Associative and Endophytic Nitrogen-fixing Bacteria
and Cyanobacterial Associations

Nitrogen Fixation: Origins, Applications, and Research Progress

VOLUME 5

The titles published in this series are listed at the end of this volume.

Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations

Edited by

Claudine Elmerich
Institut Pasteur,
Paris, France

and

William E. Newton
Department of Biochemistry
Virginia Polytechnic Institute and State University
Blacksburg, Virginia, U.S.A.

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"Colonization of a wheat root hair by *Azospirillum*. Photograph courtesy of Claudine Elmerich, Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette, and Institut Pasteur, Paris, France, and reproduced with permission."

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PREFACE TO THE SERIES

Nitrogen Fixation: Origins, Applications, and Research Progress

Nitrogen fixation, along with photosynthesis as the energy supplier, is the basis of all life on Earth (and maybe elsewhere too!). Nitrogen fixation provides the basic component, fixed nitrogen as ammonia, of two major groups of macromolecules, namely nucleic acids and proteins. Fixed nitrogen is required for the N-containing heterocycles (or bases) that constitute the essential coding entities of deoxyribonucleic acids (DNA) and ribonucleic acids (RNA), which are responsible for the high-fidelity storage and transfer of genetic information, respectively. It is also required for the amino-acid residues of the proteins, which are encoded by the DNA and that actually do the work in living cells. At the turn of the millennium, it seemed to me that now was as good a time as any (and maybe better than most) to look back, particularly over the last 100 years or so, and ponder just what had been achieved. What is the state of our knowledge of nitrogen fixation, both biological and abiological? How has this knowledge been used and what are its impacts on humanity?

In an attempt to answer these questions and to capture the essence of our current knowledge, I devised a seven-volume series, which was designed to cover all aspects of nitrogen-fixation research. I then approached my long-time contact at Kluwer Academic Publishers, Ad Plaizier, with the idea. I had worked with Ad for many years on the publication of the Proceedings of most of the International Congresses on Nitrogen Fixation. My personal belief is that congresses, symposia, and workshops must not be closed shops and that those of us unable to attend should have access to the material presented. My solution is to capture the material in print in the form of proceedings. So it was quite natural for me to turn to the printed word for this detailed review of nitrogen fixation. Ad's immediate affirmation of the project encouraged me to share my initial design with many of my current co-editors and, with their assistance, to develop the detailed contents of each of the seven volumes and to enlist prospective authors for each chapter.

There are many ways in which the subject matter could be divided. Our decision was to break it down as follows: nitrogenases, commercial processes, and relevant chemical models; genetics and regulation; genomes and genomics; associative, endophytic, and cyanobacterial systems; actinorhizal associations; leguminous symbioses; and agriculture, forestry, ecology, and the environment. I feel very fortunate to have been able to recruit some outstanding researchers as co-editors for this project. My co-editors were Mike Dilworth, Claudine Elmerich, John Gallon, Euan James, Werner Klipp, Bernd Masepohl, Rafael Palacios, Katharina Pawlowski, Ray Richards, Barry Smith, Janet Sprent, and Dietrich Werner. They worked very hard and ably and were most willing to keep the volumes moving along reasonably close to our initial timetable. All have been a pleasure to work with and I thank them all for their support and unflagging interest.

Nitrogen-fixation research and its application to agriculture have been ongoing for many centuries – from even before it was recognized as nitrogen fixation. The Romans developed the crop-rotation system over 2000 years ago for maintaining and improving soil fertility with nitrogen-fixing legumes as an integral component. Even though crop rotation and the use of legumes was practiced widely but intermittently since then, it wasn't until 1800 years later that insight came as to how legumes produced their beneficial effect. Now, we know that bacteria are harbored within nodules on the legumes' roots and that they are responsible for fixing N_2 and providing these plants with much of the fixed nitrogen required for healthy growth. Because some of the fixed nitrogen remains in the unharvested parts of the crop, its release to the soil by mineralization of the residue explains the follow-up beneficial impact of legumes. With this realization, and over the next 100 years or so, commercial inoculants, which ensured successful bacterial nodulation of legume crops, became available. Then, in the early 1900's, abiological sources of fixed nitrogen were developed, most notable of these was the Haber-Bosch process. Because fixed nitrogen is almost always the limiting nutrient in agriculture, the resulting massive increase in synthetic fixed-nitrogen available for fertilizer has enabled the enormous increase in food production over the second half of the 20^{th} century, particularly when coupled with the new "green revolution" crop varieties. Never before in human history has the global population enjoyed such a substantial supply of food.

