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CHARACTERIZATION OF A MAJOR CLUSTER OF GENES INVOLVED IN NITROGEN FIXATION AND ANOTHER REQUIRED FOR INDOLE-3-ACETIC ACID BIOSYNTHESIS IN THE SUGARCANE ENDOPHYTE, ACETOBACTER DIAZOTROPHICUS

by

Sunhee Lee

A Dissertation Submitted to the Faculty of the DEPARTMENT OF PLANT PATHOLOGY
In Partial Fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

2001
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Sunhee Lee entitled Characterization of a major cluster of genes involved in nitrogen fixation and another required for Indole-3-acetic acid biosynthesis in the sugarcane endophyte, Acetobacter diazotrophicus and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Christina Kennedy
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March 1, 2001
STATEMENT BY AUTHOR

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SIGNED: ___________
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DEDICATION

Affectionately dedicated to my family

my parents WonHyung Lee and Eunju Kim

my sister, Ran Lee and brothers, Dongho Lee and Donghyun Lee
TABLE OF CONTENTS

LIST OF FIGURES ................................................................. 8
LIST OF TABLES ............................................................... 9
ABSTRACT ........................................................................... 10

I. INTRODUCTION .................................................................. 12
   1. Rational and significance .................................................. 12
   2. Review of literature ....................................................... 16

2.1. Nitrogen cycle .................................................................. 16
     2.1.1. Importance of Biological Nitrogen Fixation .............. 17
     2.1.2. Nitrogenase and regulation ................................... 22
     2.1.3. $nif$ and nitrogen fixation-associated genes ............ 29
     2.1.4. Nitrogen control system ....................................... 33

2.2. *Acetobacter diazotrophicus* .......................................... 36
     2.2.1. Characteristics .................................................. 38
     2.2.2. Infection, colonization and transmission ................. 40
     2.2.3. Genetic diversity ................................................. 43

2.3. Sugarcane ....................................................................... 45
     2.3.1. Botany .............................................................. 45
     2.3.2. Sugarcane production ......................................... 48
     2.3.3. Nitrogen requirements for sugarcane growth ........... 50
     2.3.4. Industrial usage of sugarcane ............................... 52

2.4. Indole-3-acetic acid (IAA) ............................................. 54
     2.4.1. IAA biosynthetic pathways .................................. 56
         2.4.1.1. Indole-3-acetic acid pathway ......................... 58
         2.4.1.2. Indole-3-acetamide pathway ....................... 61
         2.4.1.3. Trytamine pathway ................................... 63
         2.4.1.4. Indole-3-acetonitrile pathway .................... 63
     2.4.2. Regulation of bacterial IAA production ................. 64
     2.4.3. Physiological effects of microbial IAA ................... 68

2.5. Cytochrome c biogenesis genes ($ccm$ genes) .................. 71
     2.5.1. Common principles of cytochrome c biogenesis .......... 71
2.5.2. Molecular mechanisms of cytochrome c biogenesis ....... 72
  2.5.2.1. System I ........................................ 72
  2.5.2.2. System II ....................................... 83
  2.5.2.3. System III ..................................... 84

2.5.3. Function of cytochrome c .............................. 85

3. Dissertation Format ........................................ 86
  3.1. Analysis of nif gene operon in Acetobacter diazotrophicus
       (Appendix A) ........................................ 88
  3.2. Characterization of the cytocrome c biogenesis gene (ccm gene) operon
       of Acetobacter diazotrophicus and IAA production (Appendix B) ... 88
  3.3. Characterization of indole-3-acetic acid production in Acetobacter
diazotrophicus and assessment of its role in sugarcane growth
       enhancement (Appendix C) ................................... 90

II. PRESENT STUDY ........................................... 91
  1. Significant Results ..................................... 91
  2. Summary and Concluding Remarks ......................... 94

REFERENCES .................................................................. 98

APPENDIX A. SUNHEE LEE, ALEXANDER RETH, DIETMAR MELETZUS,
       MYRNA SEVILLA AND CHRISTINA KENNEDY. 2000.
       CHARACTERIZATION OF A MAJOR CLUSTER OF NIF, FIX, AND
       ASSOCIATED GENES IN A SUGARCANE ENDOPHYTE, ACETOBACTER
       DIAZOTROPHICUS. J. BACTERIOL. 182:7088-7091 ................. 127

APPENDIX B. SUNHEE LEE, EUDORADO ESCAMILLA, AND CHRISTINA
       KENNEDY. 2001. CHARACTERIZATION OF THE CYTOCHROME C
       BIOGENESIS GENE (CCM GENE) OPERON OF ACETOBACTER
       DIAZOTROPHICUS AND IAA PRODUCTION .......................... 134

APPENDIX C. SUNHEE LEE, ELIZABETH PIERSON, AND CHRISTINA
       KENNEDY. 2001. CHARACTERIZATION OF INDOLE-3-ACETIC ACID
       PRODUCTION IN ACETOBACTER DIAZOTROPHICUS AND ASSESSMENT
       OF ITS ROLE IN SUGARCANE GROWTH ENHANCEMENT ............ 183
# LIST OF FIGURES

Fig. 1. The nitrogen cycle ................................................................. 18

Fig. 2. Estimates of amount of fixed nitrogen on a global scale ..................... 19

Fig. 3. World nitrogen fertilizer consumption, 1960-1999 .................................. 21

Fig. 4. Regulation of nitrogen fixation ...................................................... 25

Fig. 5. Comparison of the organization of the \textit{nif} gene cluster in different diazotrophs .......................................................... 34

Fig. 6. Sugarcane growing areas of the world .............................................. 46

Fig. 7. World sugarcane production by continent, 1999 .................................... 47

Fig. 8. Sugarcane plant showing different parts ........................................... 49

Fig. 9. Sugarcane and raw sugar production, and area of sugarcane harvest in the world .......................................................... 51

Fig. 10. Pathways of IAA biosynthesis ..................................................... 59

Fig. 11. Possible scheme of cytochrome c biogenesis in typical Gram-negative bacteria .......................................................... 73

Fig. 12. A working model of the system II for cytochrome c biogenesis .............. 74

Fig. 13. A working model of the system III for cytochrome c biogenesis .............. 75

Fig. 14. Comparison of the bacterial cytochrome c maturation operon ............... 82
LIST OF TABLES

Table 1. Strategy to protect nitrogenase from oxygen ........................................... 27

Table 2. The proposed function of nitrogen associated genes and their mutant phenotypes .......................................................... 30

Table 3. Physiological effect of IAA ........................................................................ 55

Table 4. IAA producing bacteria and their biosynthetic pathway .......................... 57

Table 5. Regulation of IAA levels .......................................................................... 65

Table 6. Cytochrome c biogenesis genes (ccm homologs) identified in Gram-negative bacteria .......................................................... 76

Table 7. Conserved motifs of cytochrome c biogenesis genes ............................... 80
ABSTRACT

*Acetobacter diazotrophicus* is a true endophyte of sugarcane and is often found in plants grown in agricultural areas of low nitrogen fertilizer input. Results from our laboratory, using mutant strains of *A. diazotrophicus* unable to fix nitrogen, have shown that there are two beneficial effects of *A. diazotrophicus* on sugarcane: one dependent on nitrogen fixation, and the other independent of nitrogen fixation. A plant growth promoting substance like indole-3-acetic acid (IAA) may represent the latter effect that accounts for improved plant growth.

My first project was to characterize the genes responsible for nitrogen fixation, and determine their regulation. In summary, I have isolated, sequenced, and analyzed the major 31.5 kb nif gene cluster, including both nif and associated genes of *A. diazotrophicus*. This cluster of 33 genes represents the largest and most complete assembly of contiguous nif/fix and associated genes characterized in any diazotrophic bacterial species.

My second project has been to determine whether nitrogen fixation and/or IAA production are important for the ability of *A. diazotrophicus* to stimulate plant growth. In order to determine the role of IAA directly, mutants of *A. diazotrophicus* producing reduced amounts of IAA were generated by Tn5 mutagenesis. Among IAA⁻ candidates, one excreting less than 6% of IAA compared to the parent strain was further characterized. The mutation was mapped to genes involved in cytochrome c biogenesis (ccm genes-cytochrome c maturation genes). A Nif⁺/Iaa⁻ double mutant and Nif⁻ mutant were constructed by inserting a chloramphenicol cassette into nifD region.
Plant inoculation experiments using mutant strains also demonstrated that *A. diazotrophicus* could stimulate plant growth regardless of N availability, as evidenced by the significant growth difference between plants inoculated with wild type and uninoculated plants. Under N-limiting conditions plants inoculated with wild type had greater height and biomass than plants inoculated with Nif' or Nif'/Iaa' mutants, suggesting nitrogen fixation by *A. diazotrophicus* stimulates sugarcane growth. Plants inoculated with Iaa' mutants were always comparable to uninoculated plants regardless of N availability, indicating that IAA biosynthesis is a major bacterial factor influencing sugarcane growth, particularly under N-sufficient conditions.
I. INTRODUCTION

1. Rational and Significance

Throughout the world agriculture depends on chemical fertilizers to achieve and maintain the high yields that are possible with modern crop cultivars. Industrial production of nitrogen fertilizer demands large inputs of energy. Synthetic nitrogen use has grown from 3 million to 80 million tons over the last 40 years (134). The current annual worldwide expenditure for fertilizer nitrogen exceeds $20 billion (201). Recent reports of negative effects from these chemicals highlight the importance of developing new production methods that do not require synthetic nitrogen (6, 137). Such methods may also be sustainable, agronomically and economically, as well as environmentally safe.

The natural process of Biological Nitrogen Fixation (BNF) plays a critical role in the achievement of harmless nitrogen fertilizer. Increased use of BNF may help alleviate global warming and ground- and surface-water contamination (6). Several species of microorganisms are responsible for converting atmospheric nitrogen into forms that are usable by plants and animals. The most important and extensively studied system for agriculture is the legume-rhizobia symbiosis (45, 121). In this relationship many legumes can grow well even under nitrogen-deficient conditions, with the contributions of nitrogen fixing bacteria.
Cereals and other nonlegumes usually require heavy applications of nitrogen fertilizer for good yields. If these species could be genetically manipulated to form effective symbiotic or associative nitrogen-fixing systems, it would decrease or remove the major dependence on nitrogen fertilizer. Several approaches are possible to maximize the use of BNF for economically important crops (163, 235). First, the symbiotic genes in legumes could be transferred to cereals to enable them to have nitrogen-fixing symbioses (56, 66). Second, transferring nitrogen-associated genes into cereals to construct ‘nitrogen fixing’ cereals (70, 113). Third, the host range of rhizobia could be widened. Fourth, endophytic nitrogen-fixing microorganisms found in cereals or monocots may be manipulated to colonize important cereals (147, 277, 280). Recent research efforts have focused on the development of endophytic diazotrophs that are able to provide BNF. A variety of endophytic diazotrophs have been found that colonize the interior roots of rice, maize and grass (18, 58). Several bacteria are believed to be capable of contributing nitrogen to sugarcane (43), rice (164, 311), and wheat (298). However, so far no BNF microbial associations or symbioses are known to produce significant amounts of fixed nitrogen for the major cereals.

The focus of the work described in this dissertation is part of an effort to accomplish the fourth approach mentioned above. A sugarcane endophyte, _Acetobacter diazotrophicus_, is a strong candidate for use in forming effective symbiotic or associative nitrogen-fixing systems in other crops. This bacterium was isolated from surface sterilized sugarcane plants in relatively high numbers (53, 107, 169). Moreover, since the initial isolation of the bacterium in Brazil, it has been found that _A. diazotrophicus_
colonizes the interior of sugarcane varieties grown in Australia, Mexico, Cuba, Argentina, and the USA (9, 26, 78, 101, 101, 169, 170). Additionally, the colonization of crops such as *Pennisetum purpureum*, pineapple, sweet potato and coffee (72, 140, 216) strengthens the evidence that the association between sugarcane and *A. diazotrophicus* is widespread and likely benefits both plant and microbial partners. This observation may suggest that the beneficial association of this bacterium is common, and thereby improving the potential of using genetically engineered *A. diazotrophicus* strains with other crops. However, since other diazotrophs were also isolated with *A. diazotrophicus* from sugarcane and many more unculturable species could be present, it is not clear whether *A. diazotrophicus* is the major contributor responsible for sugarcane growth promotion. Furthermore, although it is evident that *A. diazotrophicus* can fix nitrogen inside sugarcane plants and nitrogen fixation may represent one of the possible benefits (260), it has not been proven yet whether fixed N is transferred or utilized by the sugarcane plants. Before attempting to develop this bacterium as a candidate to extend the nitrogen-fixing symbiosis to currently non-nodulated plants, we must obtain a better understanding of the molecular basis of the sugarcane-*A. diazotrophicus* interaction.

This work is focused on understanding the endophytic nitrogen-fixing bacterium, *Acetobacter diazotrophicus*, and its beneficial association with sugarcane (*Saccharum* spp.). An initial objective includes characterization of the genes responsible for nitrogen fixation and their regulation. I was able to isolate, sequence, and analyze the major 31.5 kb *nif* gene cluster which includes both *nif* and associated genes of *A. diazotrophicus*. DNA sequence analysis indicated that individual gene products are generally most
similar to those found in other known members of the α group of Proteobacteria to which
*A. diazotrophicus* belongs, based on the 16S rRNA phylogenetic tree. This *nif* gene
cluster represents the largest assembly of contiguous *nif/fix* and associated genes
characterized in any diazotrophic bacterial species. Sequence information could be used
to study the function of individual genes, the evolution of the *nif* cluster, and the
differentiation of strains in different habitats and hosts, and their regulation.

This work also reports the successful development of molecular genetic methods
such as complementation and Tn5 mutagenesis of *A. diazotrophicus*. An operon
responsible for indole-3-acetic acid biosynthesis was identified by Tn5 mutagenesis. The
operon contains genes involved in cytochrome *c* biogenesis (*ccm* genes). Mutations in
these genes resulted in loss of functional cytochrome *c* synthesis and a significant
reduction of IAA production by *A. diazotrophicus*. In this work, using Ccm* mutants it
was clearly demonstrated that *c*-type cytochromes of *A. diazotrophicus* are not involved
in a respiratory system. To our knowledge, this is the first demonstration of *ccm* genes
being related to IAA biosynthesis. This work also showed the presence of indole-3-acetic
acid and indole-3-ethanol in the culture of *A. diazotrophicus*, suggesting that IAA is
synthesized by an indole-3-pyruvic acid pathway; however, other intermediates involved
in alternative IAA biosynthetic pathways were not detected. A decarboxylated form of
IAA, indole-3-carboxylic acid, was also detected by HPLC chromatography, indicating
that regulation of the endogenous IAA level in *A. diazotrophicus* might occur through
decarboxylative oxidation. Conditions for IAA extraction, and detection by HPLC and
TLC, were established and the methods were developed to obtain consistent results.
This work also describes the construction of Nif⁺, Iaa⁺, and Nif⁻/Iaa⁻ strains of A. *diazotrophicus* that are essential in studying two possible benefits of the bacterium. Although the relationship of nitrogen fixation and IAA production in sugarcane growth was not clearly established, sugarcane inoculation experiments demonstrated that both nitrogen fixation and IAA production are equally important for improving the height and biomass of plants, at least at the early stage of sugarcane growth. The results of plant experiments also suggested that the effect of IAA production is more significant when nitrogen is not limited. The conditions and techniques established in this work will be valuable in evaluating the persistence of the longer-term beneficial effects, and examining the possible effects on other crops such as rice, wheat, or sorghum.

2. Review of literature

2.1. Nitrogen cycle

One of the aims of the current work was to characterize genes for nitrogen fixation in *Acetobacter diazotrophicus*. Nitrogen is an essential nutrient for all organisms since it is a constituent of all proteins and nucleic acids, as well as other nitrogen containing molecules. The low content of nitrogen in most soils is in contrast to the abundance of nitrogen in air. Nitrogen (N₂) accounts for 78% of Earth's atmosphere. However, N₂ is unavailable for use by most organisms because few are equipped with an enzyme able to make the triple bonded molecule. For nitrogen to be useful for organisms it must be "fixed" or converted from N₂ take one of several forms in which the nitrogen is...
bonded to hydrogen (for example, $\text{NH}_4^+$) or oxygen (for example, $\text{NO}_3^-$). The former process is defined as Biological Nitrogen Fixation (BNF) and the ability to fix $\text{N}_2$ is restricted to a few organisms called diazotrophs. Mechanistic details of nitrogen fixation mediated by an enzyme called nitrogenase appear quite similar among the different species examined to date. The stoichiometry of the overall reaction is as follows.

\[
\text{nitrogenase} \\
\text{N}_2 + 16 \text{ ATP} + 8e^- + 16 \text{ H}_2\text{O} \rightarrow 2\text{NH}_3 + 16 \text{ ADP} + 16 \text{ Pi} + \text{H}_2
\]

Other bacteria bring about transformations of ammonia to nitrate, and of nitrate to $\text{N}_2$ or other nitrogen containing gases such as NO or $\text{N}_2\text{O}$. Many bacteria and fungi degrade organic matter, releasing fixed nitrogen for reuse by other organisms. All these processes contribute to the nitrogen cycle (Fig. 1).

2.1.1. Importance of BNF

Nitrogen fixation occurs both biologically and non-biologically. Asymbiotic and symbiotic biological systems fix an estimated 175 million metric tons of nitrogen annually (Fig. 2) (30). Two means of non-biological nitrogen fixation are known, occurring through the effects of lightning in the atmosphere, and in industry by the Haber-Bosch process requiring high levels of fossil fuel combustion for energy (229, 271). Synthetic nitrogen use has grown from 10 million to 80 million tons over the last 40 years in response to the needs for high yielding crop production (Fig. 3). This
Nitrification

\[ \text{Nitrification} \rightarrow \NO_2^- \]

Nitrobacter

Nitrosomanas

\[ \text{Nitrogen fixing bacteria: } \]
\[ \text{Cyanobacteria} \]
\[ \text{Rhizobium} \]
\[ \text{Azotobacter} \]
\[ \text{Beijerinckia} \]

\[ \text{Ammonification} \rightarrow \NH_3 \]

\[ \NO_3^- \rightarrow \text{Assimilation} \rightarrow \text{PROTEINS} \rightarrow \NH_3 \]

\[ \text{Denitrification} \rightarrow \NO_2^- \rightarrow \text{Denitrifying bacteria: } \]
\[ \text{Pseudomonas} \]
\[ \text{Bacillus licheniformis} \]
\[ \text{Paracoccus denitrificans} \]

\[ \text{Clostridium pasteurianum} \]
\[ \text{Phototrophic bacteria} \]

\[ \text{Nitrogen Fixation} \rightarrow \text{N}_2 \]

\[ \text{N}_2O \rightarrow \text{N}_2 \]

Figure 1. The nitrogen cycle
Fig. 2. Estimates of the amount of fixed nitrogen on a global scale. Data from Bezdicek & Kennedy, in Microorganisms in Action (1998).
increase has occurred in both developed and developing countries. The current annual worldwide expenditure for nitrogen fertilizer exceeds 20 billion dollars (US), an amount comparable to that for synthetic chemical pesticides (201). Modern industrial production of nitrogen fertilizer demands large inputs of energy in the form of natural gas, and fertilizer constitutes a major energy cost in the production of high-yield corn or rice crops (30). By the year 2050, the world population is expected to double from its current level of more than 5 billion. It is reasonable to expect that the need for fixed nitrogen for crop production will also at least double. If this is supplied by industrial sources, synthetic nitrogen fertilizer use will increase to about 160 million tons of nitrogen per year, about equal to that produced by the biological process. This amount of nitrogen fertilizer will require burning some 270 million tons of coal or its equivalent. In addition to the cost effect, there are several significant environmental reasons to seek alternatives to chemically fixed nitrogen fertilizer: it affects the balance of the global nitrogen cycle, pollutes groundwater, increases the risk of chemical spills, and increases atmospheric nitrous oxide (N₂O), a potent "greenhouse" gas (201). Atmospheric chemists estimate that fixation by lightning delivers about 3 million tons of nitrogen to the Earth's surface, while bacteria add about 140 million tons to soils (297). Therefore the most important source of fixed nitrogen occurs from the activity of certain bacteria that convert atmospheric nitrogen (N₂) to ammonia (NH₃) catalyzed by the enzyme nitrogenase (160, 221). The total biological nitrogen fixation is estimated to be twice as much as the total
Fig. 3. World nitrogen fertilizer consumption, 1960-19999 (Source: FAO)
nitrogen fixation by non-biological processes (Fig. 3) (30). Biological Nitrogen Fixation (BNF) occurs in almost 100 prokaryotic genera distributed over many of the major phylogenetic divisions. The nitrogen-fixing bacteria are categorized into three groups based on their association with host plants; growing in association with the roots of higher plants, widespread in soils, or free-living (12, 55, 87, 219). Those microbes that fix nitrogen independently of other organisms are termed free-living. Free-living diazotrophs require a chemical energy source if they are non-photosynthetic, whereas the photosynthetic diazotrophs utilize light energy. The free-living diazotrophs contribute little fixed nitrogen to agricultural crops. Associative nitrogen-fixing microorganisms are those diazotrophs that live in close proximity to plant roots (that is, in the rhizosphere), and can obtain energy from the plants (228). The symbiotic relationship between diazotrophs called rhizobia, and legumes (ex. *Rhizobium meliloti* and alfalfa) can provide large amounts of fixed nitrogen to the plant and can have a significant impact on agriculture. In this relationship, the plant supplies the bacteria with the energy for bacterial growth as photosynthates, while the bacteria supply the plant with fixed nitrogen (294). A variety of endophytes have been reported to colonize non-legume plants and are believed capable of contributing to the nitrogen requirement in sugarcane (41), rice (163, 235, 312), and wheat (201).

