1986: Centenary of the Isolation of Denitrifying Bacteria

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Art historians remember that the French Impressionists exhibited together for the last time in 1886, but microbiologists have their own reason for taking special centennial notice of that year. It was then, a short time before the appearance of Hellriegel and Wilfarth's pioneering paper on recognition of the necessary role of "lower organisms" in symbiotic nitrogen fixation (7), that two French investigators first reported the isolation of a pure culture of nitrogen-releasing bacteria, the denitrifiers (6), and the 19th century's most outstanding investigator, Louis Pasteur, contributed both directly and indirectly to the breakthrough. During a 3-year period a century ago, microbiologists unveiled the procaryotes operating at the entry and exit points in the nitrogen cycle.

Useful as denitrification may be globally, its impoverishing effects fall heavily on agriculture. Financially expensive and labor-intensive remedies for the scarcity or depletion of nitrogen resulting from denitrification in crop and pasture soils include purchase and spread of nitrogen fertilizer and rotation of legumes with crop plants in fallow soil to encourage symbiotic nitrogen fixation. Yet, despite their unrelenting activity, the diazotrophs may fail to provide full replacement of nitrogen. The denitrifiers are equally relentless.

Fermentation chemists and soil scientists were aware of the phenomenon of microbial depletion of nitrogen more than 100 years ago. Recognition preceded isolation of the responsible agents in pure culture by 30 years. As early as 1856, Reiset (14) reported that decaying plant and animal materials incessantly pour out (*déversent*) nitrogen into the atmosphere. Although he has received little credit for his insight, Reiset was the first to issue an experiment-based statement that nitrogen is cycled through the biological world. (Dubbing the nitrogen cycle the Reiset cycle would not be inappropriate.)

Pasteur soon followed with what must certainly be the least known of his scientific observations. Concerned as he was in 1859 with establishing the microbial origin of fermentation, he erroneously ascribed to "lactic yeast" the gas-producing reduction of naturally occurring nitrates* at the expense, he presumed, of hydrogen produced during fermentation of beetroot juice (12). He may certainly be excused his error. Isolating pure cultures was not yet a standard process, and, after all, he was right about the suspected origin of the gas-nitrate.

During the next 15 years, Smith (19), Schloesing (16), and Reiset (15) further determined that "nitric acid" was indeed the source of the nitrogen released during "fermentation." I use quotation marks around these terms because, even at this early stage, the authors had cleverly differentiated true fermentation from reduction of nitrates and related the latter process to the apparent use of the oxygen atoms of the nitrates for the combustion of carbon compounds and the release of carbon dioxide. In 1868, Schloesing (16) observed the release of nitrogen oxide gases and rejoiced that a worker as renowned as Pasteur had affirmed that "*le gaz nitreux*" liberated during apparent fermentation arose from the reduction of nitrates. (Although he gave no reference, Schloesing must certainly have had Pasteur's note from 1859 in mind.) Schloesing soon associated anaerobic conditions with the process (17).

Meusel (10) first associated microorganisms unequivocally with nitrogen losses when he noted that antiseptic-sensitive agents identified as mixed populations of bacteria in soil and natural waters reduced nitrates to nitrites and beyond. Three years later, Schloesing and Müntz established the bacterial etiology of nitrification, thus explaining a property of soil that had been exploited for nitrate manufacture for decades (18). It was left to Gayon and Dupetit (5) to introduce the term denitrification in 1882 and further to demonstrate the antagonistic effect of heat as well as oxygen on the process. They also showed that individual organic compounds such as sugars, oils, and alcohols could supplant complex organic materials and serve as reductants for nitrate. That same year, Déherain and Maquenne (1) (formally presented by Pasteur when they read their paper before members of the Academy of Sciences in Paris) carefully confirmed the evolution during denitrification of small amounts of the nitrogen oxide gases along with elemental nitrogen. More significantly, these workers also first correlated the quantity of organic substrate consumed and the volume of gas released. The year 1883 brought Duclaux's dismaying realization that bacteria that produced nitrates and those that destroyed them developed *c&e-h-c&e* in plots employed for the commercial manufacture of nitrates (2). This meant that the denitrifiers were normal, not enthetic, residents of soil and were not to be controlled by prior treatment of fertilizer or by other exclusionary methods.

