MICROBIOLOGY

BEIJERINCKIA, A NEW GENUS OF NITROGEN-FIXING BACTERIA OCCURRING IN TROPICAL SOILS

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Almost half a century ago these Proceedings contained a paper, written by M. W. BEIJERINCK and entitled: „On oligonitrophilous bacteria". [3]. It was in this paper that the author published his discovery of a group of mobile microbes of unusual dimensions and of a very singular aspect. He decided to give them the name of *Azotobacter*, because they were evidently capable of assimilating atmospheric nitrogen, but so peculiar was their image, that BEIJERINCK hesitated between the names *Azotobacter* and *Parachromatium* for the new microbes; the latter name was intended to indicate a relationship with the genus *Chromatium*, well known among the *Thiorhodaceae*. This idea of a relationship remained active in BEIJERINCK's mind throughout his further work on *Azotobacter* and, as will appear further, it has received an unexpected justification quite recently by the work of WILSON. [17].

It is well nigh superfluous to mention that BEIJERINCK's important discovery has particularly stimulated research on microbial nitrogen-fixation all the world over.

Yet, research on the microbes, bringing about fixation of atmospheric nitrogen in the tropics, has been rather inadequate for more than one reason; especially in Indonesia. Moreover, the results obtained in this country have been rather conflicting.

In papers, dating from 5 to 8 years after BEIJERINCK's discovery of *Azotobacter*, one of his pupils, DE KRUYFF, concluded from investigations of Javanese soils that *Azotobacter chroococcum* „is very rare in tropical countries". [9]. The author mentions the presence, however, of several nitrogen-fixing bacteria, among which *Bacterium Krakataui* [8] and *Micrococcus* No. 1 [9]. These organisms have been described very superficially, but it is stated that they do not produce the well known wrinkled *Azotobacter*-pellicle on BEIJERINCK's classical mannitol-medium. This medium gradually turns into a thick, viscous mass; a striking phenomenon, also shown by the organisms, which form the object of the present paper. Of course, exact identification of DE KRUYFF's organisms is not possible with his scanty data.

Some years later the scarcity of *Azotobacter* in Javanese- and other soils was contested by GROENEWEGE [5], who was able to show the presence of typical *Azotobacter* in several kinds of soil from Java and

also from Hawai. Since then, *Azotobacter* has been found to be present in arable soils of suitable p_H, i.e. 6 or more, almost everywhere. Soil-deficiencies of some kind (for which see e.g. VAN NIEL [12]) may well inactivate *Azotobacter* in the soil and even prevent its development in BEIJERINCK's mannitol-medium, but their *presence* in these (rare) cases can as a rule be demonstrated by the addition of the necessary trace-elements to the medium mentioned.

BEIJERINCK's medium, viz. a solution made up of tap water, mannitol 2 % and K_2HPO_4 0.02 %, in thin layers, has been almost exclusively used in *Azotobacter*-studies, sometimes with some minor modifications (e.g. ASHBY's medium), until 1932. In that year WINOGRADSKY [18] published a new and very efficacious procedure for the isolation of nitrogen-fixing organisms, which consists in the distribution of a small quantity of sieved soil-particles on the surface of a nutrient silica gel-, or purified agar-plate. As will presently be seen, this method has led to an important deepening of our knowledge of nitrogen-fixing organisms.

Using sodium benzoate 0,5 % as a source of carbon, as proposed by WINOGRADSKY, some tentative experiments were made with the view of demonstrating the presence of *Azotobacter* in samples of arable soil from the Royal Botanic Garden in Buitenzorg. Without exception, typical *Azotobacter chroococcum* could be detected in such soils within a couple of days and several strains were isolated, differing in production and intensity of the well known black colour. An aberrant strain, not darkening on staying and producing a green fluorescent substance, soluble in the medium, was also isolated and provisionally labelled *Azotobacter vinelandii*.