2.1.2. Nitrogenase and its regulation

BNF is catalyzed by the nitrogenase enzyme complex. The best-characterized $N_2$-fixing organism is the facultative anaerobic enterbacterium *Klebsiella pneumoniae*, which
carries a cluster of 20 genes (termed nif genes) required for the synthesis and function of the molybdenum nitrogenase (8). Molybdenum (Mo) nitrogenase is a complex metalloenzyme composed of two component proteins called the Fe protein and the MoFe protein (49, 88, 236, 290). The MoFe protein is a α2β2 tetramer; the α- and β-subunits are products of the nifD and nifK genes respectively. The Fe protein is a homodimer, the monomer being coded by the nifH gene. Nitrogenase binds and hydrolyses 2MgATP, yielding 2MgADP and 2Pi for each electron that is transferred from the Fe protein to the MoFe protein (247, 248, 259). Fe protein has ATP-binding site(s) and one [Fe4S4] cluster per homodimer: it supplies energy by ATP hydrolysis, and transfers electrons from reduced ferredoxin or flavodoxin to FeMo protein (54, 195, 259).

In addition to the Mo nitrogenase, some diazotrophs have alternative nitrogenases. In the absence of molybdenum the alternative nitrogenases, containing either vanadium or iron, are expressed (32-34, 141). In contrast to K. pneumoniae, the aerobic soil bacterium A. vinelandii is able to synthesize a molybdenum nitrogenase (nifHDK), a vanadium nitroogenase (vnfH, vnfDGK), and an iron-containing nitrogenase (anfHDK) (35). When Mo is absent, Azotobacter vinelandii synthesizes an alternative nitrogenase in which vanadium is used instead of Mo. Mo, if available, represses the less efficient vnf system. If vanadium is also absent A. vinelandii can make a third nitrogenase (anf) that uses only iron, but is an even less efficient system under the laboratory conditions used for its study. All three nitrogenases are composed of two components: the FeMo-, FeV-, or FeFe-cofactor containing FeMo protein (component I) and Fe protein (component II) (150, 151, 182).
Despite the considerable taxonomic diversity among nitrogen-fixing bacteria, there is a surprising degree of uniformity in the mechanisms underlying the regulation of \textit{nif} genes in many diazotrophs. \textit{nif} transcription starts at promoters recognized by a RNA polymerase holoenzyme containing alternative sigma factor, $\sigma^N (\sigma^{54})$. A \textit{nif}-specific transcriptional activator, NifA, acts with $\sigma^{54}$-RNA polymerase holoenzyme to initiate transcription at \textit{nif} promoters for \textit{nif} gene transcription and, subsequently, nitrogenase synthesis in all the proteobacterial diazotrophs (69, 71, 94, 161, 209, 288, 289). Most \textit{nif} promoters have a highly conserved sequence between positions -12 and -24 with respect to the point of transcription initiation; the consensus sequence of the \textit{nif} promoter is TGG-N8-TTGCA (19, 29). Buck et al (1986) (48) first reported the consensus motif (upstream activator sequence; UAS) of \textit{nif}A, TGT-N10-ACA, which is present in almost all NifA-activated promoters between 80 and 150 bp upstream of the transcription start site. NifA binds to its palindromic motif (UAS) and contacts $\sigma^N$ bound to \textit{nif} promoter by means of DNA loops (Fig. 4) (188, 196, 197). In most cases this looping is assisted by binding of the Integration Host Factor (IHF) in the intervening region, thus promoting interaction between the upstream-bound activator and the RNA polymerase bound downstream (129). To activate transcription, NifA catalyzes the isomerization of closed complexes between RpoN and a \textit{nif} promoter to open complexes using ATP or GTP as an energy source (28). Control of \textit{nif} transcription is mediated by regulating the expression and activity of the NifA protein in response to oxygen levels and the level of fixed nitrogen.
Figure 4. Regulation of Nitrogen Fixation
In the \( \gamma \)-group of Proteobacteria, including *K. pneumoniae* and *A. vinelandii* nif gene expression is controlled by another protein, NifL, which inhibits NifA activity in response to fixed nitrogen and external concentration of oxygen. The inhibition of NifA activity by NifL requires direct protein-protein interaction, rather than catalytic modulation of NifA activity by NifL (69). The nitrogen regulators NtrC and NtrB determine whether or not the nifLA operon is expressed in *K. pneumoniae* (depending on the presence of ammonia or organic nitrogen). In the absence of ammonia or organic nitrogen the NtrC protein is phosphorylated by the NtrB protein. NtrC-P then binds to the upstream region of the nifLA operon and activates transcription (38, 39, 126, 189, 264) (Fig. 4).

Environmental cues are important in determining nitrogenase synthesis and its activities since BNF is a high-energy consuming process. In the presence of \( \text{NH}_4^+ \) or/and excess \( \text{O}_2 \), nitrogenase synthesis and transcription of nif genes are inhibited (91, 165, 245). Due to the marked oxygen sensitivity of nitrogenase, the environmental oxygen tension is a major regulatory factor for most diazotrophs (Table 1). Aerobic diazotrophs have evolved mechanisms to protect the nitrogenase enzymes from oxygen damage. Some aerobes only fix nitrogen under microaerobic condition (when oxygen concentration are low). Others such as *Azotobacter* spp. have evolved a complex respiratory system that consumes oxygen rapidly to maintain a low oxygen concentration inside of the cells (180, 223). The best-known examples are Rhizobia and cyanobacteria. The nitrogenase of *Rhizobium* spp. is sequestered in root nodules where nodule
Table 1. Strategy to protect nitrogenase from Oxygen

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avoidance: no nitrogen fixation when exposed to air</td>
<td>Obligate anaerobes: &lt;br&gt; <em>Clostridium pasteurianum</em>&lt;br&gt; <em>Desulfovibrio gigas</em></td>
</tr>
<tr>
<td>Facultative nitrogen fixation: only diazotrophic in anaerobic conditions</td>
<td>Facultative anaerobes: &lt;br&gt; The genera of <em>Klebsiella</em>, <em>Enterobacter</em>, <em>Bacillus</em>, and photosynthetic bacteria such as <em>Rhodospirillum rubrum</em></td>
</tr>
<tr>
<td>Microaerophily: grow aerobically, yet sensitive to oxygen; only diazotrophic in microaerobic condition</td>
<td>Aerobic bacteria &lt;br&gt; <em>Rhizobia</em>, <em>Bradyrhizobium japonicum</em>, <em>Xanthobacter flavus</em>, <em>Azospirillum lipoferum</em>, <em>Xanthobacter autotrophicus</em>, and methane-oxidizing bacteria</td>
</tr>
<tr>
<td>Respiratory protection: organisms' high respiration scavenge oxygen from nitrogen fixing site</td>
<td>Facultative and microaerophilic bacteria &lt;br&gt; <em>Azotobacter chroococcum</em>, <em>A. vinelandii</em>, <em>X. flavus</em></td>
</tr>
<tr>
<td>Slime: slime layer made of extra-cellular polysaccharides impedes diffusion of oxygen</td>
<td>Aerobic nitrogen-fixing bacteria &lt;br&gt; <em>Beijerinckia indica</em></td>
</tr>
<tr>
<td>Conformational protection: the nitrogenase reversibly changes the conformation to an oxygen inaccessible form</td>
<td><em>A. chroococcum</em>, <em>A. vinelandii</em> and some anaerobes</td>
</tr>
<tr>
<td>Heterocysts: a compartment from which the photo-generation of O₂ is excluded; spatial separation</td>
<td>Cyanobacteria &lt;br&gt; <em>Anabena cylindrica</em></td>
</tr>
<tr>
<td>Nodules: a barrier which slows down diffusion of oxygen and contains leghaemoglobin which has a high affinity for oxygen</td>
<td>Leguminous plants &lt;br&gt; <em>Rhizobium</em> spp.</td>
</tr>
</tbody>
</table>
Table  Continued

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane structure: act as a subcellular compartment and separate photosynthesis from nitrogen fixation</td>
<td>Cyanobacterium Gloeotheca</td>
</tr>
<tr>
<td>Mobile trichomes: rod-like microbes which can aggregate in bundles and central cells in the bundle gain nitrogen-fixing activity</td>
<td>Trichodesmium spp.</td>
</tr>
</tbody>
</table>

Parenchymal cells and leghemeoglobin regulate the free oxygen concentration (23, 63, 193). In the case of cyanobacteria, nitrogenase is protected by spatially separating nitrogen fixation from photosynthesis through special cells called heterocysts (109, 314). Likewise, the availability of fixed nitrogen in the environment is a significant regulatory effector in free-living diazotrophs, but is of less importance for symbiotic organisms exporting fixed nitrogen to their host.

An additional layer of nitrogenase regulation occurs through reversible ADP-ribosylation of Fe protein to prevent unproductive nitrogen fixation during energy limiting or nitrogen sufficient conditions (119, 120). ADP-ribosylation of Fe protein at a specific arginine residue prevents the association of Fe protein with the MoFe protein, rather than blocking electron transfer between the two structural proteins (175). The NAD⁺-dependent dinitrogenase reductase ADP-ribosyltransferase (DRAT) encoded by draT and dinitrogenase reductase-activating glycohydrolase (DRAG) encoded by draG

are directly involved in ADP-ribosylation, and deribosylation in photosynthetic
diazotrophs such as *Rhodospirillum ruburum* and *Rhodobacter capsulatus* (118, 174, 175, 184).

2.1.3. *nif* genes and nitrogen fixation-associated genes

An analysis of the genes necessary for nitrogen fixation in several bacteria has
shown that while certain features of *nif* gene structure and function are common to most,
the degree of linkage and specific arrangement of genes can vary considerably (67, 183).
There are approximately twenty genes that are necessary for the synthesis, assembly, and
proper functioning of nitrogenase (67). The known genes and their functions are listed in
Table 2. The most conserved genes are *nifHDK* encoding the structural subunits of the
Mo-containing nitrogenase enzyme. Other common *nif* genes encode proteins that serve
to modify nitrogenase polypeptides (*nifU, nifS*) or to form the FeMo-cofactor (*nifE, nifN,
nifB, nifQ, nifV*), all of which are essential for nitrogenase activity. The transcriptional
activator encoded by *nifA* is also conserved among Proteobacterial diazotrophs. Several
other *nif* genes found variously distributed among diazotrophs include: *nifF, nifJ* (for
electron transfer to nitrogenase); *nifY, nifM* (for subunit modification); *nifT, nifZ, nifX,
nifW* (functions not well-characterized), but only *K. pneumoniae* has been shown to
contain all of these genes (3, 176).

In *K. pneumoniae* and *A. vinelandii*, the majority of *nif* genes are clustered in one
and two segments of the bacterial chromosome respectively; in other organisms, up to
two or five genetically unlinked groups of *nif* (and/or *fix*) genes may occur (67). The
Table 2. The proposed function of nitrogen associated genes and their mutant phenotypes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Products and Proposed functions</th>
<th>Mutant Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogenase structure</td>
<td><strong>nifH</strong></td>
<td>Nitrogenase structure; Fe protein subunit and FeMo cofactor biosynthesis; homodimer contains a single [4Fe-4S] cluster; multiple nifH polypeptides found in some diazotrophs and also found in a eukaryote (<em>Marchantia polymorpha</em>) chloroplast genome; contains ATP-binding motif, Gly-X-Gly-X-X-Gly-Lys-Ser</td>
</tr>
<tr>
<td></td>
<td><strong>nifD</strong></td>
<td>MoFe protein α-subunit, forms tetramer with β-subunit; holoprotein contains 2FeMo-cofactor molecules and four [4Fe] or two [8Fe] clusters; a FeMo-cofactor binding motif, His-Gly-X-X-Gly-Cys</td>
</tr>
<tr>
<td></td>
<td><strong>nifK</strong></td>
<td>MoFe protein β-subunit; a FeMo-cofactor binding motif, His-Gly-X-X-Gly-Cys</td>
</tr>
<tr>
<td>FeMo cofactor synthesis</td>
<td><strong>nifV</strong></td>
<td>Homocitrate synthetase; required for homocitrate (an organic component of FeMo-cofactor) synthesis; high sequence similarity to α-isopropyl malate synthase gene (<em>leu4</em>) from <em>Saccharomyces cerevisiae</em>; in <em>Caulobacter pasteurianum</em> the nifV gene is split into two separate genes</td>
</tr>
<tr>
<td></td>
<td><strong>nifE</strong></td>
<td>FeMo-cofactor synthesis; has a significant similarity to nifD</td>
</tr>
<tr>
<td></td>
<td><strong>nifN</strong></td>
<td>FeMo-cofactor synthesis; has a significant similarity to nifK</td>
</tr>
<tr>
<td></td>
<td><strong>nifB</strong></td>
<td>FeMo-cofactor synthesis; shows a clustering of Cys residues located near the N-terminus, which is a proposed FeMo-cofactor precursor binding site; in <em>C. pasteurianum</em>, nifB and nifN are expressed as fused proteins</td>
</tr>
</tbody>
</table>
Table *Continued*

<table>
<thead>
<tr>
<th>Genes</th>
<th>Products and Proposed functions</th>
<th>Mutant Phenotypes$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>nifQ</strong></td>
<td>Initial step of FeMo-cofactor synthesis; NifQ mutant phenotype can be suppressed by the addition of elevated levels of Mo; a clustering of four Cys residues in the C-terminus (Cys-X$_4$-Cys-X-X-Cys-X$_5$-Cys)</td>
<td>Nif$^+$</td>
</tr>
<tr>
<td><strong>Maturation or activation nifU</strong></td>
<td>Fe protein stabilization; assembly of iron-sulfur clusters; also found in truncated form in <em>R. capsulatus</em> and <em>B. japonicum</em>; Cys-X-X-Cys motif</td>
<td>Nif$^+$</td>
</tr>
<tr>
<td><strong>nifS</strong></td>
<td>Fe protein stabilization; homodimeric cysteine desulfurase; mutants show slower diazotrophic growth in <em>K. pneumoniae</em> and <em>A. vinelandii</em>, but no diazotrophic growth in <em>R. capsulatus</em></td>
<td>Nif$^+$</td>
</tr>
<tr>
<td><strong>nifW</strong></td>
<td>Homocitrate processing; associates with the MoFe Protein; oxygen protection of the MoFe protein; interacts with the NifZ</td>
<td>Nif$^+$, but slow diazotrophic growth</td>
</tr>
<tr>
<td><strong>nifZ</strong></td>
<td>Accumulation of active FeMo protein; formation of FeMo-cofactor or insertion of FeMo cofactor into immature MoFe protein; interact with the NifW; only found in <em>Azotobacter</em> sp., <em>K. pneumoniae</em>, <em>Frankia</em>, and <em>Anabaena</em> sp.</td>
<td>Nif$^+$, but slow diazotrophic growth</td>
</tr>
<tr>
<td><strong>nifM</strong></td>
<td>Activation of Fe protein; insertion of the Fe-S center into an apo-Fe protein; NifH and NifM form homodimers; only a very low level of interspecies sequence identity in the C-terminal third of the polypeptide; the transcriptional activators, VnfA and AnfA, may regulate the nifM expression.</td>
<td>Nif$^+$</td>
</tr>
<tr>
<td><strong>nifX</strong></td>
<td>FeMo cofactor processing; shows a significant sequence similarity to NifY; a possible negative regulatory element.</td>
<td>Nif$^+$</td>
</tr>
<tr>
<td>Genes</td>
<td>Products and Proposed functions</td>
<td>Mutant Phenotypes$^1$</td>
</tr>
<tr>
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<tr>
<td><strong>Electron transport to nitrogenase</strong></td>
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<td></td>
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<tr>
<td><em>nifF</em></td>
<td>Flavodoxin, physiologic reductant of the Fe protein</td>
<td>Nif$^+$ in Av, but Nif$^-$ in Kp and Rc</td>
</tr>
<tr>
<td><em>nifJ</em></td>
<td>Pyruvate-flavodoxin reductase; composed of two identical subunits; active only in iron-depleted medium in Anabanea sp.</td>
<td>Nif$^+$ in Kp and Av; Nif$^+$ in Rr; Nif$^-$ in low iron condition in An</td>
</tr>
<tr>
<td><em>fixA</em></td>
<td>Electron transfer; electron transfer flavoprotein β-subunit</td>
<td>Fix$^+$</td>
</tr>
<tr>
<td><em>fixB</em></td>
<td>Electron transfer; electron transfer flavoprotein α-subunit</td>
<td>Fix$^+$</td>
</tr>
<tr>
<td><em>fixC</em></td>
<td>Electron transfer; electron transfer-quinone oxidoreductase</td>
<td>Fix$^+$</td>
</tr>
<tr>
<td><em>fixX</em></td>
<td>Electron transfer; ferredoxin like protein</td>
<td>Fix$^+$</td>
</tr>
<tr>
<td><strong>Transcriptional regulator</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>nifL</em></td>
<td>Negative regulatory element; inactivates <em>nifA</em>; a redox-sensitive flavoprotein; no <em>nifL</em> homolog found in any rhizobia</td>
<td>Nif$^+$</td>
</tr>
<tr>
<td><em>nifA</em></td>
<td>Transcriptional activator; responds to two environmental stimuli, O$_2$ and fixed nitrogen; Cys-X-X-X-X-Cys domain responding to O$_2$</td>
<td>Nif$^+$</td>
</tr>
<tr>
<td><strong>Unknown</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>nifT</em></td>
<td>Function unknown; not essential for nitrogen fixation</td>
<td>Nif$^+$</td>
</tr>
<tr>
<td><em>nifY</em></td>
<td>Function unknown; show a significant sequence similarity to NifX; a possible involvement of the protein in this sensing of environmental stimuli; only found in <em>K. pneumoniae</em>, <em>A. vinelandii</em>, and <em>Azospirillum brasilense</em></td>
<td>Nif$^+$</td>
</tr>
</tbody>
</table>
Products of fix genes allow the bacterium to live and fix nitrogen in nodules (a special structure of legume roots where rhizobial bacteria reside and fix nitrogen). In *R. capsulatus*, nitrogen fixation genes are located in three unlinked regions of the chromosome (98, 153, 183, 302) (Fig. 5).

The *fixABCX* operon has been identified in all Rhizobia, *Azotobacter* species and *Azospirillum brasilense* (7, 90, 97, 116, 128, 133, 194). Although these gene products are thought to be involved in some aspect of electron transfer, their precise biochemical roles have not yet been established (83). Mutants of *fixA, B, C, or X* show defects in symbiotic nitrogen fixation (Fix⁻ phenotype) (116, 117, 127, 143). These genes are also present in the genome of *E. coli* (*fixA,B,C,X*) and *Mycobacterium tuberculosis* (*fixA,B*), neither of which are nitrogen-fixing organisms. The influence of these *fix* genes in *E. coli* is upon carnitine metabolism, encoding the structural components of the *E. coli* carnitine pathway and expressed under anaerobic growth conditions only in the presence of L-carnitine or crotonobetaine as inducers (47, 84, 86). However, their function in diazotrophic organisms is unknown (85). Transcription of *fix* genes also requires NifA and σ54 (117, 125).

2.1.4. Nitrogen control system
Nitrogen metabolism in prokaryotes involves the coordinated expression of a large number of enzymes related to both utilization of extracellular nitrogen sources and intracellular biosynthesis of nitrogen-containing compounds. Complex regulatory networks involving regulation at both the transcriptional and posttranslational levels affect the control of this expression (16, 5891). While the most detailed studies to date have been performed with enteric bacteria, there is substantial evidence to demonstrate that the nitrogen regulation (*ntr*) systems found in the enteric bacteria are conserved in many other genera. Moreover, new regulatory mechanisms are also being discovered as nitrogen control is examined in detail in other bacterial species (95, 149, 190, 227).

