17.

Reprinted from SCIENCE, July 10, 1953, Vol. 118, No. 3054, pages 34–36.

Observations on the Formation and Behavior of "Conjugation" Cells and Large Bodies in *Azotobacter agile*[1]

Norman C. Dondero and Max R. Zelle
Laboratory of Bacteriology, Cornell University, Ithaca, New York

DURING SINGLE-CELL STUDIES on *Azotobacter agile,* strain M.B. 4.4,[2] the development of two unusual cell types—"conjugation" cells and large bodies—was observed. Löhnis and Smith (*1*) reported cellular fusion in Azotobacter, but this was considered by Lewis (*2*) to be incomplete division. The formation of large bodies in *A. agile* M.B. 4.4 was believed by Eisenstark *et al.* (*3*) to be caused by the stimulus of substances in the culture medium. The formation of large bodies was followed by time-lapse, phase-contrast, photomicrography in a *Bacillus* by Schaechter (*4*) to the point of formation of apparently motile granules, and small, nonmotile, occasionally budding elements.

So far as the authors are aware, this is the first report of observations of these forms at close intervals under conditions such that some previously advanced interpretations of the causes could be ruled out. These observations are reported because they offer support to the contention of Lewis and a possible alternative to the interpretation of Eisenstark *et al.*

The strain was routinely carried in stock on slants of modified Karlsson and Barker (*5*) selective medium in which 1 per cent glucose was substituted for 1 per cent ethyl alcohol. The medium was nitrogen free except for that contained in 0.02 per cent yeast extract. An inoculum was transferred in water of syneresis from the base of a slant, by means of a micropipet and micromanipulator (*6*), to the surface of a film of the same medium on a coverslip inverted on a moist chamber, where it could be watched with the microscope. For the initial experiments the moist chamber slides were incubated at 30° C in closed cans containing wet filter paper. For observation, the chambers were removed from the tins and placed on the microscope, which rested on a bench at room temperature. There was considerable loss of water during observation, to the extent that it became difficult to express moisture from the agar with the microneedle. Under these conditions, the viability of the microcultures was, in general, rather low. Many cells either failed to grow and divide, or divided once or twice in the usual manner and then became vacuolated and nonviable on transfer to fresh medium shortly after removal from the tin. It was noticed, in more than six separate instances, that a cell would start to divide in an unusual manner, the first sign of which appeared as a refractile granule near the center of a swollen cell. The granule elongated to become a line across the cell, and shortly after, a cleft appeared in the center of the line and proceeded along it so that the cell divided as in Fig. 1, a–d. Uneven division resulted in earlier separation at one end, thus leaving the two sections at the incompletely divided end attached by a narrow strip and giving the impression of two cells joined by

[1] From investigations supported jointly by Cornell University and the Atomic Energy Commission under contract No. AT(30-1)-1244.

[2] Obtained from A. E. Eisenstark, Oklahoma A. and M. College, Stillwater.

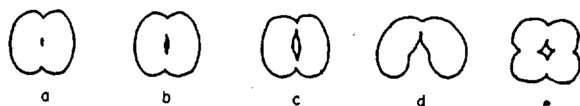

FIG. 1. Stages of division in Azotobacter in the formation of "conjugation" cells.

a tube. These forms, if seen in a stained smear, might easily be interpreted as conjugating cells, since the developmental sequence would not then be apparent. Because of the deterioration of the slide culture it was necessary to test the viability of the paired individuals by transfer to fresh medium by means of a micropipet; none of the transfers showed any signs of viability.

Still more rarely, a ring of four cells (Fig. 1e) was seen to arise by a process similar, in the early stages, to that just described. Before separation at the ends of the cleft, a new division was seen to start in a plane perpendicular to that of the division already in progress. Completion of this type of division was never observed, although it might have occurred.

It is believed that these abnormal divisions were the result of unfavorable environmental conditions induced by the method of handling the cultures, since they were never again seen after the complete apparatus was installed in an incubator room with provision for humidification.

The appearance of large bodies occurred under conditions which seemed favorable to the overwhelming majority of cells among which the large bodies arose. The cells were grown on the same agar as previously mentioned and examined *in situ* in a moist chamber in a humidified room. In populations which were otherwise normal in appearance, there would appear sporadically the abnormally large and irregularly shaped cells from which other normal- and abnormal-appearing cells stemmed. Several such incidents were closely followed and recorded. An account of one of these follows in detail.

Cells from a 48-hr microcolony grown on a coverslip at 30° C were transferred to a fresh coverslip. Abnormal forms did not appear during the first 12 hr of incubation, although a considerable number of normal cells had grown. Single cells were isolated to unoccupied areas of the agar according to routine (*6*) and further incubated for 4 hr. In one of the subcultured microcolonies of about 8 cells, 2 cells about three times normal length were seen. Each cell was again isolated to another portion of the agar. Twenty-one hours later the microcolonies formed from the long cells each contained both normal cells and large bodies. Ten hours later some of the large forms had divided to form more large cells, while others continued to enlarge without division. Large bodies often exceeded 20 μ in length and 10 μ in breadth. Lateral budding was observed in some large bodies, others constricted off the ends, and some divided into several cells simultaneously (Fig. 3a). Most of the cells were limp, flexible, and delicate. Upon rupture by light touches of the microneedle, the content of the cell, consisting of large refractile granules in a liquid of low viscosity, was released upon the surface of the agar where it would persist for a few minutes before disappearing. The released granules had no motion of their own.