Unfortunately, this bright shiny coin has a slightly tarnished side! The abundance of nitrogen fertilizer has removed the necessity to plant forage legumes and to return animal manures to fields to replenish their fertility. The result is a continuing loss of soil organic matter, which decreases the soil's tilth, its water-holding capacity, and its ability to support microbial populations. Nowadays, farms do not operate as self-contained recycling units for crop nutrients; fertilizers are trucked in and meat and food crops are trucked out. And if it's not recycled, how do we dispose of all of the animal waste, which is rich in fixed nitrogen, coming from feedlots, broiler houses, and pig farms? And what is the environmental impact of its disposal? This problem is compounded by inappropriate agricultural practice in many countries, where the plentiful supply of cheap commercial nitrogen fertilizer, plus farm subsidies, has encouraged high (and increasing) application rates. In these circumstances, only about half (at best) of the applied nitrogen reaches the crop plant for which it was intended; the rest leaches and "runs off" into streams, rivers, lakes, and finally into coastal waters. The resulting eutrophication can be detrimental to marine life. If it encroaches on drinking-water supplies, a human health hazard is possible. Furthermore, oxidation of urea and ammonium fertilizers to nitrate progressively acidifies the soil – a major problem in many agricultural areas of the world. A related problem is the emission of nitrogen oxides (NO_x) from the soil by the action of microorganisms on the applied fertilizer and, if fertilizer is surface broadcast, a large proportion may be volatilized and lost as ammonia. For urea in rice paddies, an extreme example, as much as 50% is volatilized and lost to the atmosphere. And what goes up must come down; in the case of fertilizer nitrogen, it returns to Earth in the rain, often acidic in nature. This uncontrolled deposition has unpredictable environmental effects, especially in pristine environments like forests, and may also affect biodiversity.

Some of these problems may be overcome by more efficient use of the applied fertilizer nitrogen. A tried and tested approach (that should be used more often) is to ensure that a balanced supply of nutrients (and not simply applying more and more) is applied at the right time (maybe in several separate applications) and in the correct place (under the soil surface and not broadcast). An entirely different approach that could slow the loss of fertilizer nitrogen is through the use of nitrification inhibitors, which would slow the rate of conversion of the applied ammonia into nitrate, and so decrease its loss through leaching. A third approach to ameliorating the problems outlined above is through the expanded use of biological nitrogen fixation. It's not likely that we shall soon have plants, which are capable of fixing N_2 without associated microbes, available for agricultural use. But the discovery of N_2-fixing endophytes within the tissues of our major crops, like rice, maize, and sugarcane, and their obvious benefit to the crop, shows that real progress is being made. Moreover, with new techniques and experimental approaches, such as those provided by the advent of genomics, we have reasons to renew our belief that both bacteria and plants may be engineered to improve biological nitrogen fixation, possibly through developing new symbiotic systems involving the major cereal and tuber crops.

In the meantime, the major impact might be through agricultural sustainability involving the wider use of legumes, reintroduction of crop-rotation cycles, and incorporation of crop residues into the soil. But even these practices will have to be performed judiciously because, if legumes are used only as cover crops and are not used for grazing, their growth could impact the amount of cultivatable land available for food crops. Even so, the dietary preferences of developed countries (who eats beans when steak is available?) and current agricultural practices make it unlikely that the fixed-nitrogen input by rhizobia in agricultural soils will change much in the near-term future. A significant positive input could accrue, however, from matching rhizobial strains more judiciously with their host legumes and from introducing "new" legume species, particularly into currently marginal land. In the longer term, it may be possible to engineer crops in general, but cereals in particular, to use the applied fertilizer more efficiently. That would be a giant step the right direction. We shall have to wait and see what the ingenuity of mankind can do when "the chips are down" as they will be sometime in the future as food security becomes a priority for many nations. At the moment, there is no doubt that commercially synthesized fertilizer nitrogen will continue to provide the key component for the protein required by the next generation or two.