The *ntr* system comprises a web of interconnected cascades, resulting in the phosphorylation of NtrC under conditions of nitrogen depletion (178, 190, 240). Central to the *ntr* system is a two-component regulatory system that activates transcription of σ^N^-dependent promoters. Phosphorylation of the response regulator NtrC by the NtrB kinase is controlled by the PII signal transduction protein in response to the intracellular ratio of glutamine and 2-ketoglutarate. During nitrogen-limited growth, the intracellular levels of glutamine decline, NtrC is phosphorylated and high-level expression of Ntr-regulated genes occurs (233, 262, 316). The *ntr* system is comprised of four enzymes: a uridylyltransferase/uridylyl-removing enzyme (UT ase/UR) encoded by *glnD*, a small trimeric protein, PII encoded by *glnB*, and a two component regulatory system composed of the histidine protein kinase NtrB and the response regulator NtrC. UTase catalyzes the uridylylation and deuridylylation of PII protein. Adenylytransferase catalyzes the adenylylation and deadenylylation of GS. In enteric bacteria, several genes are
transcriptionally regulated by the NtrBC system. These include: genes encoding transport systems for glutamine, arginine, and histidine; genes required for nitrate and nitrite assimilation; the nitrogen fixation regulatory genes \textit{nifLA}; and the nitrogen regulatory genes (178, 190, 240). Not all nitrogen-regulated genes are controlled directly by NtrC. Regulation of some genes in the Ntr regulon requires the secondary transcriptional regulator Nac, whose expression is activated by NtrC, and regulates histidase, proline oxidase, urease, glutamate dehydrogenase, and glutamate synthase (177, 258).

Nitrogen metabolism genes of \textit{Bacillus subtilis} are regulated by the availability of rapidly metabolizable nitrogen sources, but not by any mechanism analogous to the two-component Ntr regulatory system found in enteric bacteria. Instead, at least three regulatory proteins (CodY, GlnR and TnrA) independently control the expression of gene products involved in nitrogen metabolism in response to nutrient availability (95). The TnrA protein is active only during nitrogen limitation (200, 304), whereas GlnR-dependent repression occurs in cells growing with excess nitrogen (303, 305). A third regulatory protein, CodY, controls the expression of several genes involved in nitrogen metabolism, competence and acetate metabolism in response to growth rate. The highest levels of CodY-dependent repression occur in cells growing rapidly in a medium rich in amino acids, and this regulation is relieved during the transition to nutrient-limited growth (92, 96, 267, 303).

2.2. \textit{Acetobacter diazotrophicus}
Sugarcane has been grown continuously for more than 100 years in some areas in Brazil without the addition of any nitrogenous fertilizer, and it has long been suspected that substantial N fixation occurs in such a system (76). The results of $^{15}$N dilution studies, $^{15}$N$_2$ incorporation or nitrogen balance experiments, as well as acetylene reduction with sugarcane demonstrated that sugarcane can apparently obtain 70% of its nitrogen from Biological Nitrogen Fixation (BNF) (40, 42, 171). At least 11 genera of N$_2$-fixing bacteria including species of *Beijerinckia, Erwinia, Azotobacter, Derxia, Enterobacter*, and *Azospirillum* (73, 75, 77) have been isolated from sugarcane, but none occur in large enough numbers to account for the high rates of N fixation found in this crop (78).

Recently, an endophytic organism, *Acetobacter diazotrophicus*, was isolated from surface-sterilized macerated sugarcane stems, roots, leaves and rhizosphere soil samples from Brazil in large numbers (53). The bacterium was not found between rows of sugarcane plants or 11 weed species associated with sugarcane, and it also does not survive well when artificially inoculated into soil in Brazilian sugarcane fields (12, 44, 238). Another study of the endophytic nature and specificity of *A. diazotrophicus* was investigated using 12 field-grown Australian sugarcane cultivars and several other grasses by Li and MacRae (1991, 1992) (169, 170). In contrast to the Brasilian data, *A. diazotrophicus* was isolated in low concentration in the Australian sugarcane rhizosphere, possibly due to sucrose exuded into the soil or to small pieces of root being included in samples (12, 169).
*A. diazotrophicus* has also been isolated from sugarcane in Mexico, Australia, Argentina, Uruguay, and the United States of America, strengthening the notion that the association between sugarcane and *A. diazotrophicus* is widespread and beneficial (53, 101, 169). *A. diazotrophicus* has also been isolated from other plants such as coffee, sweet potato, tea, and Cameroon grass, but its distribution among these crops is not known (12, 140, 185).

2.2.1. Characteristics

*A. diazotrophicus* is a small, Gram-negative, rod-shaped bacterium (dimensions of 0.7-0.9 by 1-2 μm) with peritrichous or lateral flagella. The cells can occur singles, in pairs, or chains, but never form endospores. *A. diazotrophicus* belongs to the α group of Proteobacteria, and is the only *Acetobacter* species able to fix nitrogen (107). Genomic GC content is 61 mol %. *A. diazotrophicus* grows best in a semisolid medium (LGIP medium) (53) that provides the microaerobic conditions necessary for N₂ fixation and growth, as well as allowing for bacterial movement and pellicle formation (74). *A. diazotrophicus* forms distinct dark brown colonies with lighter brown margins on solid potato agar supplemented with 10 % sucrose. Dark orange colonies appear on nitrogen-poor medium supplemented with bromthymol blue. The bacterium can grow in sucrose concentrations up to 30% and at pH 3.0, but fails to grow at pH 7.0 (53, 275). The bacterium will also grow well on monosaccharides such as glucose, fructose, and galactose, and will grow in glycerol, ethanol, and mannitol (50). However, it dose not
grow on many other carbon (C) compounds, such as dicarboxylic acids or maltose, due to the lack of transport mechanisms for these compounds (53, 107, 169, 292).

Despite its preference for living within sucrose-rich plants, Ureta et al. (1995) have shown that *A. diazotrophicus* grows best in high concentrations of gluconate, glucose, glycerol, and sucrose (in descending order), probably because *A. diazotrophicus* is unable to transport or respire sucrose (4). The ability of *A. diazotrophicus* to grow on sucrose can be explained as being due to extracellular saccharolytic enzyme activity that provides the bacteria with glucose and fructose for growth. One of the key enzymes, levansucrase, that hydrolyzes sucrose, releasing fructo-oligosaccharides and levan, is probably responsible for this saccharolytic activity (9). Therefore, its ability to grow on a range of C substrates suggests that *A. diazotrophicus* is not restricted to sucrose-rich environments (299, 300) and can grow within the xylem where sucrose and other potential C substrates are very low (0 to 9 %) (179, 300).

Although *A. diazotrophicus* needs O₂ to grow, it fixes nitrogen in microaerobic conditions. It is relatively tolerant to O₂, and will continue to fix N₂ at a pO₂ of 4% in 10% sucrose (41, 44). However, when compared with *Azocarcus indigens* and *Azotobacter paspali* in a recent study using NifH expression and acetylene reduction, *A. diazotrophicus* was classified as being sensitive to O₂ (295). The mechanisms to maintain a low O₂ intercellular concentration have not been characterized in *A. diazotrophicus*. However, it has been suggested that *A. diazotrophicus* can maintain intracellular oxygen tension at very low levels by a rapid respiratory electron transport system. The consumption of O₂ at the surface of diazotrophic prokaryotes protects
nitrogenase from inactivation by O₂. This could be accomplished through the high expenditure of ATP in nitrogen fixation and a physiological mechanism that carries out a high rate of uncoupled electron transport. This kind of O₂ protection has been generally accepted in *Azotobacter vinelandii*. However, recent data demonstrated that O₂ consumption at the cell surface is less effective than generally assumed in *A. vinelandii* for protection of nitrogenase from O₂ damage (208). It is proposed that alternative factors like the supply of ATP and reducing equivalents are more important.

Ammonium may be used as an N-source for *A. diazotrophicus* (107), and nitrogenase activity is only partially inhibited by ammonium and amino acids, with inhibition decreasing at high sucrose levels (99, 239, 276). Unlike most diazotrophs, *A. diazotrophicus* contains no nitrate reductase and high levels of nitrate (up to 80 mM) do not affect its nitrogenase activity (169). This implies that *A. diazotrophicus* can continue to fix nitrogen inside the sugarcane plant even when nitrate-containing nitrogen fertilizer is applied in the field.

2.2.2. Infection, colonization and transmission

The exact location of *A. diazotrophicus* within sugarcane plants has not been conclusively established. However, evidence indicates that the bacteria inhabit the intercellular space, xylem sap, and intercellular apoplast (51). Several researchers have suggested several possible mechanisms that *A. diazotrophicus* utilizes to enter sugarcane seedlings/plantlets, and move within the adult plants. It is likely that the bacteria can spread from generation to generation via the vegetative propagation of setts, pieces of
sugarcane stems (170). However, the fact that the bacterium is not always present in setts suggests that other mechanisms of plant infection must also occur. James et al. (1994) (139) have shown that the bacteria entered the root apoplast via lateral root junctions and the loose cells of the root cap, but not through root hairs. In addition, it was suggested that the bacteria also entered the root tip cells through "infection thread-like structures", and the bacteria are subsequently lysed by the host, which was also reported by Bellone et al. (1997) (26) and Fuentes-Ramirez et al. (1997) (5). However, Bellone et al. (1997) stated that *A. diazotrophicus* cells were not lysed, but were surrounded by a host cell membrane similar to rhizobia-legumes.

The xylem and/or developing vascular tissue at lateral root junctions may also be sites of the entry. Sprent and James (1995) (272) isolated *A. diazotrophicus* from xylem sap and have suggested that the xylem is a suitable location for *A. diazotrophicus* due to the low pO₂, allowing for high nitrogenase expression (104). In fact, Boddey et al. (1991) have shown that *A. diazotrophicus* will grow and fix N₂ considerably on sucrose concentrations as low as 1%. The occurrence of *A. diazotrophicus* in stem xylem vessels and the adjacent intercellular spaces have been confirmed using GUS-labeled bacteria that were inoculated into one-node setts prior to germination. The microscopic examination of the aerial tissues revealed that *A. diazotrophicus* was found within xylem and intercellular apoplast (100, 260). It appears that once it is in the xylem, *A. diazotrophicus* then moves up the stem as the sugarcane plant grows. Such movement has also been shown by *Herbaspirillum, Calibacter xyli* subsp. *xyli*, and *Xanthomonas albilineans* (61, 122, 144).
Another mechanism mediating *A. diazotrophicus* transmission is mycorrhizal fungi. Paula et al. (1991) (216) were able to infect sugarcane, sweet potato, and sweet sorghum with *A. diazotrophicus* when co-inoculated with the mycorrhizal fungus *Glomus clarum* or *Gigaspora gigantea* (25). The mycorrhizal spores carry the bacteria and introduce them into the plant, when spores are germinated and penetrate the root surface. Interestingly, it was observed that plants co-inoculated with mycorrhiza and *A. diazotrophicus* had greater N content, root length and branching than plants inoculated with mycorrhiza alone (138).

The pink sugarcane mealy bug (*Saccharococcus sacchari*) has been suggested as another method of transmission of the bacteria, since *A. diazotrophicus* and other Acetobacter spp. can be isolated from the insect (11). Recently, the bacterium has been isolated from mealy bugs in Mexico, Brasil, and Australia (51). This insect feeds on the tissue between sugarcane leaf sheath and the stem, and *Acetobacter* were particularly abundant in aerial storage tissue in the summer. The honeydew of the pink mealy bug is very high in sugars such as fructose and glucose but low in sucrose, with a low pH of 2.9 to 3.2, which makes the insect's gut a potential habitat of *A. diazotrophicus* outside of host plants (11, 16). However, when Ashbolt and Inkermant (1990) tested portions of mealy bug-infested stems for nitrogenase activity, they were all negative, indicating that the bacteria within the insects are not fixing nitrogen or not providing fixed nitrogen to the insect. The sugarcane leafhopper (*Perkinsiella saccaricida*) feeds on the same sugarcane tissue as the mealy bugs and is another insect that harbors *Acetobacter* spp. in its gut (135). However, it has to be emphasized that the presence of the bacteria within
insects is not by any means conclusive evidence that they are actually transmitted by the insects.

2.2.3. Genetic diversity

Considerable diversity exists within individual soil bacterial species depending on the habitat. Less variability has been observed in bacteria that predominantly or exclusively live under constant conditions inside organisms. Caballero-Mellado et al (1994) (52) have shown that *A. diazotrophicus* isolates from different sugarcane cultivars growing in diverse geographic regions of Mexico and Brasil have limited genetic diversity, using multilocus enzyme profiles, plasmid, and *nifHDK* restriction fragment patterns. Later this study was confirmed by Caballero-Mellado et al. (1995) (51) using 55 isolates of *A. diazotrophicus* recovered from diverse sucrose-rich host plants and from mealybugs associated with sugarcane plants; electrophoretic mobilities of 12 metabolic enzymes were measured. They explained that the limited diversity could be explained by two reasons: the limited diversity of the modern commercial sugarcane varieties and the relatively constant environment inside sugarcane stems.

There is no evidence that *A. diazotrophicus* is dispersed by sugarcane seeds. Long-distance dispersal of *A. diazotrophicus* can be explained by two characteristics of cultivation practice of sugarcane: first, sugarcane is normally propagated vegetatively from stem cuttings, where *A. diazotrophicus* exists endophytically; second, many commercial varieties from one country are often cultivated in another country. For example, cultivars CP 72 2086 and My 55 14 from the United States and Cuba are
cultivated in Mexico. Although *Saccharum* species have diverse chromosome number and geographic origins, the monophyletic features can be explained by the recent evolution of this group of plants. It might be true that *A. diazotrophicus* is spread among cane cultivars by the mealybugs associated with sugarcane (11) as well as by the spores of the vesicular-arbuscular mycorhizal fungus (217). Therefore, it is likely that the same *A. diazotrophicus* clones could be isolated from many cane varieties cultivated in many different geographic areas. Interestingly, *A. diazotrophicus* was also proposed to have evolved recently (237). The similar maternal host background of sugarcane may have pressured sugarcane to select similar *A. diazotrophicus* strains. This may represent co-evolution of the host and its endophyte. Such studies are worth extending to determine the genetic variability of *A. diazotrophicus* isolates from other sugarcane varieties in widely separated geographic regions, from other host plants such as coffee, tea, and banana.

A genetic variation was found in the number of plasmids in some strains of *A. diazotrophicus*. Some strains have two to three plasmids with size ranging from 2 to 170 kb (52). No gene functions have been determined for any of the plasmids harbored by *A. diazotrophicus* yet. The existence of plasmidless strains (e.g. strain PA15 used in this study) suggests that the fundamental phenotypic characteristics of this species are not plasmid encoded. Moreover, it was established that the *nif* genes are chromosomally located (52, 167, 281). Nevertheless, it is important to note that the majority of isolates contain highly conserved plasmids, implying that plasmids may play an important role in this plant-microbe interaction. Plasmids have been shown to be beneficial in bacterium-
plant or bacterium-insect relationships (27, 181). Therefore, these plasmids may provide some fitness advantage of *A. diazotrophicus*-sugarcane association.

2.3. Sugarcane

Sugarcane is a genus of tropical grasses that requires strong sunlight and abundant water for best growth. Sugarcane is composed of six species of perennial grasses of the genus *Saccharum* L., in tribe *Andropogoneae* of the *Gramineae*. There are two wild species, *S. spontaneum* L. and *S. robustum* Brandes & Jeswiet ex Grassl, and four cultivated species, *S. officinarum* L., *S. barberi* Jeswiet, *S. sinense* Roxb., and *S. edule* Hassk. The four cultivated species are complicated hybrids, and all intercross readily. All commercial canes grown today are inter-specific hybrids (307). Sugarcane originated in the South Pacific. *S. spontaneum* occurs in the wild from eastern and northern Africa, through the Middle East, to India, China, Taiwan, and Malaysia, and through the Pacific to New Guinea. Sugarcane is currently grown primarily in tropical and subtropical regions (Fig. 6) (136). World sugarcane production by continent in 1999 is shown in Fig. 7.

2.3.1. Botany

In an optimal climate sugarcane will grow to maturity in 12 months. After cutting, it will re-grow for another 12 months. Depending on the variety and the growing conditions, the mature stems may vary from 4 to 12 feet in height, and in commercial varieties stems are from 0.75 to 2 inches in diameter (10). At each node a broad leaf rises
Fig. 6. Sugarcane growing areas of the world. •• shows the region where sugarcane is cultivated. Modified from Blackburn (1984).
Fig. 7. World sugarcane production by continent, 1999 (Source: FAO)
consisting of a sheaf or base and the leaf blade. The sheaf is attached to the stem at the node, surrounding the stem with overlapping edges. The leaf blade is very long and narrow, varying in width from 1 to 3 inches (Fig. 8) (10, 36). A cross section of the sugarcane stem reveals widely spaced vascular bundles scattered through the middle of the section. In the periphery, the bundles are smaller and arranged closer with each other forming a solid ring. The matrix of an internode is made of the parenchyma cells in which the vascular bundles are embedded. The parenchyma cells are thin-walled cells separated by smaller intercellular space. These cells increase in size towards the center and are the storage organs for the sweet sugarcane juice.

2.3.2. Sugarcane production

Sugarcane propagation is through stem cuttings of immature canes 8-12 months old. These are called "setts", "seed", "seed-cane" or "seed-pieces". The best setts are taken from the upper third of the sugarcane because the buds are younger and less likely to dry out. The setts can be planted at a 45 degree angle or laid horizontally in a furrow. It takes 12,500 - 20,000 setts to plant one hectare. The setts are lightly covered with soil until they sprout (10-14 days) and then the sides of the furrow are turned inward. Sugarcane is a perennial crop that usually produces crops for about 3-6 years before fields must be replanted. The first crop is called the "plant crop" and takes 9-24 months to mature, depending on location. The cane is cut close to the ground because the lower stem has the highest sugar content and it aids in ratooning, the emergence of new crops from the stems and trash (leaves and tops) left behind. Ratoon crops take approximately
Fig. 8. Sugarcane plant showing different parts. Modified from Magness et al. (1971) and Van Dellenwijn (1952)
one year to mature. As many as four or more ratoon crops may be produced before replanting is necessary, mostly due to the slow decline in yields (37).

Sugarcane yields the highest number of calories per unit area of any plant (Heiser 1981), producing up to 10 tons of sucrose per hectare. 90-120 tons of millable cane per hectare is produced from the plant crop and 45-90 tons from ratoons. Recovery of raw sugar from cane varies from 11-13 percent (215). In 1999 the world production of sugar from cane and beets was about 1,274 million tons (Fig. 9). Almost 65% of this production came from sugarcane. Substantial increases in yields have occurred over the past 100 years due to improved cultural practices, fertilizer use, disease and pest control, field and factory mechanization, and breeding of higher-yielding varieties (131).

2.3.3. Nitrogen requirements for sugarcane growth

Nitrogen demand is very high for plant growth in most crops. It is also the major nutrient influencing the yield and quality of sugarcane. Sugarcane absorbs both \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) (142). Under an adequate water supply, \( \text{NO}_3^- \) is better absorbed than \( \text{NH}_4^+ \), possibly due to the greater mobility of \( \text{NO}_3^- \). Based on this observation, it has been suggested that 40 ppm \( \text{NO}_3^- \) should be supplied constantly throughout the crop cycle for optimum growth and quality of canes. Adequate nitrogen availability resulted in increased leaf area, tiller numbers, stalk length, stem diameter and number of millable canes. Nitrogen also improved succulence, as evidenced by a higher leaf water status (1). Symptoms of N deficiency are clearly discernible. Humbert and Martin (1955) (130) demonstrated that N-deficient sugarcane shows a yellowing of the leaves, retarded
Fig. 9. Sugarcane and raw sugar production, and area of sugarcane harvest in the world, 1990-1999
(Source: FAO)
growth, stalks with smaller diameter, premature drying and senescence of old leaves. However, it should be mentioned that the percentage N of sugarcane is very low compared with legume plants such as soybean. Therefore, demands for N are not as high. For example, the N content of green sugarcane leaves is between 1.0 and 1.2 % of total weight, and this can be reduced to as low as 0.2 to 0.4 % in stems and senescent leaves (293); in contrast, soybean can have from 2 to 6 % N per plant (246).

Sugarcane demands significant amounts of soil nutrients (especially N). However, soil fertility is dramatically decreased every year, due to monocropping, inadequate fertilization and non-recycling of the crop residue, if no synthetic fertilizer is provided (131). Barnes (1974) showed that a 50 ton crop removes, on average, 30-40 kg N, 22.7-27.2 kg P2O5 and 68 kg K2O. More recently, Zende (1988) (103) demonstrated that the 0.56-1.2 kg of N per ton of cane is removed from soil during sugarcane growth. Therefore, the observation that low nitrogen fertilizer inputs can sustain yields of certain sugarcane varieties in some regions for many years is very surprising. It strongly suggests that bacteria associated with sugarcane are responsible for N supply to sugarcane plants through BNF.