* "Les nitrates, que renferme naturellement le jus de la betterave, se décomposent, et il se forme de grandes quantitks de vapeur nitreuse à la surface des cuves" **(12).** [Nitrates, which the juice of beetroot naturally includes, decompose, and great quantities of nitrous vapor form at the surface of the vats."]

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Informative as these earlier experiments were, the modern era of studies of denitrification began in 1886 with Gayon and Dupetit's report of the isolation in pure culture of two strains of denitrifying bacteria (6). We thus commemorate the centenary. Although the original isolates are no longer extant, many strains like them have since been isolated. Today we realize that no clear and certain distinction separates denitrifiers from diazotrophs. Even though nondiazotrophic *Pseudomonas* and *Alcaligenes* species are the most numerous of the denitrifiers we find in 30 genera, some diazotrophic *Azospirillum, Rhizobium,* and *Rhodopseudomonus* species can also denitrify (13).

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In the best tradition of scientific discovery, the pioneering team that isolated the denitrifiers consisted of a well-established investigator with wide-ranging interests and a young and energetic worker, seemingly on the threshold of a brilliant career. Born in Bouëx, Charente, in 1845, the elder partner, Leonard-Ulysse Gayon (he abandoned his first name in all his writings), is best known and widely acknowledged as the originator of modern enology. He began his higher education in 1867 as a student of École Normale Superieure in Paris and was named *agrégé préparateur* in Pasteur's laboratory in 1871. He was awarded the Doctor of Science degree in 1875. After leaving Paris and Pasteur, with whom he maintained a lifelong friendship, Gayon served for many years as professor of chemistry at the University of Bordeaux and as chief chemist for the Customs Office for the Port of Bordeaux. With Pasteur's strong backing (9), he founded in 1880 the Agronomy and Oenology Station at Bordeaux and served for 40 years as its director. A member of the Administrative Council of the Society for Physical and Natural Sciences of Bordeaux when his collaboration with Dupetit began, he then served as vice president of that society from 1889 to 1892. The Academy of Sciences, Belle Lettres, and Arts of Bordeaux also inducted him to membership in 1884. From 1900 to 1905 he was dean of the faculty of sciences of the university. The city of Bordeaux honored Gayon by naming a street for him.

Gayon's development, with Millardet (11), of Bordeaux mixture, a copper sulfate and lime preparation first used in 1883 to treat mildew infections of grapevines, stands as a significant and lasting contribution to 19th century science and agriculture. Again with Pasteur's strong support, Gayon was named to fellowship in the National Academy of Agriculture in 1884 (9). A few years later, he was named Chevalier de la Legion d'Honneur. One of his graduate students, Laborde, proposed naming a newly isolated fungus in his honor, *Eurotiopsis gayoni* (9). Gayon is best remembered for the work that occupied the last half of his life, that is, the many and varied studies of the quality and validity of the wines of his region. His contributions did not stop there, for in 1949 his grandson, Jean Ribereau-Gayon, was appointed director of the organization now called the Institute of Oenology, and in 1976 Ribereau-Gayon was succeeded in turn by his own son, Pascal Ribéreau-Gayon, the current director.

In contrast with Gayon, who led so long and distinguished a life in science, Auguste-Gabriel Dupetit (who also abandoned his first name) could manage only a brief career and died tragically young. Born in 1861 in Auch, Gers, he went to Bordeaux in 1878 as a pharmacy student. Upon completion of his studies in 1881, he was named *préparateur* in chemistry, and at the Agronomy Station of the Southwest, he began his short-lived but telling colleagueship with Gayon. Together they published a range of short scientific reports before bringing out their classic paper on the initial axenic culturing of denitrifiers.

