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ENDOSYMBIOTIC BACTERIA ASSOCIATED WITH PLANT SEEDS AND BIRDS' EGGS

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FOREWORD

During the last three years, in cooperation with a colleague of mine at the University of São Paulo – Brazil, we have been coordinating a research project on 'Nitrogen Fixing Bacteria in non-legume plants'.

Three labs were involved in the development of the project. My group in Lab 1 was responsible for finding, isolating and purifying the strains of bacteria collected in nature and agricultural plantations. The two other laboratories were respectively responsible for the taxonomical classification and the molecular characterization of the bacteria species collected. During the first two years, after the start of the cooperative project, our group collected and purified over 50 species of nitrogen fixing bacteria from different non-leguminous plants, along with several other bacteria that were used in the master theses of two students involved in the project. Unfortunately, the taxonomical identification and the molecular characterization of the collected nitrogen fixing bacteria, for which the two other labs were responsible, were not done for various reasons and this motivated me to enhance a habit I normally have which is to individually analyze, in addition to the basic problems of the main project, which I was part of, anything interesting that occurs as a sideline. This had already happened to me in the 1950s, when I was working with Prof. Theodosius Dobzhansky on 'Natural population of Drosophila in Brazil'. At that time I discovered *Rhyncosciara angelae*, an extraordinary insect not related to Drosophila, but excellent to cytogenetics and gene action studies. In that case the work on Drosophila was proceeding very successfully and later we published excellent papers, which was expected having Dobzhansky as the leader of the groups. But even so during the development of the main project I was looking for any other interesting thing that appeared in our field excursion. In one of them I discovered groups of larvae of *Rhyncosciara angelae* and with this exceptional insect I later published alone or with colleagues several papers which are among the most important in my scientific carrier.

In the research with fixing nitrogen bacteria the result was a failure, but the sideline observations resulted in an important discovery. I have discovered the existence of endosymbiotic bacteria living normally in great number inside seeds of plants and eggs of birds which I believe is a discovery important enough to be described here.

Introduction

The title of this article is: 'Endosymbiotic bacteria associated with plant seeds and birds' eggs' which is part of a more general project that we are organizing entitled: 'Endosymbiotic bacteria in plants and birds'.

Endosymbiotic bacteria are part of the body of the organism with which they are associated and are present not only in the seeds or eggs of the respective organisms.

In plants it is easier to follow the distribution of the endosymbiotic bacteria in the body of the host, as parts of the embrions in development, roots, stem, leaves and finally in the flowers which are responsible for the origin of new seeds.

Bacteria are the most ancient, the structurally simplest, and the most abundant of organisms, and are the only ones characterized by a prokaryotic organization of their cells (Raven & Johnson, 1992). The contact of bacteria with other organisms is very common, and in plants and animals these associations may have cases of negative, neutral or positive consequences.

Plants are normally associated with different types of bacteria, externally (exophytic) and internally inside the body (endophytic). Plants or animals and bacteria may present a symbiotic relation that is the living together in close association of two dissimilar organisms in which both partners have advantages in living together. They can also present an endosymbiotic process when one of the partners live intimately and permanently inside the host (plant or animal) in a symbiotic association.

A great number of bacteria in nature have the ability to fix the nitrogen, which constitutes about 80% of the earth's atmosphere. This fixed nitrogen is very important for the survival of these bacteria and any excess can be and is frequently used by plants in different types of contact with these bacteria. For example, leguminous plants have a specific association with the nitrogen fixing bacteria of the genera Rhizobium as a symbiont, that is, it may live inside nodules in the root of leguminous plants but it can also survive and develop in nature in the soil. In each generation the nodules symbionts are produced by Rhizobium contamination through newly formed nodules in the plant roots.

Rhizobium in nature, when in contact with leguminous plants, is able to induce the formation of nodules in the root of the plant inside of which the bacteria develop, survive and produce nitrogen compounds which serve as fertilizer for the plant. This process has been known for more than a century and in the last decades it is in worldwide use in agricultural cultures with great success.

Nitrogen fixing bacteria endophytics, different from the Rhizobium group, living inside non-leguminous plants, were first described by Dobereiner and Pedrosa (1987) and presently there are several groups working in this field.

Until now, among the fixing nitrogen endophytic bacteria, there has not been found any association of a specific bacteria to a specific plant in such a way that the system could be exploited in agriculture. But even so this unspecified association is very important for the natural vegetation and for agriculture itself. As mentioned in the pre-introduction of this paper this is what we are working on in our laboratory in Brazil and we have found that a great number of the non-leguminous plants analyzed have endophytic nitrogen fixing bacteria associated with them.