2.3.4. Industrial usage of sugarcane

Sugarcane was originally grown for the sole purpose of chewing in southeastern Asia and the Pacific. The rind was removed and the internal tissues sucked or chewed. Production of sugar by boiling the cane juice was first discovered in India, most likely
during the first millennium BC (222). Today, sugarcane has many industrial uses, and is one of the most widely used domestic products. In sugar factories the harvested cane is shredded and crushed with heavy rollers to retrieve the juice. About 1 ton of raw sugar can be extracted from 8-9 tons of sugarcane. This raw brown sugar can be further refined to produce white sugar.

Sugarcane has many other uses besides the production of sugars. Molasses is a by-product of the manufacturing of sugarcane, which is residual syrup. Its use as a stockfeed in Germany can be dated back to at least 1811. Today, at least 600,000 tons of molasses is used as stockfeed annually in the U.S. alone, and is valued for its high carbohydrate contents (215). Molasses can also be distilled and fermented to produce various items (for example, rum). Ethyl alcohol (ethanol) is another alcohol produced from molasses, which has many uses such as in production of vinegar, cosmetics and pharmaceuticals, cleaning preparations, and coatings. Ethanol is currently being studied as a gasoline extender for future use in automobile. In Brasil, approximately 14.2 billion liters of ethanol are produced each year (75). At present, four million cars run on gasahol that contains about 80% ethanol (41). Other products produced from molasses include butanol (a solvent), lactic acid (a solvent), citric acid (mostly for foods and beverages), and glycerol (75). Another useful by-product of sugar production is bagasse, the fibrous residue, which can be used to make paper, cardboard, fiberboard, and wallboard (252). It is possible that other uses of sugarcane will be developed in the future, but even now it can be viewed as a very important and useful plant crop worldwide.
2.4. Indole-3-acetic acid (IAA)

Our hypothesis explored in this research is that auxin produced by *A. diazotrophicus* is a factor for sugarcane growth promotion. Auxin (from Greek term, auxein, to increase) was the first phytohormone recognized and chemically detected. The discovery of auxin was originally reported in Darwin’s book, *Power of Movement in Plants* (1880) (65). His work on the phototropic response of grass coleoptiles demonstrated that the transfer of a signal from the tip to the lower part of the plant, which was later identified as IAA. Many indole compounds and phenylactic derivatives have been reported to have auxin-like activity. Among these, indole-3-acetic acid (IAA) is considered the most physiologically active indole compound in plants. IAA influences various aspects of plant growth and developmental responses. Many functions attributed to IAA overlap with other phytohormones, particularly ethylene. The influence of IAA is concentration-dependent; i.e., a low concentration may be stimulatory, whereas a high concentration is frequently inhibitory. The physiological actions of exogenous IAA are summarized in Table 3.

IAA biosynthesis is not restricted to higher plants. Microorganisms such as bacteria, fungi, and algae are able to produce physiologically active IAAAs that may have a pronounced effects on plant growth and development. In fact, it has been suggested that up to 80% of bacteria isolated from the rhizosphere have the capacity to synthesize IAA *in vitro* in the presence or absence of physiological precursors (tryptophan-TRP) (101, 168). Microbial isolates from the rhizosphere of different crops appear to have a greater potential to synthesize and release IAAAs as secondary metabolites because of the rich
Table 3. Physiological effect of IAA

<table>
<thead>
<tr>
<th>Plant response to IAA</th>
<th>Stimulation</th>
<th>Inhibition</th>
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<tbody>
<tr>
<td></td>
<td>Apical dominance</td>
<td>Leaf and fruit abscission</td>
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<td></td>
<td>Callus tissue morphogenesis</td>
<td>Lateral buds growth when IAA is supplied from the apical bud</td>
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<td></td>
<td>Cell division in cambium</td>
<td>Leaf senescence</td>
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<td></td>
<td>Cell enlargement</td>
<td>Root growth</td>
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<td></td>
<td>Differentiation phlom and xylem</td>
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<td></td>
<td>Enzyme activity</td>
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<td></td>
<td>Femaleness in dioecious flowers</td>
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<td></td>
<td>Flowering</td>
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<td></td>
<td>Fruit abscission/ripening</td>
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<td></td>
<td>Fruit setting and growth</td>
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<tr>
<td></td>
<td>Growth rate</td>
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<td></td>
<td>Lateral root development in tissue culture</td>
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<tr>
<td></td>
<td>Production of ethylene at high concentration</td>
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<tr>
<td></td>
<td>Respiration</td>
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<td></td>
<td>RNA/ protein synthesis</td>
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<td></td>
<td>Root initiation</td>
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<tr>
<td></td>
<td>Tropistic response toward gravity and light</td>
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</table>

supply of substrates from root exudates (46, 226, 278).

2.4.1. IAA biosynthetic pathways

Production of IAA by microbial isolates varies greatly in amount among different species and strains, and varies due to the availability of the substrate(s). Several different biosynthetic pathways for IAA production are distributed among these bacteria. Comprehensive lists of microorganisms capable of synthesizing IAA are given in Table 4. Although for many years it was assumed that tryptophan is the precursor of IAA, in vitro studies have demonstrated that some microbes can produce small amounts of IAA in the absence of the precursor (TRP). Additionally, it was confirmed with tryptophan auxotrophic mutants of plants, which showed IAA biosynthesis can also occur via a tryptophan-independent route (203, 204, 206, 241, 306). Approximately 90% of IAA synthesis occurs independently of tryptophan in the case of Azospirillum spp. (15, 231). However, in the presence of TRP, the microbes release much greater quantities of IAA and IAA derivatives (145). The conversion of TRP into IAA occurs by different routes, including deamination, decarboxylation, and hydrolysis (Fig. 10) (214). Biochemical and genetic evidence, along with radiolabelled precursor [\(^{\text{2}}\)H]tryptophan and [\(^{\text{3}}\)H]indolacetamide feeding experiments, led to the conclusion that individual organisms could operate one or multiple biosynthetic pathways for IAA production (172, 214, 230).
Table 4. IAA producing bacteria and their biosynthetic pathway

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Organism</th>
<th>Genes and genetic, biochemical evidences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole-3-acetamid</td>
<td><em>Pseudomonas syringae pv. savastanoi</em></td>
<td><em>iaaM, iaaH</em></td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas syringae pv. syringae</em></td>
<td><em>iaaM, iaaH</em></td>
</tr>
<tr>
<td></td>
<td>Agrobacterium tumefaciens</td>
<td><em>tms-1, tms-2</em></td>
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<td></td>
<td><em>Agrobacterium rhizogenes</em></td>
<td><em>aux-1, aux-2</em></td>
</tr>
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<td></td>
<td><em>Erwinia herbicola pv. gypsophila</em></td>
<td><em>iaaM, iaaH</em></td>
</tr>
<tr>
<td></td>
<td><em>Bradyrhizobium japonicum</em></td>
<td><em>bam</em></td>
</tr>
<tr>
<td></td>
<td><em>Rhizobium fredii</em></td>
<td>idoleacetamide hydrolase activity</td>
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<tr>
<td></td>
<td><em>Rhizobium leguminosarum</em></td>
<td><em>bam</em></td>
</tr>
<tr>
<td></td>
<td><em>Azospirillum brasilense</em></td>
<td>Southern hybridization, TRP-monooxygenase activity</td>
</tr>
<tr>
<td></td>
<td><em>Arthrobacter spp.</em></td>
<td>HPLC</td>
</tr>
<tr>
<td></td>
<td><em>Flavobacterium spp.</em></td>
<td>HPLC</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas spp.</em></td>
<td>HPLC, TLC, MS, GC-MS</td>
</tr>
<tr>
<td></td>
<td><em>Xanthobacter spp.</em></td>
<td>HPLC</td>
</tr>
<tr>
<td>Indole-3-pyruvic acid</td>
<td><em>Agrobacterium tumefaciens</em></td>
<td>PC (paper chromatography), TRP-transaminase activity</td>
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<tr>
<td></td>
<td><em>Azospirillum brasilense</em></td>
<td><em>ipdc</em></td>
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<td></td>
<td><em>Azospirillum lipoferum</em></td>
<td>aminotransferase activity, Southern</td>
</tr>
<tr>
<td></td>
<td><em>Azospirillum halopraeferens</em></td>
<td><em>ipdc</em></td>
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<tr>
<td></td>
<td><em>Enterobacter cloacae</em></td>
<td>Southern hybridization</td>
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<tr>
<td></td>
<td><em>Erwinia herbicola pv. gypsophila</em></td>
<td><em>ipdc</em></td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas fluorescens</em></td>
<td>HPLC</td>
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<tr>
<td></td>
<td><em>Pseudomonas pv. savastanoi</em></td>
<td>TRP-transaminase activity</td>
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<tr>
<td></td>
<td><em>Pseudomonas spp.</em></td>
<td>TRP-transaminase activity</td>
</tr>
<tr>
<td></td>
<td><em>Rhizobium spp.</em></td>
<td>HPLC, TLC, GC-MS, MS</td>
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<tr>
<td></td>
<td><em>Rhizobium meliloti</em></td>
<td>GC-MS</td>
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<td></td>
<td><em>Xanthomonas spp.</em></td>
<td><em>aat</em> genes, alcohol dehydrogenase activity,</td>
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<td></td>
<td></td>
<td>aminotransferase activity</td>
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<td></td>
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<td>HPLC, TLC, MS, GC-MS</td>
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Table *Continued*

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Organism</th>
<th>Genes and genetic, biochemical evidences</th>
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</thead>
<tbody>
<tr>
<td>Tryptamine</td>
<td><em>Acetobacter xylinum</em></td>
<td>PC</td>
</tr>
<tr>
<td></td>
<td><em>Arthrobacter spp.</em></td>
<td>HPLC</td>
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<td></td>
<td><em>Bacillus cereus</em></td>
<td>TRP-decarboxylase activity,</td>
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<tr>
<td></td>
<td><em>Azospirillum brasilense</em></td>
<td>TLC, PC, GC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TRP-decarboxylase activity</td>
</tr>
<tr>
<td>Indole-3-acetonitrile</td>
<td><em>Agrobacterium tumefaciens</em></td>
<td>nitrile hydratase and amidase activity</td>
</tr>
<tr>
<td></td>
<td><em>Rhizobium spp.</em></td>
<td>nitrile hydratase and amidase activity</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas chlororaphis</em></td>
<td>Southern hybridization</td>
</tr>
<tr>
<td></td>
<td><em>Klebsiella ozonae</em></td>
<td>nitrilase gene</td>
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<td></td>
<td><em>Rhodococcus rhodochrous</em></td>
<td>aliphatic nitrilase gene</td>
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<tr>
<td></td>
<td><em>Rhodococcus spp.</em></td>
<td>Southern hybridization</td>
</tr>
<tr>
<td></td>
<td><em>Rhodococcus erythropolis</em></td>
<td>nitrile hydratase gene</td>
</tr>
</tbody>
</table>

2.4.1.1. Indole-3-pyruvic acid pathway

In both higher plants and most microorganisms, the indole-3-pyruvic acid (IpyA) pathway seems to be the major IAA biosynthetic pathway, whereas other pathways operate in only certain species. The formation of IpyA from TRP is catalyzed by multispecific aminotransferases, followed by spontaneous or enzymatic decarboxylation to indole-3-acetaldehyde (IAAld), which is then further oxidized by an IAAld oxidase to IAA. In a side reaction, IpyA may be reduced to indole-3-lactic acid (ILA) by lactate dehydrogenase, a reaction that requires NADH. Indole-3-ethanol (TOL)
Figure 10. Pathways of IAA biosynthesis. → represent pathways present in both bacteria and plant. --- is a proposed tryptophan-independent biosynthetic pathway. --> shows bypass pathways.
is produced by a side reaction (reduction) from IAAld. This reaction is carried out by an alcohol dehydrogenase, a flavoprotein, and is sensitive to IAAld and IAA, and acts as a regulator of IAA levels.

The gene for a key enzyme in the IpyA pathway, indole-3-pyruvate decarboxylase (ipdC), has been isolated from Azospirillum brasilense (62). The ipdC gene of A. brasilense showed similarity to that of Zymomonas mobilis and Saccharomyces cerevisiae (38 %). Based on DNA hybridization results, this gene seems to be present in A. lipoferum and A. halopraeferens but not in A. irakense, a low IAA producer. Screening a cosmid library of Enterobacter cloacae for the ability to convert tryptophan to IAA revealed a single E. cloacae ipdC gene (159), the gene product having 43% similarity to that of A. brasilense. Clark et al. (1993) (57) concluded that Erwinia herbicola pv. gypsophilae strains can produce IAA via the IpyA pathway, evidenced by the chemical identification of intermediates and by assaying for production of IAA after treatment with selected intermediates. The aromatic aminotransferase (AAT) activity of Rhizobium meliloti 102F34 was detected, and further, two genes (aatJ and aat2) encoding the enzyme were isolated (244).

Estimates obtained from HPLC and capillary gas chromatography-mass spectrometry analyses revealed that A. diazotrophicus strains produced from 0.14 to 2.42 μg IAA/ml in culture medium through the IpyA pathway. Considering that A. diazotrophicus is found within plant tissues, the biosynthetic ability to synthesize IAA suggests that the bacteria could promote sugarcane root development and/or improve sugarcane growth by direct effects on metabolic processes (22).
2.4.1.2. Indole-3-acetamide pathway

Another well-known pathway of tryptophan to IAA is carried out by the plant pathogen, *Psuedomonas syringae* pv. *savastanoi*, as well as other microorganisms. This pathway first involves the four electron oxidative decarboxylation of tryptophan to indole-3-acetamide (IAM) by a TRP monooxygenase and, then, hydrolysis of IAM by an IAM hydrolase to yield IAA. The presence of this pathway in higher plants has also been reported (145, 146, 232). In IAA-producing strains of *P. syringae* pv. *savastanoi* isolated from oleander, a 52 kb plasmid is required for IAA biosynthesis (59, 60). This plasmid contains two genes, *iaaM* and *iaaH*, both of which are involved in IAA biosynthesis. The plant pathogen, *Agrobtwterium tumefaciens* carries the Ti plasmid, which encodes two genes, *tms-1* and *tms-2*, for IAA biosynthesis and is required for formation of crown galls (172). Over-expression of the *tms-2* gene encoding indoleacetamide hydrolase resulted in the production of a 49 kDa protein that could convert indole-3-acetamide to IAA (256, 282). Using biochemical analysis and a lacZ-*tms-1* gene fusion, Thomashow et al. (1986) and Van Onckelen et al. (1986) (296) confirmed that *tms-1* encodes the enzyme tryptophan-2-monooxygenase. Comparison of nucleotide sequences shows there is 54% and 38% similarity between the *tms-1* and *iaaM* gene products, and the *tms-2* and *iaaH* gene products, respectively (152, 308).

A second set of *iaa* genes (TB-iaa), showing weak homology to *tms-1* and *tms-2* were located within the T-DNA (132), which appears to have the same basic function as the other set of IAA genes, but do not produce sufficient levels of IAA to cause tumor
and root induction. *A. rhizogenes*, the causative agent of hairy root disease on dicotyledonous plants, possesses genes for IAA biosynthesis *(aux1 and aux2)* localized in the Ri-plasmid T-DNA region (63). The *aux* genes and their regulatory signals are similar to those of *A. tumefaciens* (105). Another subspecies of *P. syringae*, pv. *syringae*, causing brown spot disease of bean, also produces IAA by the IAM pathway. Genes for this pathway are on the chromosome rather than a plasmid (187). However, not all pathogenic strains of *Pseudomonas syringae* synthesize IAA via the indoleacetamide pathway. DNA hybridization probed with *iaaM* and *iaaH* genes from *Ps. pv. savastanoi* failed to detect these genes in several strains isolated from host plants (301). It could be explained that the sequences of *iaaM* and *iaaH* in these strains have significantly diverged or that IAA is synthesized by a different pathway.

*iaaM* and *iaaH* genes were found only in pathogenic strains of *Erwinia herbicola* and *E. herbicola* pv. *gypsophilia* using *P. syringae* pv. *savastanoi* *iaaM* and *iaaH* genes as Southern hybridization probes (105). The *Bradyrhizobium japonicum* gene (*bam*) was isolated by complementation and its gene product shows significant similarity to those of the *iaaH* and *tms-2* genes. However, regions upstream and downstream from the *bam* gene did not indicate the similarity to *iaaM* and *tms-1* (a tryptophan 2-monoxygenase gene). Tryptophan 2-monoxygenase activities of *A. brasilense* were detected on a non-denaturing gel and DNA hybridization analysis also indicated the presence of the IAM pathway. However, the principal IAA biosynthesis route of *A. brasilense* is not the IAM pathway (14).
2.4.1.3. Tryptamine pathway

Tryptophan can be converted into tryptamine (TAM) by TRP decarboxylase. followed by its conversion into IAA through IAAd by amine oxidase (224). Although this pathway appears to be widespread among plants and fungi, very little attention has been focused on tryptamine as an intermediate in bacterial IAA biosynthesis. However, *Bacillus cereus* and *Azospirillum brasilense* have been reported to synthesize a decarboxylase that can act on tryptophan to produce tryptamine (123, 222).

2.4.1.4. Indole-3-acetonitrile pathway

A minor pathway that has been postulated for IAA production is the conversion of TRP to IAA through indole-3-acetonitrile (IAN) (124, 162, 198, 205, 315). IAN can be hydrolyzed directly to IAA or via a two-step process involving an initial conversion of an amide by nitrile hydratase, followed by hydrolysis of the acetamide to IAA by amidase (157). The reduction of indole acetonitrile for IAA synthesis is involved in conversion of tryptophan to indoleacetaldoxime and then to indole-acetonitrile either directly or via glucobrassicin (156).

The gene for nitrilase has been cloned from *Alcaligenes faecalis* JM3 and sequenced (156). The deduced amino acid sequence shares 34 % similarity with that of *Klebsiella ozonae* and 27 % of *Arabidopsis thaliana* respectively (21, 273). Interestingly, an *A. thaliana* nitrilase gene, nit-2 is dramatically induced by virulent strains of *P*. 
syringae; in contrast, avirulent strains stimulate only modest expression of nit-2 (20), which may imply the role of IAA as a plant defense response. Although nitrilase activity could not be detected in several strains of Agrobacterium and Rhizobium spp., these strains showed nitrile hydratase and amidase activity involved in converting indole-3-aceonitrile to IAA via IAM (158). The nitrile hydratase and amidase genes are organized as an operon in several strains of Rhodococcus and in Pseudomonas chlororaphis (82, 157).