The schematic summary of cell relationships (Fig. 2) is based on several pedigrees and illustrates various observed relationships in the lineage of normal and abnormal cells. To sum up the observations, the normally ellipsoidal cells were very stable with respect to morphology, and formed large bodies only at rare intervals. The apparently spontaneous appearance of large bodies was seen, however, in several independent instances. Many of the large bodies died before producing any offspring. However, when offspring were produced, normal cells, large bodies, or both could result. The resulting large bodies would behave in a manner similar to the primary large bodies in that they, in turn, would be nonviable or produce a mixed lineage. The normal-appearing ellipsoidal cells derived from large bodies were of two types—those from which a normally stable line of ellipsoidal cells arose, rarely producing large bodies, and those producing an unstable line of apparently normal cells which formed large bodies at much higher than normal rate. The incidence of large bodies, therefore, was clonal rather than general.

The similarity of the normal-cell large-body relationship to the pedigrees of Hansen and Smith (*7*) for the segregation of types of *Botrytis cinerea* through repeated monospore subculturing is sufficiently striking to suggest that the phenomenon in Azotobacter similarly may have resulted from segregation of dissimilar nuclei in a heterocaryon. Stained preparations of large bodies lent plausibility to this hypothesis since they contained numerous chromatinic structures, presumably nuclei (Fig. 3, a–c). The normal ellipsoidal cells (Fig. 3d) may also be multinucleate, as suggested by the apparently multiple chromatinic structures and inheritance pattern.

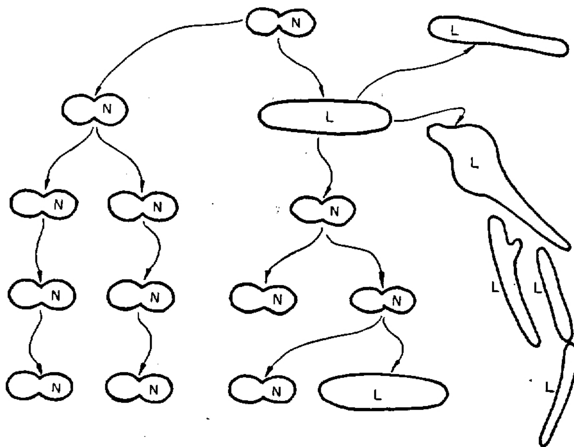

FIG. 2. Schematic relationship of large bodies and normal cells; n = normal, l = large bodies.

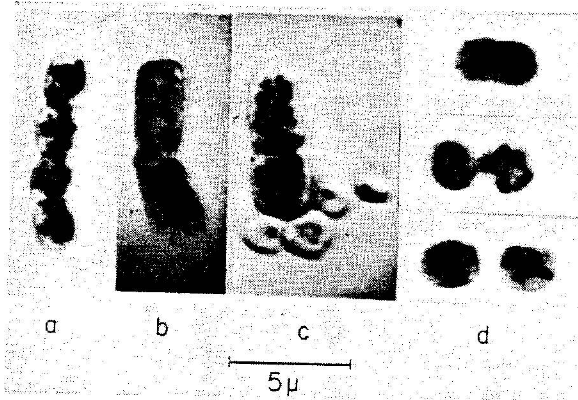

FIG. 3. a–c, Large bodies of *A. agile* M.B. 4.4. In b, the indentation in the side of the cell represents collapse of the cell wall, not incipient cell division. In c, a large body and some smaller cells from the same microcolony. Note the very small cells to the right which have resulted from irregular cell division. In d, normal cells in various stages of cell division. All preparations giemsa stained after treatment with N/1 HCl at 58° C for 8 min.

No cell fusions were observed. However, a heterocaryon could be formed by mutation in a multinucleate cell. A satisfactory explanation of the observed phenomena can be made by postulating a mutation which has no effect at low ratios of mutant to normal nuclei, but which interferes with cell division and not with nuclear division when the nuclear ratio exceeds a critical value.

Toxic factors, as the agents responsible for large body formation, were not entirely ruled out by these observations. However, the physical disposition of the cells on the surface of the agar was such that a large body could arise from a cell in direct contact with a normal cell. Because the incidence of large bodies was clonal rather than general, it seems more likely that the expressed effect was genetic rather than environmental.

Since the reported observations were subsidiary to the main purposes of the experiments being undertaken at the time, no further efforts were made to investigate alternative possibilities.

References

1. LÖHNIS, F., and SMITH, N. R. *J. Agr. Research,* **23**, 401 (1923).
2. LEWIS, I. M. *J. Bacteriol.,* **34**, 191 (1937).
3. EISENSTARK, A., WARD, C. B., and KYLE, T. S. *Ibid.,* **60**, 525 (1950).
4. SCHAECHTER, M. *Science,* **116**, 637 (1952).
5. KARLSSON, J. L., and BARKER, H. A. *J. Bacteriol.,* **56**, 671 (1948).
6. ZELLE, M. R. *Ibid.,* **61**, 345 (1951).
7. HANSEN, H. H., and SMITH, R. E. *Phytopathology,* **22**, 953 (1932).