Quite negative results, however, were obtained at the examination of forest-soils, these being either humus-rich soils from woody parts of the Buitenzorg garden (250 m. above sea-level), or soil from the virgin-forest in the neighbourhood of the Tjibodas mountain-garden (1400 m. above sea-level). The reaction of these soils was slightly acid (p_H 4.5—5.5) and, as could be anticipated, *Azotobacter* failed completely in these samples. This makes it probable that the principal reason of DE KRUYFF's failure to demonstrate *Azotobacter* in Javanese soil was an unfortunate choice of his soil-samples with regard to the p_H. It should be considered indeed, that the notion of p_H and the knowledge of its great influence on microbial life were still embryonic in the years 1906—1909, when the pioneer work of SÖRENSEN, MICHAELIS and CLARK was only just making a start (1909—1914—1915)!

However, when the benzoate-agar plates sprinkled with forest-soil, were re-examined after two or three weeks standing, very remarkable colonies had made their appearance. They are almost porcelain-white and rise high above the surface of the agar (some of them are almost spherical) and consist of microbes embedded in a tough elastic matter. Microscopic examination reveals the extremely uncommon appearance

of the microbes themselves: at first sight they resemble strongly refractive diplococci of about 1.5 μ in diameter, *lying some distance* (0.3—0.7 μ) *apart*. Closer examination shows these "cocci" to be united in pairs by an enveloping membrane, which often appears to be more or less constricted between them. The "cocci" are coloured yellow-brown by LUGOL's iodine-solution; methylene-blue and gentian-violet leave them colourless, but the intermediate space between two "cocci" is strongly stained; at the same time, a well defined capsular slime-mass is revealed in some strains.

Of course, it soon became clear that the organisms are in reality straight, or slightly curved, rods of about 3 μ by 1—1.5 μ, containing two polar globular bodies of lipoid character, which occupy the total breadth of the microbe and eventually cause a distinct local dilatation of the cell wall. The lipoid bodies are mostly of equal, but sometimes of different dimensions and may in older cultures become so voluminous as to completely distend the bacterial cells into large, misshapen and branched bodies.

The description has been given in such detail to make it clear that, though working on aerobic nitrogen-fixing organisms and in particular with *Azotobacter*, in no stage of the experiments with these newly unveiled organisms the slightest thought occurred to me that they might be a particular species of *Azotobacter*, or even an organism in some way related to this genus. In fact, I was quite puzzled!

I was aware, it is true, of the work of ALTSON [1] who, in 1936, had isolated "*Azotobacter*" from acid Malayan soils — a quarzite soil of p_H 4.5 and an alluvial soil of p_H 4.6 — *by means of the same sieved-soil distribution method of* WINOGRADSKY (on silica-gel-mannitol plates). But ALTSON states emphatically — and without giving further particulars on the morphology of his microbes — that „he studied the morphology of the organism during a period of about eighteen months" (l.c. p. 274) and that „microscopic examination showed that this (gelatinous growth) consisted of typical cells of *Azotobacter*" (l.c. p. 269). For this reason, ALTSON's paper was not given much attention at first. Yet, the manner in which this author found his organism, as well as its cultural and physiological behaviour, agree so closely with the corresponding properties of the organisms found in Buitenzorg-soils, that I am now quite convinced of the identity, or at least the very close parentage, of his — unnamed! — microbes and the organisms found here. Notwithstanding the fact that the latter most decidedly do not consist of typical *Azotobacter*-cells!

It will not be difficult now to imagine my astonishment in finding in the 6th edition of BERGEY's manual, which had just reached me, the description of *Azotobacter indicum* STARKEY and DE; *a description fitting in all details the organisms, which I had isolated* and which have been just described.

STARKEY and DE's original publication [16], containing two good microphotographic images and a subculture of the original strain of their *Azotobacter indicum* (kindly put at our disposal by KLUYVER from the collection at the Laboratory of Microbiology at the Technical University of Delft) sufficed to remove any doubt as to the identity of some of the Buitenzorg strains with *Azotobacter indicum* STARKEY and DE.

However, I am unable to understand, how ALTSON (whose earlier work is not mentioned by STARKEY and DE) as well as STARKEY and DE could include the organism in the genus *Azotobacter*, a genus showing such distinctive morphological and cultural characters. Neither has HOFER [6] in his investigation on the type of flagellation of several *Azotobacter* species, *among which Azotobacter indicum*, expressed any doubt on the systematic position of the latter organism.