2.4.2. Regulation of bacterial IAA production

The number and type of IAA biosynthetic pathways, as well as several other factors, acting in concert, will have a significant effect on the output of IAA by a microbe. The amount of microbial IAA available to influence a plant is subject to several levels of regulation. On the part of the microbe, the pathway for IAA biosynthesis, the regulatory sequences, the location of the biosynthetic genes, the presence of precursors, and the presence of enzymes converting free IAA to conjugated forms are directly involved in expression of the IAA biosynthetic genes. The regulation strategies and the examples are listed in Table 5.
Table 5. Regulation of IAA levels

<table>
<thead>
<tr>
<th>Regulation</th>
<th>High production of IAA</th>
<th>Examples/Notes</th>
<th>Low production of IAA</th>
<th>Examples/Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location of genes</td>
<td>Plasmid-encoding genes</td>
<td><em>Agrobacterium</em> strains (Ti and Ri plasmid) and <em>P. syringae</em> pv. <em>savastanoi</em></td>
<td>Chromosome-encoding genes</td>
<td><em>P. syringae</em> pv. <em>syringae</em></td>
</tr>
<tr>
<td>Expression</td>
<td>Constitutive expression</td>
<td>Indoleacetamide pathway; most of microorganisms</td>
<td>Inducible expression</td>
<td>Indoleacetonitrile, indolepyruvic acid, indoleacetonitrile pathways; most of microorganisms</td>
</tr>
<tr>
<td>TRP concentration in root exudates</td>
<td>High</td>
<td>Most organisms</td>
<td>Low</td>
<td>Most microorganisms; for example, <em>Enterobacter cloacae</em>, <em>Rhizobium phaseoli</em>, <em>Bradyrhizobium japonium</em>, and <em>Azospirillum brasilense</em></td>
</tr>
<tr>
<td>Feed back inhibition</td>
<td>Tryptophan-feeds back to inhibit anthranilate synthase</td>
<td>Enhance IAA production; <em>Azospirillum brasilense</em></td>
<td>Anthranilate, a tryptophan precursor</td>
<td>Inhibit IAA production; <em>Azospirillum brasilense</em></td>
</tr>
<tr>
<td>Regulation</td>
<td>High production of IAA</td>
<td>Examples/Notes</td>
<td>Low production of IAA</td>
<td>Examples/Notes</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------</td>
<td>----------------</td>
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<td>----------------</td>
</tr>
<tr>
<td><strong>O₂</strong></td>
<td>Aerobic conditions</td>
<td>Produce high levels of IAA; <em>Enterobacter cloacae</em></td>
<td>Less aerobic conditions</td>
<td>Produce IAA with side chain pathways (ILA and TOL), which are considered as storage products; low levels of IAA; <em>Enterobacter cloacae</em></td>
</tr>
<tr>
<td>Multiple IAA biosynthetic pathways-employ a particular pathway at certain condition</td>
<td>Symbiosis state</td>
<td><em>Rhizobium</em> spp. utilize indoleacetamide pathway only when associated with the host plant</td>
<td>Free-living state</td>
<td>Free-living <em>Rhizobium</em> uses the indole pyruvic acid pathway</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>High pH</td>
<td><em>Pseudomonas crescens</em> utilize main IAA biosynthetic pathways</td>
<td>Low pH</td>
<td>Low pH causes <em>Pseudomonas crescens</em> to utilize a side chain pathway for IAA biosynthesis; reduction of IAA production</td>
</tr>
<tr>
<td>Regulation</td>
<td>High production of IAA</td>
<td>Examples/Notes</td>
<td>Low production of IAA</td>
<td>Examples/Notes</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>-----------------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>IAA state</td>
<td>Free IAA</td>
<td><em>P. syringae</em> pv. <em>savastanoi</em>; IAA-lysine synthetase (<em>iaaL</em>) mutant accumulate free IAA</td>
<td>Conjugated with amino acid or sugar moieties</td>
<td><em>P. syringae</em> pv. <em>savastanoi</em> has IAA-lysine synthetase, which is involved in catalyzing the formation of 3-indole-acetyl-ε-L-lysine (a conjugated form of IAA)</td>
</tr>
<tr>
<td>Regulatory sequence</td>
<td>Eukaryotic expression signal</td>
<td>IAA biosynthetic genes are directly transferred to plant genomes and expressed from the regulatory sequences similar to well-known eukaryotic expression signals; <em>A. tumefaciens</em> and <em>A. rhizogenes</em></td>
<td>Typical prokaryotic organization</td>
<td>All microorganisms except <em>A. tumefaciens</em> and <em>A. rhizogenes</em></td>
</tr>
</tbody>
</table>
2.4.3. Physiological effects of microbial IAA: growth promotion versus pathogenesis.

The role of IAA in plant-microbe interactions is not well understood. Although it is easy to imagine that the plant could benefit from the IAA, it is not clear why bacteria produce IAA, an energy-requiring process. Several speculations are followed: first, by stimulating plant growth, microorganisms can also increase the production of plant metabolites that they can utilize for their own growth (107). Second, more subtle benefits could be the detoxification of tryptophan analogues, which are deleterious to bacterial cells. Shinshi et al. (1987) (263) and Gaffney et al. (1990) (103) demonstrated that auxin and cytokinin caused accumulation of β-1,3-glucanase and chitinase mRNAs and inhibit these enzyme activities in cultured tobacco tissues. Additionally, it was shown that the expression of the tms genes in bacteria inhibit the development of the hypersensitive plant response (243). Therefore, a third benefit of IAA production could be the inhibition of plant defense enzymes, which would facilitate bacterial invasion. Finally, IAA production was shown to be directly involved in the regulation of in planta growth and in the expression of other factors that affect the host-pathogen interaction of Pseudomonas syringae pv. syringae (187).

Generally, bacterial phytopathogens either transform their host plants (ex. A. tumefaciens) or colonize intercellular spaces in plant tissues (P. syringae pv. savastanoi). Moreover, beneficial bacteria appear to affect plants while inhabiting the external surface of a plant (68). One of the exceptions to this rule is the Rhizobium-legume relationship, in which bacterial IAA seems to be essential in the root nodulation process and beneficial
while bacteria are found in both the intercellular and intracellular space of legume plants (89). An important consideration in predicting whether bacterial IAA will stimulate beneficial growth or pathogenesis in a plant is the level of IAA synthesized by the plant itself. In plant roots endogenous levels of IAA may be either suboptimal or optimal for growth. However, an additional input from bacterial sources into the existing IAA pool could modify endogenous IAA to either optimal or super-optimal levels, resulting in the induction of plant growth or pathogenesis, respectively. It has also been documented that in some cases IAA biosynthetic genes in pathogenic strains are encoded on a plasmid, while these genes in nonpathogenic strains are encoded on the chromosome, examples being Xanthomonas campestris pv. glycines (93) and P. syringae pv. savastanoi (187, 270). Sitbon et al. (1992) (266) proposed that the IAM pathway might work as a regulatory element. The IAM pathway is rarely found in plants, therefore constitutive expression of a bacterial IAM pathway may result in high levels of IAA. In contrast, many beneficial bacteria such as Enterobacter cloacae and Azosprillum spp. synthesize IAA mainly via the IpyA pathway.

When Agrobacterium species transform plant cells, the direct delivery of high levels of IAA into the host cells results in gall formation. This is correlated with a stimulation of plant growth to the point of gall formation (105). In the case of P. syringae pv. savastanoi, the colonization of intercellular tissues and secretion of IAA causes tumorous growths (173). The IAA response may also depend on the stage of plant root development. Application of a low level of IAA promoted growth in young maize
roots, but inhibited growth in older roots (225). Another factor to be emphasized is that IAA may not act alone, but rather it may interact with other plant growth hormones in a variety of complex ways (220). Finally, it has been shown that the presence of enzymes involved in converting free, active IAA into a conjugated, inactive form are involved in determining IAA levels, subsequently determining the fate of the beneficial or pathogenic effect of IAA. For example, the oleander strain of *P. syringae pv. savastanoi* can catalyze free IAA into a conjugated form, while the mutant strains that accumulate five times more free IAA than the parental strain did not incite typical gall formation when inoculated into oleander plants (108). Therefore, it is the extent to which bacterial sources of IAA modify endogenous levels of free IAA in the plant that ultimately determines whether the relationship is beneficial or pathogenic. It appears that optimal levels of IAA enhance the plant growth, but high levels cause a disease response.

Several genetic and biochemical methods have been used to characterize the genes directly involved in IAA biosynthesis in other organisms. Available methods include southern hybridization, PCR, random mutagenesis of a genome, complementation, and the detection of enzymatic activities. To isolate the IAA biosynthetic genes of *A. diazotrophicus* all the methods mentioned above were attempted, but only random Tn5 mutagenesis successfully identified genes that might be involved in IAA biosynthesis. One Tn5 mutant produced significantly less IAA as compared with the wild type strain. These genes turned out to encode proteins involved in cytochrome c biogenesis.
2.5. Cytochrome c biogenesis genes (ccm genes)

Many different types of c-type cytochromes are found in eubacteria, archaeabacteria, mitochondria and thylakoids of algae or plants, some of which catalyze not only electron transfer, but also other types of chemical reactions. The hallmark of c-type cytochrome synthesis is the covalent attachment of heme vinyl groups to two cysteiny1 residues of the apocytochrome at a CxxCH motif. The biological significance of the covalent linkage is not clear, however its formation involves posttranslational steps that vary between cell types. In addition, it involves in some bacteria complex machinery to connect to systems for forming and reducing disulphide bonds (212).

2.5.1. Common principles of cytochrome c biogenesis

Bacterial c-type cytochromes are synthesized with an N-terminal signal sequence that is recognized by the Sec system for protein export (285). The apopolypeptide, with its N-terminal signal sequence in the cytoplasm is transported to the periplasm via the Sec pathway, followed by formation of a disulfide bond within the CxxCH motif of the cytochrome (211, 249, 251). As the heme binding-site cysteines move into the periplasm, they become rapidly oxidized by the DsbA/DsbB system and form disulfides (191, 250). Subsequently, heme is covalently attached by cytochrome c heme lyase (CCHL) to give the holoprotein. Soluble cytochrome c is released into the periplasm by leader peptidase, which is a step independent from heme ligation. Although there is a signal sequence, the cleavage of the signal sequence is not always a prerequisite for heme attachment to water-soluble periplasmic cytochromes (285). Holocytochrome is released from CCHL
and folds into an active tertiary structure. The existence of membrane-anchored c-type cytochromes shows that heme attachment does not require a polypeptide to release it from the cytoplasmic membrane (212).

2.5.2. Molecular mechanisms of cytochrome c biogenesis: three distinct systems

Molecular biological studies of the biogenesis of c-type cytochromes have been clearly elucidated for three different systems. System I requires specific proteins encoded by at least nine genes (helABCDX, ccl12, cycJ and cycH), and has been found in most Gram-negative bacteria (Fig. 11). Genomic sequencing also indicates that system I is present in mitochondria from plants and protozoans, including the primitive protozoan, Relinomonas. System II is characteristic of Gram-positive bacteria (Fig. 12), cyanobacteria and chloroplasts and involves at least four genes (ccsA, ccs1, resA and ccdA). System I and II are predicted to be involved in heme delivery, apocytochrome presentation and thioreduction. In contrast to the complexity of systems I and II, mitochondria from fungi requires a single protein called cytochrome c heme lyase (cyc3) (System III). Vertebrates and invertebrates also possess the gene for this enzyme (Fig. 13) (160, 283).

2.5.2.1. System I

Mutational analyses of bacteria have implicated at least 12 genes in c-type cytochrome assembly (Table 6). However, all of these genes do not necessarily occur in each bacterium. Mutations in any of the genes listed in Table 6 result in the loss of all
Fig. 11. Possible scheme of cytochrome c biogenesis in a typical Gram-negative bacteria. Modified from Kranz et al. (1998) and Page et al. (1998).
Figure 12. A working model of the system II for cytochrome c biogenesis. This system is found in Chloroplast of Chlamydomonas, Gram-positive bacteria, Cyanobacteria, and Helicobacter. Modified From Kranz et al. (1998).
Figure 13. A working model of the system III for cytochrome c biogenesis. This system is found in mitochondria of Fungus, Vertebrate, and Invertebrate. Modified from Kranz et al. (1998)
Table 6. Cytochrome c biogenesis genes (ccm homologs) identified in Gram-negative bacteria

<table>
<thead>
<tr>
<th>E. coli&lt;sup&gt;c&lt;/sup&gt;</th>
<th>B. japonicum&lt;sup&gt;c&lt;/sup&gt;</th>
<th>R. leguminosarum&lt;sup&gt;c&lt;/sup&gt;</th>
<th>R. capsulatus&lt;sup&gt;c&lt;/sup&gt;</th>
<th>P. fluorescens&lt;sup&gt;c&lt;/sup&gt; (ATCC 17400)</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ccmA</td>
<td>cvcV</td>
<td>helA</td>
<td></td>
<td></td>
<td>ABC transport</td>
</tr>
<tr>
<td>ccmB</td>
<td>cvcW</td>
<td>helB</td>
<td></td>
<td></td>
<td>ABC transport</td>
</tr>
<tr>
<td>ccmC</td>
<td>cvcZ</td>
<td>helC</td>
<td>cytA</td>
<td></td>
<td>ABC transport</td>
</tr>
<tr>
<td>ccmD</td>
<td>cvcX</td>
<td>cycX</td>
<td>helD</td>
<td>cytB</td>
<td>Maturase assembly</td>
</tr>
<tr>
<td>ccmE</td>
<td>cvcJ</td>
<td>cycJ</td>
<td></td>
<td>cytC</td>
<td>CCHL subunit,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>chaperone</td>
</tr>
<tr>
<td>ccmF</td>
<td>cvcK</td>
<td>cycK</td>
<td>ccll</td>
<td>cytD</td>
<td>CCHL catalytic</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>subunit;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>facilitating</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>haem attachment</td>
</tr>
<tr>
<td>ccmG</td>
<td>cvcY</td>
<td>cycY</td>
<td>helX</td>
<td>cytE</td>
<td>Reductive function</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>in periplasm for</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>thiols and/or</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>haem; Thioredoxin</td>
</tr>
</tbody>
</table>

<sup>c</sup> Identification by 16S rDNA sequence comparison.
<table>
<thead>
<tr>
<th>E. coli</th>
<th>B. japonicum</th>
<th>R. leguminosarum</th>
<th>R. capsulatus</th>
<th>P. fluorescens (ATCC 17400)</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ccm\textsubscript{HN}\textsuperscript{a}</td>
<td>cvcL</td>
<td>cycL</td>
<td>col2</td>
<td>cytF</td>
<td>CCHL subunit, reducing; facilitating haem attach</td>
</tr>
<tr>
<td>ccm\textsubscript{HC}\textsuperscript{b}</td>
<td>cvcH</td>
<td>cycH</td>
<td>cycH</td>
<td>cytG</td>
<td>CCHL subunit, specificity; facilitating haem attachment</td>
</tr>
<tr>
<td>dsbA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Converting SH to disulphide</td>
</tr>
<tr>
<td>dsbB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Oxidizing reduced DsbA</td>
</tr>
<tr>
<td>dipZ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Transfer of reluctant to periplasm</td>
</tr>
<tr>
<td>ccdA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Transfer of reluctant to periplasm</td>
</tr>
</tbody>
</table>
a. 5' half of the gene, coding for the N-terminal domain of CcmH

b. 3' half of the gene, coding for the C-terminal domain of CcmH

c. Organisms: Bradyrizobium japonicum; Escherichia coli; Pseudomonas fluorescens; Rhizobium leguminosarum; Rhodobacter capsulatus
(more than ten in some organisms) c-type cytochromes. Interestingly, the *E. coli* genome only contains one set of general c-type cytochrome biogenesis genes, although it synthesizes several endogenous and exogenous c-type cytochromes (64, 191, 250, 285). Thus, one biogenesis apparatus can add the heme to many different polypeptides. However, it is not known how many residues flanking the CxxCH motif are important. Also, it is not clear whether there is any discrimination against particular amino acid residues in either these sequences or the spacer residues between the cysteines (212).

A supercomplex of multiple membrane and periplasmic proteins is envisioned to function as a c-type cytochrome assembly apparatus, which catalyzes heme transport (by an ABC-type transporter, CcmABC), apoprotein reduction (by a series of periplasmic thio-disulfide-type oxidoreductases, CcmG, CcmH/Ccl2), apoprotein chaperoning (by CcmH/CycH) and the lyase reaction (by CcmF) (110, 218). CcmH of *E. coli* appears to be a bifunctional protein. Its N-terminal half (CcmHN) is homologous to CycL (found in *R. leguminosarum*) whereas its C-terminal half (CcmHC) corresponds to a CycH lacking the N-terminal domain. Interestingly, the CcmHC domain is not required for cytochrome c maturation (115). A novel tryptophan-rich sequence motif is present in CcmC (the substrate binding subunit of the putative transporter) and CcmF (the putative lyase subunit), and it is proposed to represent a heme-binding domain (Table 7) (24, 102, 284, 309). Alkaline phosphatase (PhoA)-fusion analyses of CcmC and CcmF place the tryptophan-rich motif on the periplasmic side, which is compatible with its proposed heme-binding function (160, 212, 283, 286, 287). The protein CcmE has been
Table 7. Conserved motifs of cytochrome c biogenesis genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Motif</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ccmA</td>
<td>GxxGxGKT&lt;br&gt; LWxLDE</td>
<td>Nucleotide binding sites (Walker motifs)</td>
</tr>
<tr>
<td>ccmB</td>
<td>DGSL&lt;br&gt; AHWxxGGLPL</td>
<td>Membrane integral subunits</td>
</tr>
<tr>
<td>ccmC</td>
<td>WGxPMWGTxWDxRLT</td>
<td>Heme binding; tryptophan rich motif; found also in cytochrome c heme lyase</td>
</tr>
<tr>
<td>ccmE</td>
<td>YxGILPDLFRxGPGxV&lt;br&gt; VLAKHDEXYxP</td>
<td>Heme binding and/or reducing</td>
</tr>
<tr>
<td>ccmF</td>
<td>WAYYELGWGGxWFDPVEN&lt;br&gt; CxxCK</td>
<td>Heme binding and/or ligation</td>
</tr>
<tr>
<td>ccmG</td>
<td>CxxC&lt;br&gt; LDLGVYGAPETFLI</td>
<td>Heme binding</td>
</tr>
<tr>
<td>ccmH&lt;sub&gt;N&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>LRCxxC; RCVP</td>
<td>Heme binding</td>
</tr>
<tr>
<td>ccmH&lt;sub&gt;C&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>PxDGxGWxxLA</td>
<td>Selection/discrimination of specific apocytochromes c</td>
</tr>
<tr>
<td>ccsA</td>
<td>WWD</td>
<td>Heme binding</td>
</tr>
<tr>
<td>resA</td>
<td>WCEPC</td>
<td>Heme binding</td>
</tr>
<tr>
<td>resC</td>
<td>WGxxWxWD</td>
<td>Heme binding</td>
</tr>
<tr>
<td>ccdA</td>
<td>CxxC</td>
<td>Heme binding</td>
</tr>
</tbody>
</table>
shown recently to covalently bind heme to the polypeptide chain (257). The *ccm* genes and their homologs from different bacterial species are organized in one (gamma proteobacteria), two (rhizobia), or at least three (*Rhodobacter capsulatus*) gene clusters (Fig. 14).

A transmembrane protein, DipZ, has a role in reductive steps of *c*-type cytochrome biogenesis as a supplier of reductant, which is required for rearrangement of disulfide bonds in the periplasm (249). DipZ is reduced at its cytoplasmic surface by thioredoxin and may have a possible role in reductive steps required for rearrangement of disulfide bonds in the periplasm (242). The characterization of the *dipZ* gene revealed two conserved cysteines in putative transmembrane helices. In studies with *B. subtilis* it was shown that the *ccdA* gene, a transmembrane protein with a pair of conserved cysteines like DipZ, is necessary for *c*-type cytochrome biogenesis (254). *E. coli* does not possess *ccdA* and *B. subtilis* does not contain *dipZ*, whereas *P. aeruginosa* and *Hemeophilus influenzae* possess both genes. Two proteins, DsbA and DsbB, are required for both endogenous and exogenous *c*-type cytochrome biogenesis in *E. coli*. DsbA is thought to directly oxidize a target protein (e.g. alkaline phosphatase), and DsbB reoxidizes DsbA (64, 192, 250). Mutations in these two genes can be complemented by disulfide compounds, supporting their normal oxidizing role in the cytochrome *c* biogenesis. These findings could be interpreted as indicating that the two cysteines of the CxxCH motif are first converted into a disulfide bond before being processed to receive the heme group (251). A spontaneous heme attachment occurs when a gene for cytochrome *c*552 from a thermophilic bacterium is expressed in the *dipZ* or *ccm* mutants
Fig. 14. Comparison of the bacterial cytochrome c maturation operon. Organisms: (A) *Escherichia coli*; (B) *Pseudomonas fluorescens* ATCC17400; (C) *Bradyrhizobium japonicum*; (D) *Rhizobium leguminosarum*; (E) *Rhodobacter capulatus*
of *E. coli*. The apocytochrome of this thermostable c-type cytochrome adopts a folded conformation into which the heme can fit. This suggests that enzymatic catalysis is not mandatory for the addition of heme to the apocytochrome (249, 265).

2.5.2.2. System II

For system II, all c-type cytochromes possess typical Sec-dependent signal sequences and are presumably translocated as described in System I. At least four proteins, CcsA, Ccs1, ResA and CcdA, are required in cytochrome c biogenesis for Gram-positive bacteria and plastid systems. For system II, the CcsA (and possibly Ccs1) proteins replace the HelABCD and Ccl1 in system I for heme delivery, while the CcdA/ResA are probably involved in thioreduction. The ResA and Ccs1 have significant sequence similarities to certain domains of proteins known to be involved in cytochrome c maturation. ResA has a thioredoxin-like sequence WCEPC and Ccs1 has a tryptophan-rich WGxxWxWD motif that is also found in the CcmC and CcmF homologs (110, 279). The CcsA protein contains a canonical pattern for a transporter with six transmembrane domains and the highly conserved WWD domain. In addition, the *ccdA* gene is required for the biosynthesis of c-type cytochrome (transfer reductant across the cytoplasmic membrane for maintenance of heme in the reduced state), and shares sequence similarity with the central part of DipZ/DsbD (17, 166, 199, 253). The sequence CxxC is different from the thioredoxin motif WCxxC, which is present in the C-terminal part of DipZ. The cysteines in CcdA have been proposed either to be functionally active in a redox reaction or to serve as a ligand to a prosthetic group (254, 255). Overall, the CcsA and CcdA
proteins in system II organisms contain substantially more sequence conservation than the ResA and CcsL proteins. Since B. subtilis synthesizes a variety of different c-type cytochromes (four c-type cytochromes), and other ccm-like genes appear absent. Gram-positive bacteria may use a different mechanism to synthesize holocytochrome c.