So, even as we continue the discussion about the benefits, drawbacks, and likely outcomes of each of these approaches, including our hopes and fears for the future, the time has arrived to close this effort to delineate what we know about nitrogen fixation and what we have achieved with that knowledge. It now remains for me to thank personally all the authors for their interest and commitment to this project. Their efforts, massaged gently by the editorial team, have produced an indispensable reference work. The content is my responsibility and I apologize upfront for any omissions and oversights. Even so, I remain confident that these volumes will serve well the many scientists researching nitrogen fixation and related fields, students considering the nitrogen-fixation challenge, and administrators wanting to either become acquainted with or remain current in this field. I also acknowledge the many scientists who were not direct contributors to this series of books, but whose contributions to the field are documented in their pages. It would be remiss of me not to acknowledge also the patience and assistance of the several members of the Kluwer staff who have assisted me along the way. Since my initial dealings with Ad Plaizier, I have had the pleasure of working with Arno Flier, Jacco Flipsen, Frans van Dunne, and Claire van Heukelom; all of whom provided encouragement and good advice – and there were times when I needed both!

It took more years than I care to remember from the first planning discussions with Ad Plaizier to the completion of the first volumes in this series. Although the editorial team shared some fun times and a sense of achievement as volumes were completed, we also had our darker moments. Two members of our editorial team died during this period. Both Werner Klipp (1953-2002) and John Gallon (1944-2003) had been working on Volume II of the series, *Genetics and Regulation of Nitrogen-Fixing Bacteria*, and that volume is dedicated to their memory. Other major contributors to the field were also lost in this time period: Barbara Burgess, whose influence reached beyond the nitrogenase arena into the field of iron-sulfur cluster biochemistry; Johanna Döbereiner, who was the discoverer and acknowledged leader in nitrogen-fixing associations with grasses; Lu Jiaxi, whose "string bag" model of the FeMo-cofactor prosthetic group of Mo-nitrogenase might well describe its mode of action; Nikolai L'vov, who was involved with the early studies of molybdenum-containing cofactors; Dick Miller, whose work produced new insights into MgATP binding to nitrogenase; Richard Pau, who influenced our understanding of alternative nitrogenases and how molybdenum is taken up and transported; and Dieter Sellmann, who was a synthetic inorganic chemistry with a deep interest in how N_2 is activated on metal sites. I hope these volumes will in some way help both preserve their scientific contributions and reflect their enthusiasm for science. I remember them all fondly.

Only the reactions and interest of you, the reader, will determine if we have been successful in capturing the essence and excitement of the many sterling achievements and exciting discoveries in the research and application efforts of our predecessors and current colleagues over the past 150 years or so. I sincerely hope you enjoy reading these volumes as much as I've enjoyed producing them.

William E. Newton
Blacksburg, February 2004

PREFACE

Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations

This book is part of the seven-volume series that was launched a few years ago with the ambitious objectives of reviewing the field of nitrogen fixation from its earliest beginnings through the millennium change and of consolidating the relevant information - from fundamental to agricultural and environmental aspects – all in one place. Volume 5 covers the biology of bacteria that associate with non-leguminous plants. The subject matter includes a wide range of associations; it covers the bacterial species that associate either with the surface or within the tissues of grasses (often referred as plant growth-promoting rhizobacteria) and also the symbiotic associations that cyanobacteria form with fungi, algae, and both lower and higher plants. This volume does not deal with the *Frankia*-actinorhizal plant associations, which is the topic of Volume 6.

The book is divided in 13 chapters, each of which is the work of well-known scientists in the field. Just like in the other volumes of this series, the first chapter is an historical perspective. It describes how, as early as the end of the 19th century, it was shown that plant exudation favoured the proliferation of soil bacteria in the rhizosphere, and how the first nitrogen-fixing bacteria, including cyanobacteria were isolated. The chapter covers the landmarks and scientific concepts that arose from more than one century of research in this area.