Azotobacter indicum and its congeners are indeed different from the known Azotobacters in almost every respect: *morphologically* (small, single rods with hyaline protoplasm containing two (or sometimes three, in "buckled" rods) polar, strongly refractive lipoid-spherules; versus stout, short, mostly doublerods with distinctly differentiated (granulated) protoplasm in *Azotobacter sp.*); *culturally* (steadily increasing viscosity of the culture-solution by formation of a ropy to elastic slime; versus formation of a gradually thickening supernatant pellicle in *Azotobacter*-cultures) and, above all, *physiologically* (development in nutrient media with p_H 3.5—9, gradual formation of acid, which lowers the medium's initial p_H to about 3.5, versus growth only in media of $p_H > 6$ and no acid formation during growth of *Azotobacter*). These distinctive characters were, curiously enough, known to STARKEY and DE; they were established by these authors and could be confirmed on all points by our investigations.

In fact, the only feature these organisms have in common with typical *Azotobacter*, is their capacity of nitrogen-fixation under aerobic conditions!

It is therefore not surprising to find that the justness of incorporating *Azotobacter indicum* in the genus *Azotobacter* has already been called in question before.

After STARKEY's lecture on *Azotobacter indicum*, at the meeting held at New Brunswick, N.J. by the Third Commission of the International Society of Soil Science (Session of August 31, 1939), BURK put the following questions: „I would not ask if this organism just described is *Azotobacter*, but I would ask you, Dr STARKEY, this: „Is there any *doubt in your mind* that it is *Azotobacter?*" and „So I would ask, does any doubt lurk in your mind that this may not be *Azotobacter?*" [15].

From STARKEY's answer it becomes clear that it is „above all the capacity of using molecular nitrogen in the absence of available fixed nitrogen" (together with strict aerobiosis, non-sporulation, motility, etc.), which induced him to range the new organism under *Azotobacter*. Presumably ALTSON too has been primarily led by the same — or similar — arguments.

In matter of nitrogen-fixation, even with the restriction to aerobic nitrogen-fixation, it does not seem either advisable or justified to incorporate all known free living, aerobic, nitrogen-fixing organisms in one genus *Azotobacter*. Not only should such a physiological genus in that case also contain certain *Nostocaceae*, but the capacity of nitrogen-fixation has been shown in the past to be incident to several „common" microbes, belonging to very different classes and genera, such as *Bacterium lactis viscosum*, *Bact. radiobacter*, *Bact. prodigiosum*, *Bact. pyocyaneum*, *Bacillus danicus*, *Bac. malabarensis* etc. Nitrogen-fixation has even been claimed for some Saccharomycetes, such as *Torula bogoriensis rubra* DE KRUYFF [10], *Torula Wiesneri* ZIKES [19] and perhaps still other ones!

It is true that these assumptions have been regarded with some mistrust, but quite recently GEST and KAMEN have discovered an active nitrogenase-system in *Rhodospirillum rubrum*, and WILSON [17] very recently has succeeded in demonstrating nitrogen-fixation by all the species investigated of *Athiorhodaceae* and *Thiorhodaceae*; organisms not suspected hitherto of this faculty. This disclosure, by the way, involuntarily calls to mind BEIJERINCK's postulate of the parentage of *Azotobacter* and *Chromatium!*

But there are still other reasons, which induce to consider physiological genera with some circumspection:

Numerous are the instances encountered in the course of microbiological research, in which potencies, *which are not essential to the perpetuation of the species*, may not only become latent under certain conditions, but may actually be lost and vanish irreversibly. This may occur either by processes of adaptive selection within a population or by processes of — or equivalent to — mutation, spontaneous or induced, e.g., by radiation.

With special regard to *Azotobacter* e.g., it has been recognized that the development of these microbes is not essentially dependent on the ability to fix atmospheric nitrogen. This ability is not a *conditio sine qua non* for their multiplication and dispersion; they may thrive on bound inorganic nitrogen compounds as well. Consequently the nitrogenase-system can be dispensed with, without endangering the survival of the species, and it has been found indeed that *Azotobacter*-strains maintained in pure culture — i.e. under conditions artificial and very dissimilar to those prevailing in Nature — may ultimately lose the ability to assimilate free nitrogen (SCHRÖDER [13]).