Surprisingly, analysis of one species of a Proteobacterial e-group of bacteria, Helicobacter pylori (291), reveals that homologues of the system II genes are present, but not the system I genes. As in B. subtilis, ccsL, ccsA and resA genes are found in an operon, but the ccdA gene is not linked. Additionally, CcsL and CcsA orthologues in Helicobacter are fused in a single open reading frame, which supports the hypothesis that they form a functional complex (112).

2.5.2.3. System III

In fungal, vertebrate, and invertebrate mitochondria, the biogenesis of c-type cytochromes appears to be far less complicated than system I and II. In system III, cytochrome heme lyases (CCHL) are the central components of biogenesis. These proteins function as enzymes catalyzing the covalent attachment of heme to the apocytochromes. (81, 186, 202, 274). Two different CCHLs from the mitochondria of the fungi, Saccharomyces and Neurospora, have been identified, both of which are specific for cytochrome c or c$_1$ (79, 80, 317). A sequence motif CPV in cytochrome c or c$_1$ heme lyase could be involved in binding heme (274). Humans also have a heme lyase gene, indicating the presence of the lyase system in higher cells (274). However, the assembly pathway for bacterial c-type cytochrome is apparently not closely analogous to
that in fungal mitochondria, as analysis of the various bacterial genomes does not reveal any orthologues of the mitochondrial CCHL.

2.5.3. Function of cytochrome c

Bacterial c-type cytochromes participate in a variety of electron transport processes, allowing bacteria to grow on different energy sources (2). Recent renewed interest in cytochrome c has stemmed from the discovery that programmed cell death in eukaryotes (apoptosis) requires the release of cytochrome c from the mitochondrion (154, 155, 310). Additionally, defects in some of the cytochrome c biogenesis genes can result in loss of copper resistance (309) and pyoverdine (a siderophore) production (102) in Pseudomonas fluorescens. Tn3-gus mutagenesis of the ccm operon in P. fluorescens showed that the expression of the genes was constitutive, but enhanced by copper. The genes function both in copper resistance and production of active cytochrome. Gaballa et al (1996) demonstrated by TnphoA mutagenesis, and site-specific gene replacement, that the first three ORFs (cytA to cytC) were essential for cytochrome c production while only the product of cytA was needed for normal pyoverdine production.

It has been suggested that cytochrome c is also involved in synthesis and excretion of heme biosynthetic intermediates such as coproporphyrin and protoporphyrin in Rhodobacter capsulatus (31, 111). Yeoman et al., (1997) (313) have shown that cyc (cycHJKL) mutants of Rhizobium leguminosarum have pleiotropic defects. Mutations in cyc genes abolish symbiotic nitrogen fixation and result in the loss of a high affinity iron acquisition system due to the failure to make or to export siderophores. The mutants also
accumulate protoporphyrin IX, the immediate precursor of heme. In *Bradyrhizobium japonicum*, *Rhizobium phaseoli* and *Rhodobacter capsulatus*, bacterial mutants of cytochrome c biogenesis displayed defects in nitrogen fixation and photosynthetic growth (24, 234, 268). Mutants with disruptions of cycVWX genes of *B. japonicum* were completely devoid of any soluble (periplasmic) or membrane-bound c-type cytochromes; even the apo form of cytochrome c, was not detectable, neither in the membrane nor in the soluble fraction. As a consequence, the mutants had pheliotropic phenotypes, such as defects in nitrate respiration, H₂ oxidation, electron transport to cytochrome a₃, and microaerobic respiration during symbiosis (207, 234). A *Rhizobium phaseoli* cytochrome mutant, unable to oxidize N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), was isolated after Mu-dl (Kan-lac) mutagenesis of the wild-type strain CE-3. Mutant strain CFN4202 had six-fold less heme-c but similar levels of b type, o and aa₃ cytochromes than the wild-type strain. Nodules formed by this strain in *Phaseolus vulgaris* did not contain bacteroids. These results suggest that the cytochrome c-aa₃ chain, or some other respiratory chain, containing c-type cytochromes in *R. phaseoli*, is essential for bacterial division during the early steps of the symbiotic interaction with the legume-host (2, 268, 269). Such results demonstrate the surprises that remain to be uncovered with respect to the roles of cytochrome c for signaling as well as the pathways of their biogenesis.

3. Dissertation Format
All research pertaining to this dissertation has been published, submitted, or will be submitted for publication shortly. This section describes the contribution of each paper to the overall biological question addressed and my contribution to the papers with authors other than Christina Kennedy and myself. The chapter “Present Study” summarizes the major findings of these papers. All papers are photocopied as published in the respective journal, and are included as appendices. The papers in this section describe studies to understand the endophytic nitrogen-fixing bacterium, *Acetobacter diazotrophicus*, and its beneficial association with sugarcane (*Saccharum* spp.)

In the first paper entitled “Characterization of a major nif/fix gene cluster in *Acetobacter diazotrophicus*, an endophyte of sugarcane” (Appendix A), the genes responsible for nitrogen fixation and regulation were isolated and analyzed. Unlike many other diazotrophs, the *nif* gene cluster of *A. diazotrophicus* is located on one part of chromosome. The second paper, entitled “Characterization of the cytochrome c biogenesis gene (ccm gene) operon of *Acetobacter diazotrophicus* and IAA production” (Appendix B), describes the identification of the *ccm* genes, which are required not only for a functional cytochrome c, but also for indole-3-acetic acid production. The final manuscript entitled “Characterization of indole-3-acetic acid production in *Acetobacter diazotrophicus* and assessment of its role in sugarcane growth enhancement” (Appendix C) describes construction of Nif⁻, Iaa⁺, and Nif⁺/Iaa⁻ mutant strains and the sterile sugarcane inoculation experiments using these mutants. Plant experiments evaluated whether nitrogen fixation and/or Indole-3-acetic acid (IAA) production are important for the ability of *A. diazotrophicus* to stimulate sugarcane growth.
3.1. Characterization of a major \textit{nif/fix} gene cluster in \textit{Acetobacter diazotrophicus}, an endophyte of sugarcane (Appendix A)

In order to isolate the \textit{nif} gene cluster of \textit{A. diazotrophicus}, \textit{nifE} and \textit{nifV} mutants of \textit{Azotobacter vinelandii} were complemented with a cosmid library of \textit{A. diazotrophicus} constructed by Dr. Dietmar Meletzus. By this approach, I was able to isolate a cosmid containing the major part of the \textit{nif} gene cluster with 19 open reading frames. Genes located upstream of this \textit{nif} gene cluster, \textit{nifHDK}, were isolated from a lambda library by Dr. Myrna Sevilla (published in a separate paper), and genes upstream of \textit{nifH} were isolated by Dr. Dietmar Meletzus and his student, Alexander Reth. The three regions isolated by different strategies were found to be adjacent to each other on the PAI5 genome. The major 31.5 kb \textit{nif} gene cluster includes both 33 \textit{nif} and associated genes of \textit{A. diazotrophicus}. This represents the largest contiguous cluster of \textit{nif/fix} and associated genes characterized in any diazotrophic bacterial species. In addition, I was able to locate eight transcriptional units by Northern blots and promoter sequence analyses.

3.2. Characterization of the cytocrome \textit{c} biogenesis gene (\textit{ccm} gene) operon of \textit{Acetobacter diazotrophicus} and IAA production (Appendix B)

Recent results from our laboratory, using mutant strains of \textit{A. diazotrophicus} that are unable to fix nitrogen, have shown that there are two beneficial effects of \textit{A.}}
*diazotrophicus* on sugarcane: one dependent on nitrogen-fixation and the other nitrogen fixation-independent. Because *A. diazotrophicus* produces biologically active IAA, a growth promoting substance, and IAA has been known to be a major plant growth promoting factor, we hypothesized that IAA may be the factor responsible for nitrogen fixation-independent growth promote. In order to determine a role of IAA in promoting plant growth, a combination of techniques, such as Southern hybridization, PCR, complementation, and random Tn5 mutagenesis were used to isolate genes involved in IAA biosynthesis, or Iaa− mutants, of *A. diazotrophicus*. I developed a Tn5 mutagenesis technique that increased transconjugation efficiency. Mutants of *A. diazotrophicus* producing decreased amounts of IAA were successfully isolated by the Tn5 mutagenesis approach.

One of the Tn5 mutants was mapped to a gene known to be involved in cytochrome c biogenesis (*ccm* genes- cytochrome c maturation genes). Although several *ccm* gene mutant phenotypes have been described in the literature, there is no report of IAA-related phenotypes. This manuscript describes the characterization of *ccm* genes and *ccm*− mutant phenotypes. To characterize the roles of *ccm* genes in the cytochrome c biogenesis and in the bacterial respiration, I went to Dr. Eudorado Escamilla’s laboratory in Mexico to use equipment such as an oxygen electrode, 60 L fermentor, and low temperature spectrophotometer (-80 °C), and to learn the heme-staining technique. Two of his graduate students and staff members taught me the techniques to use the equipment. It was clear from the characterization of the Ccm− mutant that *ccm* genes are involved in both cytochrome c biogenesis and IAA biosynthesis. To quantify IAA production, I
learned how to operate HPLC in Dr. Hans VanEtten’s laboratory. HPLC chromatograms showed Ccm' mutants synthesize significantly reduced amounts of IAA as compared to wild type \textit{A. diazotrophicus}.

3.3. Characterization of indole-3-acetic acid production in \textit{Acetobacter diazotrophicus} and assessment of its role in sugarcane growth enhancement (Appendix C)

This study evaluated whether nitrogen fixation and/or indole-3-acetic acid (IAA) production are important for the ability of \textit{A. diazotrophicus} to stimulate sugarcane growth. I constructed a Nif' mutant and a Nif'/Iaa' double mutant by disrupting \textit{nifD} in the background of wild type and the Iaa' mutant strain. The \textit{nifD} gene was originally characterized by Dr. Myrna Sevilla, which will be published elsewhere. This double mutant has been used in sterile sugarcane plant inoculation experiments in parallel with wild type and single mutant strains (Nif' and Iaa'). I designed and set up the experiments in the growth chamber. Plant height, biomass, and the number of bacteria present were measured 30 days after inoculation. I performed all the procedures described in this paper with help from Ms. Libia Luevano for preparation of media, watering, and data collection. Results of plant inoculation experiments demonstrated significant growth differences between plants inoculated with wild type and uninoculated plants regardless of N availability, which suggested that \textit{A. diazotrophicus} stimulates plant growth. To interpret plant experiment data, Dr. Elizabeth Pierson helped me with statistical analysis.
In addition, IAA production under various conditions was investigated in order to study the possible relationships between IAA production and nitrogen fixation. To quantify the concentration of indole derivatives, I performed HPLC and TLC analysis. IAA production relies on the presence of tryptophan as a precursor, age of the culture, and N availability in liquid culture, but not on oxygen level. From the HPLC data I was able to conclude that TRP-independent pathways may not be present in \textit{A. diazotrophicus}, and sufficient N and TRP are required for the biosynthesis of IAA \textit{in vitro} condition.

\textbf{II. PRESENT STUDY}

1. Significant Results

The methods, results, and conclusions of this study are presented in the papers appended to this dissertation. The following is a summary of the most important findings in these papers.

1.1. Characterization of a major \textit{nif/fix} gene cluster in \textit{Acetobacter diazotrophicus}, an endophyte of sugarcane (Appendix A)

a. A \textit{nif} gene cluster encompassing 33 \textit{nif} and associated genes of \textit{A. diazotrophicus} was identified.

b. The arrangement of the \textit{nif} gene cluster in \textit{A. diazotrophicus} is most similar to that found in \textit{A. brasilense}.

c. The individual \textit{A. diazotrophicus} gene products are generally
most similar to those found in other α group Proteobacteria, *Rhizobium* species in particular.

d. Six potential σ^{54}-dependent promoters were identified, and four of these were proceeded by a NifA upstream activator sequences.

1.2. Characterization of the cytochrome c biogenesis gene (ccm gene) operon of *Acetobacter diazotrophicus* and IAA production (Appendix B)

a. Three IAA-negative and one IAA-overproducing mutant candidates were isolated by random Tn5 mutagenesis.

b. One Tn5 mutant, MAAd10, was mapped to genes known to be involved in cytochrome c biogenesis (ccm operon).

c. Mutations in the one of ccm genes (ccmCDFG) resulted in no cytochrome c synthesis, which was confirmed by Spectral analysis and heme-associated peroxidase activities. Significantly reduced oxidase and dehydrogenase activities with different substrates also indicated the absence of functional cytochrome c.

d. Ccm^- mutants also were affected in IAA biosynthesis: ccm^- mutants produced only about 6 % IAA compared to wild type.

e. When Ccm^- mutants were grown in both rich and minimal media, no growth defects were observed. Additionally, there were no other observable phenotypic differences between Ccm^- mutants and the wild type strain.
1.3. Characterization of indole-3-acetic acid production in *Acetobacter diazotrophicus* and assessment of its role in sugarcane growth enhancement (Appendix C)

a. A Nif" and a Nif"/Iaa" mutant that cannot fix nitrogen were generated by inserting a cassette into the *nifD* gene of wild type and the IAA-negative mutant (Ccm") chromosome, for use in plant inoculation experiments.

b. The HPLC elution profiles and TLC chromatography of acidic ethyl acetate extracts contained two intermediates of indole-3-pyruvic acid pathway (indole-3-lactic acid and indole-3-ethanol), and one indole-3-acetic acid catabolite (indole-3-carboxylic acid). This result indicated that IAA of *A. diazotrophicus* is produced by the indole-3-pyruvic acid pathway and IAA catabolism is associated with decarboxylative oxidation processes.

c. IAA production increased with increasing concentrations of tryptophan, sufficient N, and the onset of stationary growth; however, the oxygen levels did not affect IAA biosynthesis in *A. diazotrophicus*.

d. Wild type and mutant strains (Nif", Iaa", and Nif"/Iaa") colonized sugarcane plants equally.

f. Sugarcane plants inoculated with wild type *A. diazotrophicus* strain had greater height and biomass than uninoculated plants regardless of N
availability, which confirmed the benefit of this bacterium on sugarcane growth.

g. When N is limited, plants inoculated with Nif', Iaa', and Nif'/Iaa' were shorter and had less mass than plants inoculated with the wild type. This result suggested that both nitrogen fixation and IAA biosynthesis by *A. diazotrophicus* are important factors affecting sugarcane growth.

h. Regardless of N availability, plants inoculated with Iaa' were always comparable to uninoculated plants, which indicated that the effect of IAA production is more pronounced in N-sufficient conditions.

2. Summary and Concluding Remarks

My research has focused on understanding the endophytic nitrogen-fixing bacterium, *Acetobacter diazotrophicus*, and its beneficial association with sugarcane (*Saccharum* spp.). This symbiotic association between these two organisms provides a model system to examine monocot/diazotroph symbiosis. My first project was to characterize the genes responsible for nitrogen fixation, and determine their regulation. Such knowledge will assist our understanding of bacterial nitrogen fixation by *A. diazotrophicus* and the evolution of this unique bacterium. In summary, I have isolated, sequenced, and analyzed the major 31.5 kb *nif* gene cluster which includes both *nif* and associated genes of *A. diazotrophicus*. This cluster represents the largest and most complete assembly of contiguous *nif*/*fix* and associated genes characterized in any
diazotrophic bacterial species, and includes 33 genes. Northern blots and promoter sequence analyses indicate the genes are organized into eight transcriptional units.

My second project was to determine whether nitrogen fixation and/or indole-3-acetic acid (IAA) production are important for the ability of *A. diazotrophicus* to stimulate plant growth. Recent results from our laboratory, using mutant strains of *A. diazotrophicus* that are unable to fix nitrogen, have shown that there are two beneficial effects of *A. diazotrophicus* on sugarcane: one dependent on nitrogen-fixation and the other nitrogen fixation-independent. Because *A. diazotrophicus* produces biologically active IAA, plant growth promoting substance, we hypothesized that IAA may be the factor responsible for nitrogen fixation-independent growth. In order to determine a role of IAA in promoting plant growth, mutants of *A. diazotrophicus* producing decreased amounts of IAA were generated by Tn5 mutagenesis. One of the Tn5 mutants excreting significantly lower amounts of IAA compared to the parent strain in liquid culture was mapped to a gene known to be involved in cytochrome c biogenesis (*ccm* genes—cytochrome c maturation genes). The roles of *ccm* genes of *A. diazotrophicus* in producing cytochrome c and IAA were shown by a series of biochemical experiments and HPLC analysis.

Nif' and Nif'/Iaa' mutants that cannot fix nitrogen were engineered, and these mutants were utilized for sugarcane inoculation experiments in parallel with the wild type, an Iaa' mutant, and no inoculum control to determine whether IAA plays a role in bacterial enhancement of sugarcane growth. The results of plant experiments suggested that the mechanisms by which *A. diazotrophicus* improved sugarcane growth could be through
both nitrogen fixation and indole-3-acetic acid production. Similar plant responses with Nif\(^{-}\) mutants have been shown previously in our laboratory. Although the benefits of association with *A. diazotrophicus* are evident, it is necessary to further investigate the longer-term effects on sugarcane, and persistence of inoculated strains under field conditions. Furthermore, since we do not know the exact functions of the *ccm* genes in IAA production, the role of IAA in sugarcane growth should be further studied with mutants specifically defective in the ability to synthesize this compound. I tried several methods to isolate genes involved directly in IAA biosynthesis (specifically genes in indole-3-pyruvic acid pathway), but all efforts were unsuccessful. The genes of *A. diazotrophicus* may be significantly diverged from the known IAA-related genes isolated from other bacteria. If this is true, a combination of random mutagenesis and complementation will be the most desirable method to identify and isolate the possible candidate genes. So far 2500 Tn5 mutants have been screened for the altered IAA production, but further mutagenesis will accumulate more laa\(^{-}\) mutant candidates, and the complementation of the resulting mutants with a cosmid library will eventually lead to the cloning of the candidate genes.

Additionally, although it is plausible to conclude that nitrogen fixation by *A. diazotrophicus* is an important determinant of the beneficial effects of this bacterium, this work did not present direct evidence that fixed N is transferred to sugarcane plants. Direct transfer of fixed N from the bacterium to the plant could be shown by a method developed by Dr. Mike Kahn. In his \(^{15}\)N dilution method, \(^{15}\)N gas is provided to sugarcane inoculated with various inocula, and after a few days of incubation chlorophylls are
extracted and the ratio of $^{15}\text{N}/^{14}\text{N}$ is determined. Since chlorophyll is only present in the plant, the ratio of $^{15}\text{N}/^{14}\text{N}$ will be a direct indication of whether biologically fixed N is provided to sugarcane plants. Dr. David Dalton is currently continuing $^{15}\text{N}$ dilution experiments in the laboratory. This work will be the first definite evidence of the effect of BNF by \emph{A. diazotrophicus} inside of the sugarcane plants. Moreover, the exact amount of fixed N contributed by \emph{A. diazotrophicus} to sugarcane plants could be measured. Finally, since the ultimate goal of studying possible benefits of nitrogen fixation and IAA biosynthesis by \emph{A. diazotrophicus} is to provide insights of monocot/diazotroph symbiosis, and extend its benefits to other grass and cereal crops, it will be interesting to see whether there are growth enhancing effects with wild type PA15 on other agronomically important crops. The significant findings from this dissertation contributed to the understanding of the beneficial association of \emph{A. diazotrophicus} and sugarcane, and new methodology and techniques developed during this research will help to prompt new areas of interest such as host range determinants, other growth promoting factors, and effective biological nitrogen fixation.


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APPENDIX A. SUNHEE LEE, ALEXANDER RETH, DIETMAR MELETZUS, MYRNA SEVILLA AND CHRISTINA KENNEDY. 2000. CHARACTERIZATION OF A MAJOR CLUSTER OF NIF, FIX, AND ASSOCIATED GENES IN A SUGARCANE ENDOPHYTE, ACETOBACTER DIAZOTROPHICUS. J. BACTERIOL. 182:7088-7091
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February 7, 2001

To whom it may concern,

I wish to include a copy of the article “Characterization of a Major Cluster of nif, fix, and Associated Genes in a Sagnanese Endophyte, Azotobacter chroococcum.” (Journal of Bacteriology, 2000, Vol 182: 7088-7091) in my Ph.D. dissertation. I am the first author of this article and it includes research that I performed during my dissertation. Since this material is copyrighted by American Society for Microbiology, I need a release letter to include this article in the appendix of my dissertation. Could you please send the release to the address listed below as well as FAX me a copy for immediate use? The release should extend to microfilming and publication by University Microfilms Incorporated (UMI) and state that American Society for Microbiology is aware that UMI may sell, on demand, single copies of the dissertation including the copyrighted materials, for scholarly purposes.