Recently, implementation of the techniques of molecular phylogeny has led to the identification of an increasing number of N_2-fixing genera and species associated with grasses. The taxonomic status of both old and recently discovered species of the α- and β-subgroups of the Proteobacteria is the topic of the second chapter. Chapter 2 also outlines the ecology of these genera and then describes both tools and molecular probes that can be used for *in situ* localization of associated bacteria, in particular, to distinguish the bacteria located on the root surface from the endophytes resident within the plant tissues.

The genetics and regulation of nitrogen fixation in free-living bacteria is dissected in detail in Volume 2, however, it is of such importance that selected coverage of this subject is provided here in Volume 5, especially as it relates to the current understanding of the *nif* genetics of the most important grass-associated species; *Azospirillum brasilense*, *Herbaspirillum seropedicae*, *Gluconacetobacter diazotrophicus, Azoarcus sp.,* and *Pseudomonas stutzeri.* Indeed, Chapter 3 uses the established knowledge of *Klebsiella* and *Azotobacter nif* genetics as a basic framework on which to provide a comprehensive and comparative view of the grass-associated bacterial systems, while simultaneously emphasizing the unique features of each system and their regulatory networks.

Five chapters of Volume 5 focus on the molecular bases of the plant growth-promotion effect and the plant response to inoculation. Chapters 4 and 5 review more specifically the physiological and molecular bases of the root colonization. The molecular mechanisms of chemotaxis and the role of the chemotactic response in adaptation to the soil and plant rhizosphere are reviewed in Chapter 4. Chapter 5 continues the colonization process, from attachment through to root-surface colonization, with a detailed review of the involvement of flagella, pili, and surface polysaccharide components. This chapter also presents a comprehensive analysis of the factors required for rhizosphere competence both at the physiological and genetic levels. Next, the idea that plants benefit from associated bacteria as a consequence of microbial phytohormone production was launched more than 50 years ago and this is the subject of Chapter 6. It is apparent that soil bacteria produce a wide range of plant hormones and that there is a multiplicity of biosynthetic pathways. For example, the routes for indole-3-acetic acid biosynthesis differ in plants, in pathogenic bacteria, and in plant-associated N_2-fixing bacteria. Chapter 6 describes this multiplicity of pathways and discusses the role(s) of these compounds in the association.

Chapter 7 reviews the overall plant response to inoculation, including the changes in root morphology, root metabolism, and effect on plant productivity. It also includes a review of the effect of *Azospirillum* and other bacterial inoculation on legume nodulation. To complete the presentation of plant-growth promotion by inoculation, Chapter 8 deals with the role of the N_2-fixing bacteria associated with grasses as biocontrol agents, even though the amount of information in the particular case of nitrogen fixers is still limited. Biocontrol is the property of beneficial bacteria to compete with pathogens through, for example, antibiosis, iron sequestration, or aggressive root colonization. The chapter also describes the mechanisms of activation of plant defences.

Although Chapters 4 to 8 include information on the colonization ability of a range of microorganisms, the main emphasis is on *Azospirillum* as the paradigm for root-surface colonization. With the discovery some 15 years ago of endophytic associations involving N_2-fixing bacteria that did not cause disease symptoms, a new research era arrived. The example of *Azoarcus* is treated in Chapter 9, which reviews the phylogeny and physiology of *Azoarcus* and related bacteria. It describes the cytology and the molecular biology of the interaction of *Azoarcus* with rice and Kallar grass. Chapter 10 deals with sugarcane-cropping systems and focuses on the diversity of N_2-fixing bacteria associated with sugarcane. It emphasizes the modes of endophytic colonization and the molecular biology of both *G. diazotrophicus* and *H. seropedicae*.

Cyanobacteria coverage is limited to two chapters, but additional information on the physiology, genetics, and genomics of cyanobacteria is given in Volume 2, *Genetics and Regulation of Nitrogen Fixation in Free-living Bacteria*, and Volume 3, *Genomes and Genomics of Nitrogen-fixing Organisms*. Because differentiation of the non-N_2-fixing vegetative cells into N_2-fixing heterocysts is crucial for a successful cyanobacterial symbiosis, Chapter 11 summarizes current knowledge of the physiology and genetics of filamentous cyanobacteria, emphasizing the differentiation process. This chapter is followed by a comprehensive and extensive review of the various plant associations involving filamentous cyanobacteria. Chapter 12 describes the biology of the different symbioses of cyanobacteria with diatoms, *Geosiphon*, lichens, liverworts, hornworts, mosses, *Azolla*, Cycads, and *Gunnera*. Volume 5 then concludes with a chapter describing the potential of endophytic nitrogen fixers for the future and discusses the ideal model of a diazotrophic endophyte.