The endosymbiotic bacteria in plants that we are analyzing in this article are found in the seeds and other parts of the organism and apparently they breed only inside the host and are transmitted to the next generation through the newly formed seeds.

We still are unable to determine whether in each seed there is only one or more than one type of this endosymbiotic bacteria. What we know is that every seed in dozens of plants which we have detailedly tested contains a great abundance of bacteria which are transmitted to some parts of the embryos in development, and on to the mature plant in which they are found in relatively small quantity in several parts of it and in the newly formed seeds in which they are more abundant.

In our experiment with bacteria living inside the plant seeds, frequently we may isolate more than one type although this does not mean that we may have more than one endosymbiotic bacteria in a simple seed. At the moment we still do not have enough data to distinguish the endosymbiotic from the simple endophytic and exophytic bacteria that also can exist in the seeds. The problem is that to isolate the bacteria from the seed we have to clean or decontaminate the seed with a solution of sodium hypoclorite (4%), alcohol (70%) and distilled sterile water.

Besides following the recommendations of the specialist we also doubled the time for this disinfecting but even so, we have found in several cases more than one species of bacteria isolated from a single decontaminated seed. We are now attempting to determine the taxonomic and molecular classification of these bacteria to be able to separate the endosymbiotic, endophytic and infectious bacteria coming from the seeds.

The association of a Leguminous plant and the nitrogen fixing bacteria Rhizobium is very important and well used in modern agriculture. The details of this association and of the nitrogen fixing process operated by the bacteria are today very well-known and used. It is of interest that together with this association with the Rhizobium the Leguminous plants also have an association with the Endosymbiotic bacteria, in this case not related to the fixation of nitrogen that we are describing in this article. Rhizobium are related to nodules produced in the root and only in the root of the plant, while the Endosymbiotic are more concentrated in parts of the seed and less frequent in other parts of the plant which is being investigated at the present time. In beans the contamination of the seeds come from bacteria present in certain parts of the flower. The flower produces the pod inside of which are the seeds. The bean seed is formed by two attached cotiledons having one embryo in between the two. In the embryos, formed by three sections attached together; one may distinguish two pre-leaves, one pre-stem and one pre-root. A great number of bacteria can normally be found in the cotiledons and embryos of the developed seeds. Details of their appearance and behavior may be seen in the www.eca.usp.br/nucleos/njr/pavan, which we are furnishing as part of this publication.

It is of interest to analyse the development of the seeds in peanuts (*Arachis hypogaea*), a plant of the Leguminosae family. We will transcribe a summary description of peanuts found in the Encyclopaedia Britannica: 'The flowers are borne in the axils of the leaves; what appears to be a flower stalk in a slender calyx up to 40 millimeters (1.6 inches) long. After pollination and the withering of the flower, an unusual stalklike structure called a peg is thrust from the base of the flower toward the soil. In the pointed tip of this slender, sturdy peg the fertilized ovules are carried downward until the tip is well below the soil surface. Only then does the tip start to develop into a characteristic pod where the seeds are formed.

Peanuts are a concentrated food; pound for pound, peanuts have more protein, minerals and vitamins than beef liver, more fat than heavy cream and more food energy (calories) than sugar'.

When referring to peanuts in the above comparison, in reality it is meant that the seeds of the peanuts are a concentrated food. Being so, it is of interest to know that these seeds have within their parts a great quantity of live bacteria which we are studying to find out if they are only a part of the food content or, what we think is more probable, if they are responsible for part of the process of food production or of the production of substances necessary for the development of the embryos.

Peanuts, being a Leguminous plant, are also a host to the bacteria Rhizobium in its root nodules that fix nitrogen through the synthesis of nitrogen compounds which are furnished to the plant as fertilizers.

In our experience with over one hundred seeds from different species and varieties of plants analysed, we may conclude that the presence of many bacteria inside the seeds is practically the rule.

In bird eggs, we analysed eggs of domestic chickens (about nine varieties), guinea hen, pheasant and quail. In all of them we found a similarity with our description for the domestic chicken and so we will discuss these most common chicken eggs for which endosymbiont bacteria are found in large quantities in the yolk. Since the chicken eggs have few cells, plenty of food reserved for the development of the embryos and a great number of bacteria, the egg phase is one in which the proportion of bacterial genetic material in relation to the chicken genetic material must be equivalent. This also is the situation in some plant seeds that we analysed.

Since we could not breed these bacteria in artificial medium we had to study the egg bacteria in their natural habitat. We began by boiling the eggs and observing what happens with the different components of the eggs and with the bacteria they contain.