On the other hand it seems not contrary to reason to presume that *Azotobacter*-clones, which are not — or no longer — able to fix atmospheric nitrogen might very well occur in nature; yet, as long as we only have at our disposal a method for the accumulation of *Azotobacter*, which is based on an elective action with respect to the ability of development in the absence of nitrogen, it is obvious that only those individuals will grow, multiply and finally appear, which are in possession of an active

nitrogenase. Nevertheless the presumed nitrogenase-less *Azotobacter*-forms should have just as much right to the generic name *Azotobacter* as their *confrères*, which have definitely lost the nitrogenase in the course of their seclusion in culture tubes, stuffed with artificial food.

A final remark on the question of physiological genera: Certainly it would be imprudent to generalize and to assert that the fixation of free nitrogen is a distinctive character of the genus *Nostoc*, but it would be absurd to split up this genus into two genera, one for the *Nostoc*-species, able to fix nitrogen and another for the *Nostoc*-species, which lack this ability.

Without wishing, at the present stage of the investigations, to put forward more than the merest suggestion toward a working-hypothesis, I should like to indicate some rather striking points of resemblance of *Azotobacter indicum* c.s. to species of the genus *Rhizobium* FRANK.

Members of this genus (especially from the *Medicago-Melilotus*-group) also show a decided tendency to acid formation in the course of their development and often produce an abundant slimy — and occasionally viscous — gum. Morphologically the rods of *Azotobacter indicum* c.s., often irregular and buckled, are reminding of the bacteroids of some Rhizobia, in which polar lipoid-bodies, equally coloured yellow-brown by dilute iodine-solution, are common (see e.g. the original drawings of BEIJERINCK [2] of the nodule-bacterium of *Vicia Faba*).

On the other hand, of course, the Rhizobia-*bacteroids* show no motility as is manifestated by *Azotobacter indicum*; they do not multiply as such, nor do they fix atmospheric nitrogen *in vitro*. It should, however, be kept in mind that the properties of *Rhizobium* sp. may have been profoundly modified by — and adapted to — their symbiotic mode of life. As well as parasitism, symbiosis may be the cause of the loss of several qualities originally present!

However this may be, in view of the very peculiar morphology of *Azotobacter indicum* and the related species, which have been found in tropical soils during these investigations, it appears quite justified to create a new genus for these organisms. It seems only natural to the present author to give this genus the name of BEIJERINCKIA, in honour of one of the great pioniers in the field of general bacteriology, M. W. BEIJERINCK (16 March 1851—1 Jan. 1931) who, on New-Year's eve 1900, for the first time used the name *Azotobacter* for the aerobic nitrogen-fixing organisms he discovered.

The new genus may be defined as follows:

BEIJERINCKIA *nov. gen.*

Straight, or slightly curved, or irregular, locally swollen to buckled, rods, characterized by the presence, at the extremities, of highly refractive spherical bodies, presumably consisting of lipoids. No endospores. Motility

present, at least in certain stages of the development. Flagellation peritrichous (HOFER, J. of Bact. **48**, 697—701 (1944)).

Decidedly aerobic; developing in media with p_H 3.5—9 and fixing atmospheric nitrogen in the absence of nitrogen compounds. Growth is accompanied by the formation of acid and, in some species, by the formation of large amounts of a tough to elastic slime. No surface pellicle is formed on liquid media. No development on peptone-broth-agar. Gram negative.

Found in soils of tropical countries.

Species typica: *Beijerinckia indica* (STARKEY et DE) Derx comb. nov.; syn. *Azotobacter indicum* STARKEY et DE, Soil Sci. **47**, 329—343 (1939), Science **89**, 267 (1939); *Azotobacter sp.* ALTSON Journ. of Agric. Sc. **26**, 268—280 (1936).

Distinctively characterized by the formation of leavan from saccharose and by the formation of a rusty-red to fulvous colour on media kept neutral, e.g. by the addition of $CaCO_3$.

In a separate paper some other species and forms of *Beijerinckia* will be described.

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