It took several years to compile the contents of this volume and to finalize the chapters. We give special recognition to all the authors, who shared their knowledge and ideas in this fascinating field, and we hope that their invaluable contributions will promote nitrogen-fixation and related research efforts and drive us onward to more spectacular discoveries in the future.

We give a special thought to Johanna Döbereiner, a leading figure in this field, who passed away in 2000. This volume is dedicated to her memory. Many researchers learnt from her and are proud to have done so; they continue to work in her spirit. We also remember Jean-Paul Aubert, deceased in 1997, for his support of nitrogen-fixation research for more than 20 years. Finally, we thank our families, friends, and colleagues for their interest and continual support during the time spent editing this volume.

Claudine Elmerich
Gif-sur-Yvette, April 2005

William E. Newton
Blacksburg, April 2005

LIST OF CONTRIBUTORS

Gladys ALEXANDRE
Departments of Biochemistry,
Cellular and Molecular Biology,
and Microbiology,
The University of Tennessee,
Knoxville, TN 37996, USA.
Email: galexan2@utk.edu

Beatriz Eugenia BACA
Centro de Investigaciones en
Ciencias Microbiológicas,
Benemérita Universidad Autónoma
de Puebla, CP72000 Puebla,
Pue, MÉXICO.
Email: ebaca@siu.buap.mx

René BALLY
Ecologie Microbienne,
UMR CNRS 5557,
Université Claude Bernard Lyon 1,
USC INRA 1193
Bâtiment G. Mendel,
43 Bd du 11 Novembre 1918,
69622 Villeurbanne Cedex
FRANCE.
Email: bally@univ-lyon1.fr

Birgitta BERGMAN
Department of Botany,
Stockholm University,
SE-10691 Stockholm,
SWEDEN.
Email: bergmanb@botan.su.se

Sofie DOBBELAERE
Technology Transfer Office,
Department of Research Affairs,
Ghent University, Kuiperskaai 55,
B-9000 Gent, BELGIUM.
Email: sofie.dobbelaere@UGent.be

Claudine ELMERICH
Biologie Moléculaire du Gène chez
les Extrêmophiles, Département de
Microbiologie, Institut Pasteur,
75724 Paris Cedex 15, FRANCE.
Email: elmerich@pasteur.fr

Anton HARTMANN
GSF-National Research Center for
Environment and Health,
Institute of Soil Ecology,
Rhizosphere Biology Department,
Ingolstaedter Landstrasse 1,
D-85764 Neuherberg / Munich,
GERMANY.
Email: anton.hartmann@gsf.de

Robert HASELKORN
Department of Molecular Genetics
and Cell Biology,
The University of Chicago,
Chicago, Illinois 60637, USA.
Email: r-haselkorn@uchicago.edu

Thomas HUREK
Laboratory of General Microbiology,
University of Bremen,
P.O. Box 33 04 40,
D-28334, Bremen,
GERMANY.
Email: thurek@uni-bremen.de

Christina KENNEDY
Department of Plant Pathology,
University of Arizona,
Tucson, AZ 85721, USA.
Email: ckennedy@u.arizona.edu

Sunhee LEE
Albert Einstein College of Medicine,
Jack and Pearl Resnick Campus,
1300 Morris Park Avenue,
Forchheimer Building, Room 717N,
Bronx, NY 10461, USA.
Email: slee@aecom.yu.edu

William E. NEWTON
Department of Biochemistry,
Virginia Polytechnic Institute and
State University,
Blacksburg, VA 24061, USA.
Email: wenewton@vt.edu

Yaacov OKON
Department of Plant Pathology and
Microbiology, Otto Warburg Centre
for Agricultural Biotechnology,
Faculty of Agricultural, Food and
Environmental Quality Sciences,
Hebrew University of Jerusalem,
Rehovot 76100, ISRAEL
Email: okon@agri.huji.ac.il

