at any rate from that of the genus *Saccharomyces* in which, according to Guilliermond (1920) and Lindner (1930), a transverse cellwall is formed between mother and daughter cell prior to the separation of the two cells. In the polysaccharide-decomposing organism on the other hand the two cells draw away from each other leaving a connecting filament between them which eventually breaks (see fig. 1), and may remain attached to one or both cells for some time as a short stalk. Where the daughter cell has produced a daughter cell prior to separation from its mother cell a short chain of three individuals may be formed. Such structures are strikingly similar to those described by Baker (1933) under the name of "giant cocci." Baker found such giant cocci in large numbers in cavities of the cellulosic content of the intestine of the guinea pig, where he associated them with the breakdown of hemicelluloses and possibly of cellulose.

The data recorded in the previous pages have convinced the writer that the polysaccharide-decomposing organism differs fundamentally from the yeasts and that, in fact, it is impossible on the available information to group it with any existing type of microorganism, including that of the myxococci to which, as already mentioned, early investigation had indicated that it might belong. Under the circumstances, he has thought it desirable to regard it as a new species, the name of which should indicate the most characteristic property of the organism, that of reproduction by budding, and to leave it to future investigations to decide its true position among the known groups of micro-organisms, if it should be found of sufficient interest to deserve future attention.

At the suggestion of Dr. Ramsbottom of the British Museum the writer proposes that the organism be known as *Pullulomyxa botrytis* n. sp.

For the further characterisation of *Pullulomyxa botrytis* the following data have been compiled.

Motility. The organism is non-motile.

Staining properties. The ordinary aniline dyes are readily absorbed. A young culture of 2 to 3 days growth is gram posi-

tive, the stain giving good pictures of the cellular contents and of the connecting filaments between adjacent, directly related cells. A 20-day-old culture is still essentially gram positive though some of the cells will be found to take the counter stain.

Growth on agar media. No growth can be obtained on ordinary standard agar media. Even on the specially devised agar medium, growth is not visible during the first one or two days at the optimum temperature.

After 6 days incubation at 30 to 37°C. the surface colonies have attained a size of from 50 to 100 μ with an average of 80 μ. Both surface and embedded colonies are circular, with a smooth edge and a slightly granular interior. They are greyish white, transparent and moist. It is noticeable that the size of the colonies is larger on plates with a larger number of colonies than on those containing a few only. On older plates this difference is reversed and after 14 days the sparsely seeded plates may show colonies of a diameter of from 0.4 to 1.2 mm., while those on thickly seeded plates fail to exceed 0.4 mm. Older colonies are no longer transparent but retain the original moist appearance. Their colour is no longer greyish white, but faintly brownish grey.

Growth on gelatin. Growth is not easily obtained since the temperature of incubation is well below the optimum of the organism. After 17 days incubation no growth could be observed on the inoculated plates. Nevertheless, gelatin is very slowly liquefied by the organism. This was shown by adding a thick suspension of living cells to a tube of ordinary gelatin and incubating the latter at 37°C. for 17 days. By then the gelatin, when cooled, could not be made to solidify though control tubes without an addition of the organism did so.

Growth in liquid media. In the most favourable medium, described subsequently, visible growth may be noticeable at 37°C. within 24 hours. The clear liquid has become very faintly turbid and slightly bleached. Turbidity and bleaching increase slowly during the subsequent period of incubation until, after 8 days at 37°C., the medium is markedly turbid with a noticeable greyish sediment. When growth in liquid media takes place in shallow layers it is more rapid, and marked turbidity and sedi-

mentation is already noticeable after 24 hours incubation at 37°C. The more rapid development causes the cells to accumulate in clusters which may be sufficiently large within the first 48 hours of growth to be visible with a hand lens. The rapid growth in shallow layers confirms the aerobic nature of the organism. Even with the addition of suitable carbohydrates, growth is not possible under strictly anaerobic conditions.

Temperature range of growth. Initial growth, during the first 24 hours of incubation is very similar in extent throughout the range tested, between 18 and 40°C. Subsequently, however, little progress in growth is made in cultures kept at the former temperature and even at 25°C. The range from 30 to 37°C. appears to be suitable for a normal growth rate, and there is little to choose between the two extremes. The higher temperature of 40°C. has not been found suitable for the maintenance of cultures.

Lethal temperatures. A young culture kept for 30 minutes at 52°C. was found capable of propagating when subcultured into fresh medium and incubated at 37°C. An identical culture kept for the same period at 55°C. failed to do so.

pH requirements of the organism. The most abundant growth is observed when the standard medium is adjusted to a hydrogen-ion-concentration range of between pH 7.0 and 7.6. This growth can be maintained even at pH 8.0, but no growth was noticeable at a pH of 8.6. On the acid side of the neutral point, a certain amount of growth could be induced in the standard medium at a pH of 6.5 but little if any at 6.0 and below.

Composition of standard medium. When the organism was first discovered it was seen under the microscope in a medium composed of ammonium sulphate 0.01 per cent, dipotassium hydrogen phosphate 0.02 per cent and water. To this had been added 0.001 per cent of the specific polysaccharide of *Shigella dysenteriae*, Shiga. The rate of growth of the organism was very slow in this medium and it was found that the addition of a certain amount of an extract made from fresh rabbits' droppings greatly increased the rate of growth and facilitated the eventual isolation of the organism.