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Characterization of a Major Cluster of nif, fix, and Associated Genes in a Sugarcane Endophyte, Acetobacter diazotrophicus

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A major 30.5-kb cluster of nif and associated genes of Acetobacter diazotrophicus (syn. Gluconacetobacter diazotrophicus), a nitrogen-fixing endophyte of sugarcane, was sequenced and analyzed. This cluster represents the largest assembly of contiguous nif−fix and associated genes so far characterized in any diazotrophic bacterial species. Northern blots and promoter sequence analysis indicated that the genes are organized into eight transcriptional units. The overall arrangement of genes is most like that of the nif−fix cluster in Azospirillum brasilense, while the individual gene products are more similar to those in species of Rhizobiaceae or in Rhodobacter capsulatus.

Biological nitrogen fixation occurs in species of more than 100 genera distributed among several of the major phylodynamic divisions of prokaryotes (Eubacteria and Archaea) (25). Sequence and mutational analyses of the genes necessary for nitrogen fixation (nif) in many diazotrophs indicate that their products have common structures and functions, while the degree of linkage and arrangement of specific nif and associated genes vary considerably (5, 8, 17). In addition, nif genes and genes involved in plant invasion and nitrogen fixation effectiveness, such as nod and fix in species of Rhizobiaceae, are often linked.

The identification of nitrogen-fixing bacteria with endophytic habitats raises the possibility of a new classification of symbiosis (3). The relationship of a proteobacterial a group member, Acetobacter diazotrophicus (syn. Gluconacetobacter diazotrophicus), with sugarcane represents a promising model system for the study of an association between a monocot and symbiosis (3). The relationship of a proteobacteriai a group A. diazotrophicus growth-stimulating factor(s) is indicated, possibly auxin pro­duced genes vary considerably (5, 8,17). In addition, nod in spedes fix effectiveness, such as

and genes involved in plant invasion and nitrogen fixation effectiveness, such as nod and fix in species of Rhizobiaceae, are often linked.

The ability of A. diazotrophicus to enhance sugarcane growth has been doc­

umented, and while the benefit to plant growth might be due at least in part to the transfer of bacterially fixed N\(^{2}\), another plant growth-stimulating factor(s) is indicated, possibly auxin production by A. diazotrophicus (22). Because of its potential ag­

ronomic use and unique status as the only diazotroph so far studied. The Fix proteins in £. coli (2), while their fiuction in other organisms is

unknown (7). Alw of unknown function but similar to gene products in other diazotrophs are the fenedoxins FdxN and Fdxo with 17 gene products being most like those in members of the Rhizobiaceae and 9 gene products being most closely related to Rhodobacter capsulatus proteins (Table 1). NifU and NifS were most similar to the gene products of Azotobacter species, mem­

bers of the γ group of proteobacteria, McpA was most similar to the mcpA gene product of the unrelated Caulobacter cres­

tentus (67% identity).

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Although none of the individual gene products were most similar to those of Azospirillum species, the overall organization of genes in the A. diazotrophicus nif-fix cluster is most like that of Azospirillum brasilense. These are the only two diazotrophs so far characterized that have an mcpA-like gene associated with the nif-fix cluster (J. Frazzon and I. S. Schrank, personal communication; also this work). The McpA protein is involved in chemotaxis in several organisms. Because both A. diazotrophicus and A. brasilense are found naturally associated with monocot plants, it will be of interest to determine whether McpA is responsible for chemotactic responses to plant exudates for signaling or nutrition. An mcpA mutant strain of A. diazotrophicus lost chemotaxis toward a wide range of attractants (11, 23). One portion of the nif-fix cluster of A. diazotrophicus lost chemotaxis toward a wide range of attractant stimuli (unpublished result).

Differences between the clusters are that nifA and nifB of A. brasilense are not linked to the other nif genes and nifQ, nifW, and fdxB are absent from the latter (8, 15). nifY is not found in the A. diazotrophicus cluster, but its requirement for nitrogen fixation is uncertain in other diazotrophs; it may be involved in Fe-Mo cofactor insertion into nitrogenase enzyme or in fixed N sensing (11, 23). One portion of the nif-fix cluster of A. diazotrophicus is more like that of R. capsulatus (nifE to nifW) than to A. brasilense, although no ORF1-like gene is found in the latter. The nifU gene in R. capsulatus is truncated compared to most other nif genes, encoding only the C-terminal end of NifU (16).

Transcriptional and translational organization of the cluster. The transcriptional and translational organization of genes in the nif-fix cluster of A. diazotrophicus shows several interesting features. Northern analysis of mRNA was successful in identifying the cotranscription of nifHDK, as occurs in most other diazotrophs, according to size of transcripts hybridizing to a nifD probe, and also of nifENX orf4 orf3 fdxB nifQ, indicated by hybridization of RNA to a nifE probe (Fig. 1 and 2).

The former was predicted by sequence analysis, which revealed σ^r-, NifA-binding sites upstream of nifH (Sevilla and Kenneady, submitted) required, respectively, for nif promoter recognition and for nif gene transcriptional activation in all other proteobacterial diazotrophs. A σ^r recognition sequences but no NifA recognition sequence was found upstream of nifE. Northern blots hybridized with [32P]dCTP-labeled orf6, nifU, or fixA probes showed only smears or broad bands after autoradiography, suggesting rapid degradation of mRNA or a very low level of transcription. The putative transcriptional organization of the other genes was suggested by sequence analysis (Fig. 1). Locations of possibly significant σ^r, NifA recognition sites upstream of genes and of potential transcription terminators downstream of genes are given in Table 1.

An unusual degree of overlap between the 3' and 5' ends of adjacent genes, indicating translational coupling, was found for nifN-nifX (30 bp). Other cases of overlap were found for fixC-fxX (1 bp), modC-modB (3 bp), modC-modB (3 bp), modB-modA (4 bp), nifZ-fixU (3 bp), and fixU-orf1 (3 bp). Several other cases of translational coupling are found between nifY and/or related genes in other diazotrophs, including one large overlap of 34 bp between nifN and nifX in the archael diazotroph Methanococcus maripaludis and smaller overlaps in M. maripaludis (nifD-nifX, 7 bp) (14), A. vinelandii (orf7-nifW, 3 bp; nifZ-nifM, 10 bp; nifM-orf9, 7 bp) (12), and Klebsiella pneumoniae (orfB-nifQ, 1 bp; nifL-orf4, 4 bp; nifZ-nifM, 4 bp; and nifN-nifX, 14 bp) (1). Of the A. diazotrophicus genes described here, only nifN and modC had the translational initiation codon GTC. This initiation codon also occurs in several genes in various diazotrophs, for example, nifB and nifX of Frankia alni and ORF1 of Plectonema boryanum PCC 73110 (10, 21).

Characterization of genes related to nitrogen fixation and/or plant colonization is important for elucidating these processes in endophytic diazotrophs. This work examines a major cluster...
TABLE 1. Comparison of nif-associated gene products

<table>
<thead>
<tr>
<th>Gene*</th>
<th>Function</th>
<th>Regulatory feature</th>
<th>Product size (kDa)</th>
<th>Organisms with most similar gene products* (% amino acid identity *)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoN</td>
<td>α54</td>
<td></td>
<td>51.3</td>
<td>B. japonicum (34), A. cauliformis (31), C. crescentus (30)</td>
</tr>
<tr>
<td>modC</td>
<td>Molybdenum transport; ATP-binding protein</td>
<td></td>
<td>36.8</td>
<td>R. capulatus (48), A. vinelandii (47), E. coli (46)</td>
</tr>
<tr>
<td>modB</td>
<td>Molybdenum transport; permease protein</td>
<td></td>
<td>24.7</td>
<td>R. capulatus (57), A. vinelandii (40), A. eucariotic (35)</td>
</tr>
<tr>
<td>modA</td>
<td>Molybdenum transport; molybdate-binding periplasmic protein</td>
<td></td>
<td>26.9</td>
<td>R. capulatus (42), A. vinelandii (40), A. eucariotic (32)</td>
</tr>
<tr>
<td>orf1</td>
<td>Unknown</td>
<td>NiFA-, σ^a^-binding sites</td>
<td>20.2</td>
<td>A. cauliformis (51), B. japonicum (50), R. etli (47)</td>
</tr>
<tr>
<td>nifA</td>
<td>Transcriptional activator</td>
<td>NiFA-binding sites</td>
<td>62.2</td>
<td>R. capulatus (67), R. meliloti (67), Rhizobium sp. strain NGR234 (66)</td>
</tr>
<tr>
<td>nifD</td>
<td>Fe-Mo cofactor synthesis</td>
<td>NiFA-, σ^a^-binding sites</td>
<td>51.9</td>
<td>R. capulatus (67), R. meliloti (67), Rhizobium sp. strain NGR234 (66)</td>
</tr>
<tr>
<td>fdxN</td>
<td>Ferredoxin</td>
<td></td>
<td>6.8</td>
<td>R. palustris (77), Rhizobium sp. strain NGR234 (67), R. meliloti (77)</td>
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<tr>
<td>ynfI</td>
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<td></td>
<td>26.4</td>
<td>R. capulatus (31)</td>
</tr>
<tr>
<td>ynfZ</td>
<td>Maturation and activation</td>
<td></td>
<td>6.9</td>
<td>Rhizobium sp. strain NGR234 (63), R. capulatus (59), E. coli (67)</td>
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<tr>
<td>fsU</td>
<td>Unknown</td>
<td></td>
<td>8.6</td>
<td>Rhizobium sp. strain NGR234 (56), R. leguminosarum (56)</td>
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<tr>
<td>orf2</td>
<td>Unknown</td>
<td></td>
<td>36.4</td>
<td>A. brasilense (42), A. vinelandii (28)</td>
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<td>nifH</td>
<td>Nitrogenase structure; Fe protein</td>
<td>NiFA-, σ^a^-binding site</td>
<td>31.9</td>
<td>R. palustris (89), Rhizobium sp. strain NGR234 (85), R. meliloti (85)</td>
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<tr>
<td>nifD</td>
<td>Nitrogenase structure; Fe-Mo protein alpha subunit</td>
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<td>55.9</td>
<td>Rhizobium sp. strain NGR234 (80), P. palustris (75), H. seropedicae (75)</td>
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<tr>
<td>nifK</td>
<td>Nitrogenase structure; Fe-Mo protein beta subunit</td>
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<td>57.2</td>
<td>Rhizobium sp. strain NGR234 (71), B. japonicum (66), T. ferrooxidans (62)</td>
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<td>nifE</td>
<td>Fe-Mo cofactor synthesis</td>
<td>σ^a^-binding site</td>
<td>51.8</td>
<td>B. japonicum (60), Rhizobium sp. strain NGR234 (58), R. capulatus (57)</td>
</tr>
<tr>
<td>nifN</td>
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<td>49.5</td>
<td>R. meliloti (50), Rhizobium sp. strain NGR234 (49), B. japonicum (46)</td>
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<tr>
<td>nifX</td>
<td>Fe-Mo cofactor synthesis</td>
<td></td>
<td>17.4</td>
<td>Rhizobium sp. strain NGR234 (55), R. capulatus (46), H. seropedicae (37)</td>
</tr>
<tr>
<td>orf4</td>
<td>Unknown</td>
<td></td>
<td>16.9</td>
<td>A. cauliformis (53), Frankia sp. (50), F. alni (45)</td>
</tr>
<tr>
<td>orf1</td>
<td>Unknown</td>
<td></td>
<td>7.6</td>
<td>R. palustris (50), Rhizobium sp. strain NGR234 (48), Anabaena PCC7120 (39)</td>
</tr>
<tr>
<td>fdxB</td>
<td>Ferredoxin</td>
<td></td>
<td>9.9</td>
<td>R. capulatus (41), Rhizobium sp. strain NGR234 (41), P. boryanum (34)</td>
</tr>
<tr>
<td>nifQ</td>
<td>Fe-Mo cofactor synthesis</td>
<td>p-independent terminator</td>
<td>22.9</td>
<td>R. capulatus (41), A. vinelandii (40), Rhizobium sp. strain NGR234 (33)</td>
</tr>
<tr>
<td>orf6</td>
<td>Unknown</td>
<td>NiFA-, σ^a^-binding sites</td>
<td>10.9</td>
<td>R. capulatus (48), R. sphaeroides (46), B. japonicum (45)</td>
</tr>
<tr>
<td>nifU</td>
<td>Maturation and activation; assembly of iron-sulfur clusters</td>
<td></td>
<td>32.9</td>
<td>A. brasilense (55), A. vinelandii (53), A. chroococcum (52)</td>
</tr>
<tr>
<td>nifS</td>
<td>Maturation and activation; homodimeric cysteine desulfurase</td>
<td></td>
<td>43.0</td>
<td>A. brasilense (59), Rhizobium sp. strain NGR234 (59), R. sphaeroides (59)</td>
</tr>
<tr>
<td>nifV</td>
<td>Fe-Mo cofactor synthesis; homocitrate synthase</td>
<td></td>
<td>92.5</td>
<td>Z. mobilis (61), R. sphaeroides (51), F. alni (49)</td>
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<td>nifW</td>
<td>Maturation and activation; oxygen protection of the Mo-Fe protein</td>
<td>p-independent terminator</td>
<td>12.0</td>
<td>A. cauliformis (48), Rhizobium sp. strain NGR234 (42), A. brasilense (41)</td>
</tr>
<tr>
<td>fisA</td>
<td>Electron transfer; electron transfer flavoprotein beta subunit</td>
<td>NiFA-, σ^a^-binding sites</td>
<td>30.7</td>
<td>R. palustris (69), Rhizobium sp. strain NGR234 (67), B. japonicum (66)</td>
</tr>
<tr>
<td>fisB</td>
<td>Electron transfer; electron transfer flavoprotein alpha subunit</td>
<td></td>
<td>38.9</td>
<td>A. cauliformis (68), Rhizobium sp. strain NGR234 (67), B. japonicum (67)</td>
</tr>
<tr>
<td>fisC</td>
<td>Electron transfer; electron transfer flavoprotein-glutathione oxidoreductase</td>
<td></td>
<td>48.1</td>
<td>Rhizobium sp. strain NGR234 (63), B. japonicum (62), A. cauliformis (61)</td>
</tr>
<tr>
<td>fisX</td>
<td>Electron transfer; ferredoxin-like protein</td>
<td></td>
<td>10.7</td>
<td>R. leguminosarum (73), R. meliloti (71), Rhizobium sp. strain NGR234 (70)</td>
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<tr>
<td>modD</td>
<td>Molybdenum transport</td>
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<td>26.7</td>
<td>Z. mobilis (51), R. capulatus (41), E. coli (39)</td>
</tr>
<tr>
<td>mcpA</td>
<td>Chemotaxis</td>
<td></td>
<td>69.3</td>
<td>C. crescentus (63), R. sphaeroides (51), R. leguminosarum (44)</td>
</tr>
</tbody>
</table>

* Organization of gene expression is indicated by arrows in Fig. 1.

* Organisms in which the gene product most similar to that of A. diazotrophicus was found. Organisms: Agitiges eucalyticus, Azorhizobium cauliformis, Azorhizobacter chroococcum, Azorhizobacter vinelandii, Bradyrhizobium japonicum, Caulobacter crescentus, Esherichia coli, Frankia sp. strain NGR234, Herbaspirillum serendipita, Pausorhiza rhizobium, Pleconema bonnemou, Rhizobium etli, Rhizobium leguminosarum, Rhizobium meliloti, Rhizobium phaselii, Rhodobacter capsulatus, Rhodobacter sphaeroides, Rhodo- paraulmonas palustris, Thabonema ferrooxidans, and Synechococcus moelius.

* Identity of the deduced A. diazotrophicus gene product sequence to the gene product of the three organisms to which it is most related.
of genes that are certainly or potentially important for the ability of *A. diazorosicus* to fix nitrogen inside its plant host. This knowledge may be relevant for efforts to use this organism grown with low concentrations of fixed N (0.5 mM NH₅), cultures grown with high concentrations of fixed N (10 mM NH₄).

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REFERENCES


APPENDIX B. SUNHEE LEE, EUDORADO ESCAMILLA, AND CHRISTINA KENNEDY. 2001. CHARACTERIZATION OF THE CYTOCHROME C BIOGENESIS GENE (CCM GENE) OPERON OF ACETOBACTER DIAZOTROPHICUS AND IAA PRODUCTION.
Characterization of the cytochrome c biogenesis gene (ccm gene) operon of Acetobacter diazotrophicus and IAA production

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Summary

*Acetobacter diazotrophicus* is an endophyte of sugarcane often found in plants grown in agricultural areas where nitrogen fertilizer input is low. Recent results from our laboratory, using mutant strains of *A. diazotrophicus* unable to fix nitrogen, suggested that there are two beneficial effects of *A. diazotrophicus* on sugarcane growth; one dependent, and one not, on nitrogen fixation. A plant growth promoting substance, such as indole-3-acetic acid (IAA), could be responsible for the latter effect that influences and accounts for improved plant growth. In order to determine whether IAA has a role in promoting plant growth, mutants of *A. diazotrophicus* producing decreased amounts of IAA were generated by Tn5 mutagenesis. One mutant, excretes only ~ 6 % of the amount of IAA excreted by the parent strain in liquid culture. The MAd10 mutation was mapped to a gene known to be involved in cytochrome *c* biogenesis (*ccm* genes-cytochrome *c* maturation genes). The individual *ccm* genes of *A. diazotrophicus* showed 41 to 52 % identity based on amino acid sequence when compared with known *ccm* gene products. Although several *ccm* gene mutant phenotypes have been described in the literature, there are no reports of *ccm* genes being involved in IAA production. Spectral analysis, heme-associated peroxidase activities, and respiratory activities of the cell membranes revealed that the *ccm* genes of *A. diazotrophicus* are involved in cytochrome *c* biogenesis. We describe here, for the first time, the cloning of and characterization of the *ccm* genes of *A. diazotrophicus* and describe the function of these genes in cytochrome *c* biogenesis and IAA production.
Introduction

*Acetobacter diazotrophicus* is a nitrogen-fixing endophyte isolated commonly from *Saccharum* L. (sugarcane), *Ipomoea batatas* (sweet potato), *Pennisetum purpureum*, and *Coffea arabica* (coffee plants) (Paula et al., 1992; Harris and Silvester, 1992; Gillis et al., 1989; Li and MacRae, 1991; Dong et al., 1995; Bellone et al., 1997; Jimenez-Salgado et al., 1997). Isotope dilution experiments suggest that up to 80% of sugarcane nitrogen (N) can be derived from atmospheric nitrogen gas presumably through bacterial nitrogen fixation (Boddey et al., 1991; Urquiaga, et al., 1992). Additionally, a recent report from Sevilla et al. (1998) showed that *A. diazotrophicus* has two potential beneficial effects on sugarcane: one probably dependent on nitrogen fixation and the other possibly through microbial production of a plant growth promoting substance. Since *A. diazotrophicus* is known to produce indole-3-acetic acid (IAA), with particularly high amounts produced by strain PAI5 (used in the plant inoculation experiments by Sevilla et al. (1998), we speculated that IAA production may explain the plant-growth promotion of sugarcane by *A. diazotrophicus*.

Biosynthesis of IAA is not limited to higher plants. Organisms such as bacteria, fungi, and algae are able to make physiologically active IAA that may have pronounced effects on plant growth and development. In fact, it has been suggested that up to 80% of bacteria isolated from the rhizosphere have the capacity to synthesize IAA *in vitro* in the presence or absence of physiological precursors such as tryptophan (TRP) (Leinhos and
Microbial isolates from the rhizosphere of different crops appear to have a greater potential to synthesize and release IAA as secondary metabolites because of the rich supply of substrates (Brown, 1974; Jacobson et al., 1994; Barazani and Friedman, 1999; Costacurta and Vanderleyden, 1995). In addition, numerous pathogens are active producers of IAA and cause abnormal cell enlargement in infected plants (Patten and Glick, 1996; Costacurta and Vanderleyden, 1995; Arshad and Frankenberger, 1998).

Production of IAA by microbial isolates varies greatly among different species and strains, and depends on the availability of the substrate(s). Different biosynthetic pathways for auxin production exist, sometimes in parallel in the same organism (Patten and Glick, 1996; Frankenberger and Arshad, 1995). For many years it was assumed that TRP was the precursor of auxin. However, work with tryptophan auxotrophic mutants and isotope labeling have established that auxin biosynthesis can occur via a tryptophan-independent route (Wright et al., 1991; Normanly et al., 1995; Normanly and Bartel, 1999; Prinsen et al., 1993) although in the presence of TRP, microbes release greater quantities of IAA and related compounds.