Fábio O. PEDROSA
Department of Biochemistry and
Molecular Biology, Universidade
Federal do Paraná, C. Postal 19046,
81531 990 - Curitiba, PR, BRAZIL.
Email: fpedrosa@ufpr.br

A.N. RAI
Department of Biochemistry,
North-Eastern Hill University,
Shillong-793022, INDIA.
Email: rai_amar@hotmail.com

Ulla RASMUSSEN
Department of Botany,
Stockholm University,
SE-10691 Stockholm,
SWEDEN
Email: ulla.rasmussen@botan.su.se

Barbara REINHOLD-HUREK
Laboratory of General Microbiology,
University of Bremen,
P.O. Box 33 04 40,
D-28334, Bremen, GERMANY.
Email: breinhold@uni-bremen.de

Veronica REIS
EMBRAPA Agrobiologia
C. Postal 74505,
23851 970 - Seropédica, RJ,
BRAZIL.
Email: veronica@cnpab.embrapa.br

Michael SCHMID
GSF-National Research Center for
Environment and Health,
Institute of Soil Ecology,
Rhizosphere Biology Department,
Ingolstaedter Landstrasse 1,
D-85764 Neuherberg / Munich,
GERMANY.
Email: michael.schmid@gsf.de

Eric W. TRIPLETT
Department of Microbiology and
Cell Science, University of Florida,
P.O. Box 110700, Gainesville,
FL 32611-0700, USA.
Email: ewt@ufl.edu

Els VANBLEU
Centre of Microbial and Plant
Genetics, Department of Applied
Plant Sciences, K.U. Leuven,
Kasteelpark Arenberg 20,
3001 Heverlee, BELGIUM.
Email:
Els.Vanbleu@biw.kuleuven.be

Jozef VANDERLEYDEN
Centre of Microbial and Plant Genetics, Department of Applied Plant Sciences, K.U. Leuven,
Kasteelpark Arenberg 20,
3001 Heverlee, BELGIUM
Email: Jozef.Vanderleyden@biw.kuleuven.be

Igor B. ZHULIN (JOULINE)
Joint Institute for Computational Sciences, The University of Tennessee – Oak Ridge National Laboratory, Oak Ridge,
TN 37831, USA.
Email: ijouline@utk.edu

Johanna Döbereiner (1924 – 2000)

This volume is dedicated to the memory of Johanna Döbereiner in recognition of her forty-nine years of research in soil microbiology. Johanna Döbereiner was born in Czechoslovakia in 1924, she studied agronomy at the University of Munich and, in 1951, emigrated with her family to Brazil. She started work in the "soil microbiology laboratory" in the National Department for Agricultural Research of the Ministry of Agriculture in Seropédica, which later became the EMBRAPA. Johanna was at the centre of biological nitrogen-fixation research from the early discovery of *Azotobacter paspali* associated with the roots of *Paspalum notatum* until the "endophytic" associations of N_2-fixing bacteria within the tissues of forage grasses, cereals, and sugarcane. She published more than 500 scientific papers and she was ranked seventh among Brazilian scientists in the citations of her publications and the first amongst female scientists. But above all, those of us who understood her strong personality prized her friendship, her encouragement, and her capacity to face work as happy and enthusiastic as a person going on holiday. Johanna was more than a leader, she was a mother to many scientists (and a grandmother to the youngest), and she was a great friend and a source of pride for all of us. Johanna was awarded the degrees of Doctor *Honoris Causa* by both the University of Florida, USA, and the Universidade Federal Rural do Rio de Janeiro, plus the National Frederico de Menezes Viega Prize, the Bernard Houssay Prize, the UNESCO Science Prize, the Science and Technology Prize of Mexico, the Order of Rio Branco, the Order of Merit of the National Judiciary, and the Order of Merit of the Federal Republic of Germany. She was a member of the Academy of Sciences of the Vatican, the Brazilian Academy of Sciences, and the Third World Academies of Sciences. We thank V. Massena Reis, A. A. Franco, J. I. Baldani, M. C. Prata Neves, R. M. Boddey, V. L. Divan Baldani, and F. Pedrosa for supplying this dedication.