All subsequent work, and the cultivation of *Pullulomyxa*

botrytis, has been done in a medium the composition of which is based on the above observation.

The following procedure is adopted in the preparation of the standard medium: 10 gr. of fresh rabbit pellets are left over night at room temperature in 1 litre of tap water. The extract obtained is poured off, and rejected. It is replaced by a further litre of tap water, with which the pellets are given a short boil. The second extract thus obtained is filtered and to the clear liquid are added 2 g. of ammonium sulphate, 4 g. of dipotassium hydrogen phosphate, and 4 g. of a carbohydrate such as fructose or xylose. The volume of extract is finally made up to 2 litres and sterilised. When solid media are required, 1.5 per cent of agar agar are dissolved in the standard medium prior to its being sterilised. The sterilisation has usually been done fractionally on three successive days with one hour's steaming daily.

On or in the above medium, *Pullulomyxa botrytis* grows more abundantly than on any other tried. This, however, is not meant to imply that the growth of the organism, even under the most favourable conditions, is really copious, but merely that it is markedly richer than on the ordinary bacteriological culture media, which in most cases are unsuitable.

Utilisation of carbohydrates. In the preliminary note which appeared in Nature (1933) it was mentioned that *Pullulomyxa botrytis* destroyed the specific properties of a number of bacterial polysaccharides. This was thought to indicate that the saccharolytic enzyme complex of the organism would be able to function on a wide range of carbohydrates, since it is doubtful whether the organism could have met with polysaccharides of pathogenic bacteria in its natural habitats. It was thought of interest therefore, to test the action of *Pullulomyxa botrytis* on a wide range of carbohydrates, including several polysaccharides.

Of these, cellulose was found to remain unaffected. Gum arabic, however, and xylan as well as a polysaccharide isolated by Campbell (1935) from oak sapwood were suitable sources of carbohydrate. Starch and inulin did not promote growth. Among the disaccharides tested, cellobiose, maltose, sucrose and lactose could be utilised, the latter less readily than the three

former. Of the monosaccharides, fructose appeared more suitable than glucose. In fact it is questionable whether glucose can readily be utilised. The two pentoses tested, xylose and arabinose, were both suitable. Growth for prolonged periods on any of these carbohydrates did not in the least weaken the action of the organism on the specific bacterial polysaccharides which it was originally found capable of destroying.

It is of interest to note that *Pullulomyxa botrytis* is capable of utilising not only hemicelluloses which occur widespread in nature, but also an intermediate product of hydrolysis of cellulose. It will be remembered that attention was drawn above to the similarity in morphology between this organism and the "giant cocci" observed by Baker (1933) in the coecum of certain herbiverous animals. From a physiological point of view, therefore, a similarity also exists between the two types.

The action of *Pullulomyxa botrytis* on the various carbohydrates mentioned as suitable for growth did not lead to the production of visible quantities of acid or gas. It was decided, therefore, to study this action in the Barcroft manometer, and to determine the oxygen uptake of an active suspension of the organism on some of the carbohydrates which had been previously tested for their suitability as energy suppliers.

Using a suspension in saline of a 10-day old culture grown on xylose agar, xylose showed an oxygen uptake of 28 μl within 4 hours and a gas evolution of 52 μl, assuming this gas to have been carbon dioxide. On the assumption of the evolved gas being hydrogen the oxygen uptake was 80 μl within the first 4 hours and the gas evolution 52 μl as before.

The same suspension of the organism gave with fructose an oxygen uptake of 40 μl assuming the gas evolved during the reaction to have been hydrogen. In the case of this particular carbohydrate no carbon dioxide can have been evolved.

Glucose was unable to show an oxygen uptake by the suspension, even when the experiment was continued for 24 hours.

A considerable amount of work was devoted to the isolation of the enzyme system of *Pullulomyxa botrytis* which causes the destruction of the polysaccharides of bacterial toxines. These

efforts, however, were completely unsuccessful. Neither by autolysis, nor by freezing and disintegration of the living cells could the responsible enzymes be separated in active form. It has not been possible, therefore, to study the effect on infected animals of an injection of the polysaccharide-splitting enzyme of *Pullulomyxa botrytis*.

CONCLUSIONS

A description is given of the cytological, morphological and physiological characters of an organism which was isolated from decaying vegetable debris and which previous work had shown was capable of destroying the specific polysaccharides of certain bacterial toxins.

The cytological study has shown that the organism in question, for which the name *Pullulomyxa botrytis n. sp.* is suggested, represents a type which, to the writer's knowledge, has never before been described in the literature.

Its propagation proceeds by budding, but the cytological changes involved are far more complex than those met with in the yeasts.

Details are given of the morphological and cultural characteristics of *Pullulomyxa botrytis* n. sp. and a reference is made to the work which has been done on the isolation of the enzyme system by which the specific polysaccharide of the Shiga dysentry toxin must be assumed to be destroyed by the organism.

The writer wishes to place on record his thanks to Dr. Ramsbottom of the British Museum for his advice on nomenclature; to Mr. W. G. Campbell of the Forest Products Research Laboratory of the Department of Scientific and Industrial Research, for the supply of a sample of oak softwood polysaccharide; and to Mr. K. R. Butlin of his staff, for the determination of the respiratory properties of *Pullulomyxa botrytis*.

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