Gayon's early research in Pasteur's laboratory centered for a time on chemical changes generated by microbes in decaying eggs. He refuted claims of spontaneous degradation in that process and moved to a new field, the development of assays for determining the quality of beer. Afterwards, he focused his attention on fungi and a number of aspects of the fermentation of sugars (9). Once Dupetit joined him, studies of chemical control of plant diseases and the microbial reduction of nitrates occupied much but not all of their interest. Indeed, Gayon continued active collaboration with others, while Dupetit interested himself in mushroom toxins. An award-winning paper on the topic that he read in 1885 was published posthumously in 1887 (4). Dupetit's skill at constructing experimental devices added much to their success together (3).

Motivated perhaps by patriotism and loyalty to Pasteur, Gayon and Dupetit ignored Koch's use of boiled potato slices or nutrient gelatin for isolation of bacterial colonies (8) and chose instead a type of enrichment culturing for axenic selection. They installed a long, narrow, spiraled race tube in a container called Pasteur's *matras* filled with sterile nitrate broth, inoculated at the top of the spiral with a drop of sewage, and incubated at 35°C (6). A mineral salts-asparagine-citrate medium soon replaced the rich bouillon they began with. Taking frequent samples from the medium at the end of the race tube, they inoculated fresh media seratim until a microscopically homogenous population appeared.

Of several isolates obtained this way, two of the most active were selected for further studies. In a letter responding to Gayon's request for advice about naming the isolates, Pasteur revealed his annoyance with taxonomy, particularly as practiced by Germans (9). He suggested provisional naming and the following of one's own thoughtful inspiration. Clearly, study of the isolates' characteristics appealed to him more than nomenclature. Playing the role of the former major professor in classic form, Pasteur urged Gayon to show an interest in the fate of the oxygen of the nitrate utilized. If he did not, others were sure to do so. Dutifully, Gayon assigned the name *Bacterium denitrificans* to the isolates and differentiated them with the Greek letters α and β. Little that was new about the phenomenon emerged from Gayon and Dupetit's lengthy studies of the cultures, but they did anticipate possible slighting of the significance of their research by showing that the isolates could be returned to sterile soil and still function there as denitrifiers. Again heeding the master's advice, they discussed the fate of nitrate oxygen but continued to accept the seductively logical notion that the bacteria used the oxygen of nitrate for combustion of organic matter and generation of CO_2. They ingeniously dispelled any lingering notion that hydrogen generated during fermentation simply chemically reduced the nitrate when they showed that *Bacillus amylobacter,* a prodigious hydrogen producer, did not reduce nitrate. Attempting to gain insight into the mechanism of denitrification, Gayon and Dupetit noted that denitrifying cultures generated much more heat than did yeast cells carrying out fermentation. They suggested a role for the heat in driving the reaction, which was agreed to be anything but simple.

For all his success in the work with Gayon, Dupetit's life ended tragically within months of the appearance of their classic paper. Inexplicably no longer employed at Bordeaux and traveling under an assumed name, the young man took a room in the Albergo Svizzero in Savona, Italy, on the evening of 28 December 1886 and took his own life by injection of poison. Despite efforts to save him, he died on the morning of the 29th. Newspaper accounts from later that day reveal that he left a note in French indicating that strong worries drove him to suicide. Use of the name Gaston Dunault was designed to hide his identity and thus spare his parents embarrassment, but records kept by the *Anagrafe* of Savona show that the disguise was swiftly penetrated and his true identity was soon known. (I am grateful to Sergio Casella of the University of Pisa and to Nora Rossi, a student from Savona in his laboratory, for providing this information.)

With Dupetit's death, Gayon abandoned further work with the denitrifiers, owing perhaps to sorrow, perhaps to growing devotion to enology and the increasing pressure of administrative duties. Fortunately, workers in Germany, Great Britain, Italy, and The Netherlands rapidly developed an interest in the bacteria and kept the study alive well into the 20th century, with Jensen and Beijerinck assuming the most active roles (13). Today, many agricultural and ecological field studies of denitrification are under way. Moreover, species in 29 genera of true bacteria and one archebacterial genus are known to denitrify, and several enzymes unique to the process have been identified and purified-progress that would certainly have pleased Gayon and Dupetit had they lived to see it.

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