One surprise was the ability of a ponderable quantity of bacteria to survive a submergence of the egg for 30 minutes in boiling water. Under these conditions the rest of the egg had the expected reaction: the yolk and the egg white coagulate normally. After separating the egg shell, the egg white and the yolk, a certain quantity of each isolated part of the cooked egg is mixed with distilled water and placed in a blender for a few minutes then subsequently placed in the autoclave (120°C for 20 minutes under pressure). These broths of the isolated parts of the chicken egg are individually mixed with a normal medium to make a combined medium in experiments to breed new bacteria.

Another surprise ensued when the autoclaved yolk broth was studied under a microscope. A substantial number of bacteria were still alive and moving normally in the space available on the microscope slide. It looks like these bacterias, although participating in their apparent activities, do not multiply regularly. This would be expected since the normal bacteria (the non autoclaved) in the artificial medium we offer them, do not divide themselves. It seems on the other hand that we have cases in which the autoclaved culture when left in glass tubes for a week or more shows an increased amount of the condensed material in the tubes. One possible explanation is that the bacteria lost the capacity of reproducing themselves but are still alive and active in the culture medium. We are testing these possibilities.

On the other hand, there are common cases in which certain bacteria are represented by colonies in which the individuals are attached to the neighbors forming a collar. These colonies in the microscope slide are in constant movement, whether in a concentrated colony or when the collar is stretched (see Home Page).

Collar-like colonies of bacteria can be found occasionally in non-autoclaved yolk but they are rare and the colonies may have a maximum of ten to fifteen individuals. Normally they move slowly and never as rapidly as the autoclaved ones.

We still do not know the origin of these bacteria and their roles in the autoclaved yolk medium.

The pieces of coagulated yolk that are present in the autoclaved yolk broth when examined under the microscope show beside its normal yolk compounds, aglutinated in pieces, some bubbles of gas and different pieces of gel or gum eliminated from the yolk, with many different and interesting figures. The gas and the gel figures are secreted by the coagulated yolk and at least part of these products appear to come from the coagulated yolk during the time that it is between the slides in the microscope.

We still cannot say if this elimination of gas and gel from the piece of autoclaved yolk constitutes material recently synthesized or had been there in a deposit inside the pieces of coagulated yolk. There are cases of movement and elimination of gas and gel 24 hours after the microscope slide was prepared. Very interesting are the great number of structures produced by the gum or gel moving free in the medium under microscope observation.

Of great interest, beside the shape and physical structure of the bodies observed in the microscope slide, is the movement that some of these bodies exhibit and for which at present we have no explanation (see Home Page).

In conclusion, we may say that, beside the scientific discovery of this new type of association between bacteria and plants and bacteria and birds, we expect that knowing the role of the bacteria in the seeds of plants and the eggs of birds will help to understand better the processes of development of the embryo and of the organism itself. Other important practical results can be obtained after elucidating the role these bacteria have in those processes.

Intracellular bacteria of the type we presented in seeds of plants and eggs of birds are very common in many species of Arthropods. The first case described by Hertig and Wolbach (1924) deals with a rickettsiae-like organism in reproductive tissues of the mosquito *Culex pipiens*. This bacteria was later described as *Wolbachia pipientis* by Hertig (1936) and after the eighties, other genera and species of intracellular bacteria were described and a great number of others Arthropods organisms and nemotods were shown to present the symbiotic association with this type of bacteria.

Different from what we know until now about the symbiotic association of bacteria with plants and birds, the bacteria of the Genus Wolbachia and the Cytophaga-like organism (CLO) associated with Arthropods induce several reproductive abnormalities like feminization, parthenogenesis, cytoplasmic incompatibility, these override the chromosomal sex determination, to convert infected genetic males into females and to induce embryonic lethality in the embryos that result when uninfected females are mated to infected males [Werren J.H. and Windsor D. (2000); Kostas, B. and Scoll, O'Neil (1998); Selivon, D. *et al.* (2002); Rousset, F. *et al*. (1992); Werren, J.H. *et al*. (1995); Weeks, A.R. *et al.* (in press); Kyei-Poku *et al*. (2005)].

It looks as though the symbiotic association of bacteria with plants and birds is more balanced than that which occurs in Arthropods. If anything similar to what occurs in Arthropods should have occurred in certain plants like beans, or in chickens it would have been discovered long ago. Genetic studies on normal beans Phaseolus or soya beans and domestic birds like chicken have been done for a long time and in a thorough way. If any of the endosymbiotic bacteria could cause in plants or birds one of the reproductive abnormalities that Wolbachia and related species may cause in Arthropods should have been discovered long ago. As a matter of fact I am surprised that these bacteria existing in such a large number and in so many plants and birds were not discovered until now.

It looks like the endosymbiosis which occurs in plants and birds are more balanced than the ones that occur in Arthropod. With the data we have until now, we will classify the endosymbiotic bacteria present in the seeds of plants and in the eggs of birds as friendly and auspicious partners.

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