The pathways for conversion of TRP to IAA can involve deamination, decarboxylation, and/or hydrolysis reactions. In higher plants and most microorganisms, the indole-3-pyruvic acid (IpyA) pathway is the main one for auxin synthesis, whereas other pathways operate in certain species (the indole-3-acetamide pathway, the tryptamine pathway, and indole-3-acetonitrile pathway (Patten and Glick, 1996;
Frankenberger and Arshad, 1995; Kawaguchi and Syono, 1996). The formation of IpyA from TRP is catalyzed by multispecific aminotransferases, followed by spontaneous or enzymatic decarboxylation to indole-3-acetaldehyde (IAAld), which is then oxidized by an IAAld oxidase to IAA. As a side reaction, IpyA may be reduced to indole-3-lactic acid (ILA) by lactate dehydrogenase, which requires NADH. Indole-3-ethanol (TOL) is the product of a side reaction from IAAld.

In this study we used transposon mutagenesis and complementation to obtain *A. diazotrophicus* Iaa' mutant with reduced ability to produce IAA as compared to the wild type. This approach revealed a surprising discovery that cytochrome c biogenesis genes are involved in IAA production in *A. diazotrophicus*.

**Results**

*Genetic analysis of the DNA region affected the IAA production*

Following Tn5 mutagenesis, 2,500 exconjugants were analyzed for IAA production as described in the Materials and Methods. Three IAA-reduced and one IAA-overproducing mutant candidates were further characterized by HPLC and TLC (data not shown). Among IAA' mutant candidates, MAd10 was further characterized, since MAd10 excreted ~ 6% of the amount of IAA produced by parent strain in liquid culture and it is neither a Trp auxotroph nor a Nif' strain. The Tn5-linked region of MAd10 was cloned and sequenced. The Tn5-flanking region showed a high similarity to several antibiotic synthetase genes from *Bacillus subtilis* and *Streptomyces* sp., and siderophore
biosynthesis proteins from *B. subtilis* and *Pseudomonas* sp.. This result suggested that there was a possibility that the IAA' phenotype of MAd10 is not linked to the Tn5 insertion in the chromosome. When the clone containing the Tn5 region of MAd10 was inserted into the chromosome of wild-type strain PA15, and a double crossover event resulted in replacing the PA15 DNA fragment with the Tn5-flanking region, the resulting strain was IAA'. This indicated that the Tn5 insertion in the antibiotic synthetase in MAd10 was not linked to the IAA' phenotype.

**Complementation of the mutant MAd10**

To determine where the mutation causing the IAA' phenotype was located on the chromosome, MAd10 was complemented with the cosmid library of *A. diazotrophicus* (Teixeira *et al.*, 1999). After screening 2,500 transconjugants using the colorimetric method described earlier, two cosmids that complemented the IAA' phenotype of MAd10 were isolated. These two cosmids had overlapping restriction sites, which indicated that they probably contained the same gene or genes complementing reduced IAA production in MAd10. The region contained in one of the cosmids, pAd80, was further subcloned to determine the smallest fragment that complemented the IAA' phenotype. A 5.6 kb *Hind* III/EcoRI fragment (pSL5.6) successfully complemented the IAA' phenotype, restoring wild type IAA levels (Fig. 1, Table 5).
Sequence Analysis

To determine the nucleotide sequence, pSL5.6 were subcloned to smaller fragments and sequenced. Examination of the sequence revealed five contiguous open reading frames (ORFs) (Fig. 1). Further analysis revealed that these five ORFs have high similarity to proteins involved in cytochrome c biogenesis (ccm genes - cytochrome c maturation genes). The putative translation products of the A. diazotrophicus ORFs were compared with the gene products of the B. japonicum and E. coli cytochrome c biogenesis gene clusters. The degree of amino acid sequence identity between the individual A. diazotrophicus ORFs and B. japonicum cyc gene products ranged from 41 to 52 % (Fig. 2, Table 2). Because all of the A. diazotrophicus ORFs in this region have similarities to corresponding ccm genes in E. coli, the ORFs were designated as ccm genes (Fig. 3, Table 2). ccmABCDEFGH are required for the formation of holocytochrome c in E. coli; subunits of an ABC transporter (CcmABC), a heme chaperone (CcmE), a putative cytochrome c heme lyase (CcmEF), and a postulated redox system (CcmGH), as well as unknown function of CcmD (Thony-Meyer and Kunzler, 1997; Page and Ferguson, 1994; Grove et al., 1996; Schulz et al., 1999; Fabianek, Hennecke, and Thony-Meyer, 1998; Fabianek et al., 1997).

Mutations in the ccm operon affect IAA biosynthesis

To confirm that one or more of the ccm genes are responsible for the IAA phenotype, several mutant strains were generated by inserting Ω (Sm′) or KISS (Km′) cassettes or by deleting a portion of the genes to yield the strains, MAd20, MAd21, MAd
22, and MAd23 (Fig. 1). Mutagenesis results indicated that a mutation in any of the ccmCDE genes results in IAA⁻ phenotype.

IAA production by both ccm mutants and wild type has been investigated by HPLC analysis. The ccm mutants produced a barely detectable amount of IAA (4-6 % of the wild type) after 4 days of growth. The detectable IpysA pathway intermediate, ILA, was also reduced significantly compared with wild type (Table 5). The mobilization of a plasmid (pSL12) containing the functional ccm operon restored ability to produce IAA (Table 5). This indicated that defect in the cytochrome c production is responsible for the IAA mutant phenotype.

Cytochrome c measurement

The cytochrome c of A. diazotrophicus was characterized with membranes obtained from cells grown with a limited nitrogen source, since it has been shown that the concentration of cytochrome c is increased in this condition (Flores-Encarnacion et al., 1999). Non-covalently bound hemes were removed by precipitating membranes with 0.01 N HCl in acetone (Goodhew et al., 1986) to determine cytochrome c without the spectral interference of b-type cytochromes. The dithionite-reduced minus persulfate-oxidized spectra (77K) of acidic acetone treated membranes from wild type and ccm mutants were determined (Fig. 4). The spectra of wild type membranes revealed the characteristic peaks at 417, 520, and 549.5 nm originating from the c-type cytochromes while these peaks were absent or significantly reduced in the ccm mutants (Fig. 4). Difference spectra produced by cyanide treatment of the reduced preparation revealed the
presence of an α-type cytochrome in both ccm mutants and wild type, accompanied by a large enhancement of the signals at 589 nm. Additionally, b-type cytochromes were retained in ccm mutant membranes, suggested by shoulders at 430, 529, and 560 nm in reduced plus CN⁻ minus oxidized spectra (Fig. 4).

To analyze the ccm mutant phenotypes in more detail, membrane fractions of aerobically grown wild type and mutant cells were analyzed after SDS-PAGE for the presence of heme-associated peroxidase activity (heme staining). Only c-type cytochromes have covalently-bound heme; on the other hand, non-covalently bound heme can be easily removed by SDS treatment. In the wild-type cells, four c-type cytochromes with apparent molecular masses of 67, 56, 52, and 45 kDa were detected (Fig. 5). No membrane bound c-type cytochromes were detected in membranes prepared from the ccm mutants.

Respiratory activities

Since in many organisms cytochrome c is involved in respiration, oxidase and dehydrogenase activities, the wild type and mutant strains were measured polarographically with a Clark-type electrode and spectrophotometrically with potassium ferricyanide as an electron acceptor. In decreasing order, glucose, NADH, acetaldehyde, ethanol, Asc + THQ, Asc + TMPD, succinate, and lactate were the best substrates for oxidase. In the case of ccm mutants, no ethanol, acetaldehyde, and lactate oxidase activities were detected; however, similar glucose and NADH oxidase activity were retained. The oxidase activity of succinate varied among the mutants: MAd20 and
MA
d21 had no oxidase activity with succinate, whereas MA
d10 and MA
d22 had
decreased oxidase activities to that of PA15. The dehydrogenase activities of ccm
mutants were significantly decreased when acetaldehyde, ethanol, or lactate was used as
substrates, but the activities with glucose or NADH were similar to those of the wild type.

Growth of bacteria

It has been reported that ccmC or ccmG mutants of P. denitrificans have a much
slower growth rate on rich media, probably due to the lack of protection against oxidized
thiol compounds in periplasmic proteins (Gordon et al., 2000; Page et al., 1997; Page and
Ferguson, 1994). Reduced disulfide bonds provide protection against the oxidized
compounds, facilitated by CcmC and CcmG. The growth rates of wild type A.
diazotrophicus and the ccm mutants in both minimal and rich media were determined.
As shown in Figure 6, no significant growth differences were observed in either media,
unlike that observed in P. denitrificans (Page and Ferguson, 1994).

Discussion

Cytochrome c is an electron carrier with a relatively wide distribution among
prokaryotes. In contrast to other cytochromes, cytochrome c carries a prosthetic heme
group covalently attached to the apoprotein. Mature holoproteins are localized to the
periplasmic side of the bacterial cytoplasmic membrane, either membrane bound or
soluble, and, in many cases, associated in a super complex with other redox partners.
The primary structure of cytochrome c precursor polypeptides exhibits two main characteristics: an N-terminal signal sequence with typical features of an export sequence, and the CxxH motif, representing the site for covalent attachment of heme. Thus, maturation of cytochrome c involves translocation of the precursor through the membrane and covalent binding of heme. Typically, the signal sequence is cleaved during maturation, and the cytochrome c is either released into the periplasm, or is inserted into the membrane with its hydrophobic C-terminal anchor. After translocation, some cytochrome c remains bound to the membrane by an uncleaved N-terminal signal sequence (Bott et al., 1991; Preisig et al., 1993; Jenney and Daldal, 1993; von Wachenfeldt and Hederstedt, 1990).

There has been renewed interest in cytochrome c as a result of the discovery that programmed cell death in eukaryotes requires the release of cytochrome c from the mitochondria (Green and Reed, 1998; Reed, 1997). Additionally, in some prokaryotes, a defect in the biogenesis of cytochrome c leads to a dramatic increase in synthesis and excretion of heme biosynthetic intermediates (Biel and Biel, 1990; Goldman et al., 1997). Other reported phenotypes of cytochrome c mutants are loss of copper resistance (Yang et al., 1996) and pyoverdine production (Gaballa et al., 1996) in Pseudomonas fluorescens, defects in nitrogen fixation in Bradyrhizobium japonicum and Rhizobium phaseoli (Ramseier et al., 1991; Soberon et al., 1993), as well as defects in high-affinity iron acquisition in Rhizobium leguminosarum (Yeoman et al., 1997). However, there are no reports describing the relationship between IAA production and cytochrome c in literature. We describe here for the first time the cloning of genes involved in
cytochrome c biogenesis from *A. diazotrophicus* and demonstrate the involvement of these genes in both heme transport and IAA production.

The present work shows that *A. diazotrophicus* contains several *ccm* genes which are essential for the synthesis of c-type cytochromes and normal production of IAA. *A. diazotrophicus* has been reported to have cytochrome *ba* (cytochrome *a*<sub>1</sub>) and *bd* as terminal oxidases. Cytochrome *ba* seems to be the major oxidase expressed in nitrogen-fixing conditions (under low available nitrogen concentration), whereas cytochrome *bd* is abundant when excess NH<sub>4</sub><sup>+</sup> is present (Flores-Encarnacion *et al.*, 1999). A ubiquinone pool will collect electrons from NADH and succinate dehydrogenases, and the electrons are transferred to cytochrome *a*<sub>1</sub> quinol-oxidase or cytochrome *bd* quinol oxidase depending on the concentration of available nitrogen. However, electrons from glucose, gluconate, ethanol, and acetaldehyde dehydrogenase are not collected by the ubiquinone pool (personal communication). The substrate for electron transfer from the latter dehydrogenase is not known.

It has been shown that *A. diazotrophicus* has quinol oxidases in which cytochrome *c* is not involved in the respiratory system of this bacterium (Flores-Encarnacion *et al.*, 1999). Cytochrome *c* most often functions in respiratory chains by passing electrons from the *bc*<sub>1</sub> complex to terminal oxidoreductases. However, some c-type cytochromes do not move electrons from the *bc*<sub>1</sub> complex to terminal oxidases. For example, in gram-negative methylotrophic bacteria, methanol is oxidized to formaldehyde by a periplasmic methanol dehydrogenase, and the electrons are passed to the terminal oxidase via a c-type cytochrome, without going through cytochrome *bc*<sub>1</sub>...
complex (Anthony, 1992; Goodwin and Anthony, 1998). In addition, in sulfate-reducing bacteria, a tetraheme cytochrome $c_3$ plays a role in the electron transport between hydrogenase and thiosulfate reductase (Clark and Barrett, 1987). Therefore, it is possible that electrons from glucose, ethanol, and acetaldehyde are directly transferred to cytochrome $c$, and then to quinol oxidase. If this is the case, it is expected that no oxidase activities will be detected with glucose, ethanol, lactate, and acetaldehyde in $ccm$ mutants. However, the result of both oxidase and dehydrogenase enzyme assays with those substrates demonstrated that glucose and NADH were the best physiological substrates (Table 3, 4), and the activities remain the same in the case of $ccm$ mutants.

The functions of alcohol, acetaldehyde, and lactate dehydrogenase as electron donors in the respiratory chain of *A. diazotrophicus* were abolished in the $ccm$ mutant strains. It has been known that alcohol dehydrogenases, aldehyde dehydrogenases, and lactate dehydrogenase of acetic acid producing bacteria contain subunits bearing cytochrome $c$, with molecular masses ranging from 45 to 82 kDa (Ameyama and O. Adachi, 1982; Ameyama et al., 1987; Matsushita et al., 1992; Matsushita et al., 1994). Therefore, no oxidase and dehydrogenase activities with those three substrates were most likely due to the fact that these dehydrogenases are cytochrome $c$-containing enzymes, not due to the defect in respiratory system. It suggested that $c$-type cytochromes are not directly involved in the respiratory system *A. diazotrophicus*. Moreover, it was confirmed with ascorbate plus TMPD oxidase activity that $c$-type cytochromes do not seem to have a role in the respiratory system of *A. diazotrophicus* since both wild type and mutant have similar low activities.
Detailed genetic analyses have shown that cytochrome c biogenesis genes (ccm genes) are well conserved in many gram negative and positive bacteria. The _A. diazotrophicus_ genes _ccmC, ccmE, ccmF, and ccmG_ have homologs of approximately the same sizes in _B. japonicum_ and _E. coli_, which show 40-52 % identity at the level of amino acid sequences (Fig. 2, Table 2). The individual _ccm_ genes, with exception of the _ccmG_, show the highest similarity to those of _B. japonicum_. _ccmG_ has the highest similarity to the _tipB_ (_ccmG_ homolog) gene of _P. fluorescence_. The _A. diazotrophicus_ _ccmC_ contained the conserved six putative membrane-spanning helices (Page _et al._, 1997) and trypophan rich motif, WGxxWxWD, which represents the putative heme binding sites in a predicted periplasmic loop. The CcmE protein of _A. diazotrophicus_ also has three conserved motifs and a putative amino-terminal transmembrane helix, suggesting that it might anchor to the membrane, leaving the bulk of the protein to protrude into the periplasm (Sanders and Lill, 2000; Schulz _et al._, 1999; Schulz and Thony-Meyer, 2000). One of the conserved motifs, LAKHDE, contains a conserved histidine residue shown to bind heme covalently in _E. coli_ (Schulz _et al._, 1998). The CcmF protein has 11 membrane spanning α-helices, shown by Pearce _et al._ (1998) based on the sequence analysis. Moreover, the characteristic tryptophan-rich domain (WAYYELGWGGxWFWDxPVEN) was also present in the CcmF protein. Only partial sequence of _ccmG_ was obtained and analyzed. CcmG proteins are a family of proteins called the thioredoxin/protein disulfide isomerase (PDI), which catalyze the formation or cleavage of disulfide bonds in _R. leguminosarum_ and _B. japonicum_ CycY proteins, and _E. coli_ CcmG protein, _P. fluorescens_ TipB/CytE, _Hemophilus influenzae_ CcmG, and
*Paracoccus denitrificans* CcmG. The function of CcmG proteins of these bacteria is to maintain the heme-binding site of apocytochrome *c* reduced, before heme attachment occurs (Page and Ferguson, 1999). The proposed active sites of PDIs, CxxC, were also found in the *A. diazotrophicus* CcmG protein.

The distribution and order of the *A. diazotrophicus ccm* operon is quite interesting (Fig. 3). In most members of the α group of proteobacteria, *ccm* genes are located in two separate gene clusters. For example, in *B. japonicum* and *R. capsulatus*, *ccm* homologs are distributed as *cycVWZX/Y/ helABCDX* and *cycHJKL/ccl1ccl2* (Thony-Meyer, 1997). However, most members of the γ-group of proteobacteria (ex. *E. coli* and *H. influenzae*) have a single locus at which all the *ccm* genes are located (Thony-Meyer et al., 1995).

The *ccm* genes of *A. diazotrophicus* (α-group of proteobacteria) are clustered as a single unit similar to those in the γ-group of proteobacteria (Fig. 3). There is an ORF between *ccmC* and *ccmE* that has no similarity to any known proteins and it is not similar to the *ccmD* homologs found in α- and γ-proteobacteria groups. It is possible that this ORF functions as a CcmD protein, although the sequence is so divergent that it could not be recognized as a *ccmD* gene. Interestingly, a member of the Gram-negative ε-group of bacteria, *Helicobacter pylori* (Tomb et al., 1997), contains a typical cytochrome *c* biogenesis system for Gram-positive bacteria, but not one for Gram-negative bacteria. This example also represents a diverse evolutionary event in *ccm* genes (Goldman et al., 1998; Kranz et al., 1998).
To test the involvement of *A. diazotrophicus ccm* genes in cytochrome *c* maturation, several *ccm* mutant strains were constructed and analyzed with respect to the maturation of cytochrome *c*. None of the *c*-type cytochromes were detected in these mutant strains, which was shown by peroxidase staining and spectroscopic analysis with membranes isolated from cultures grown in low nitrogen conditions (Figure 4, 5). However, the *ccm* mutants have no effect on the biogenesis of *a*-type or *b*-type cytochromes (Fig. 5). Likewise, it was reported that the maturation of the membrane-bound or soluble cytochrome *c*, but not *a*- or *b*-type cytochrome, were completely abolished in the *ccm* mutants of other bacteria (Page and Ferguson, 1994). Interestingly, one exception is that a *ccmG* mutant of *P. denitrificans* which lacks both *c*-type cytochromes and also cytochrome *aa*₃, suggesting a broad function of this protein (Page and Ferguson, 1997).

Oxidase and dehydrogenase activities also confirmed that *ccm* mutants resulted in a loss of functional cytochrome *c*. All *ccm* mutants are devoid of ethanol, acetaldehyde and lactate dehydrogenases and oxidase activities, since these dehydrogenases are cytochrome *c*-containing enzymes. It is also noteworthy that for some dehydrogenases TMPD⁺ is a better electron acceptor and for others, ferricyanide works better. When ferricyanide is used as an electron acceptor, higher dehydrogenase activities with NADH and lower activities with glucose, or higher activities with glucose with TMPD⁺ were detected, which suggested that TMPD is a better electron acceptor than FeCN for glucose dehydrogenase. Because succinate is not an efficient electron donor, it is not clear why succinate gives variable oxidase and dehydrogenase activities in different *ccm* mutants.
It is possible that the methods used to detect the oxidase and dehydrogenase activities are not sensitive enough to detect very low levels.

The functions of c-type cytochromes include an electron shuttle, an apoptosis triggering factor, and chemical reactions (Kranz et al., 1998). Newly discovered functions of cytochrome c, such as apoptosis in eukaryotes, loss of copper resistance, pyoverdine production, and high-affinity iron acquisition (Yang et al., 1996; Yang et al., 1997; Goldman et al., 1997; Gaballa et al., 1996; Yang et al., 1996) suggest there might be further functions not yet determined. The involvement of cytochrome c in IAA synthesis reported here represents another function. Mutation in the ccmCEF region resulted in a dramatic decrease of IAA production. It could be speculated that cytochrome c is involved in function of the enzymes converting the intermediates to the final product, IAA. Lactate dehydrogenase and ethanol dehydrogenase are possible candidates, since both enzymes are known to have a cytochrome c as a functional unit in Gluconobacter suboxydans, Acetobacter acetii, and Acetobacter methanolicus (Ameyama and O. Adachi, 1982; Ameyama et al., 1987; Matsushita et al., 1992; Matsushita et al., 1994). These dehydrogenases are responsible for reversible enzymatic transformation: indole-3-pyruvic acid to indole-3-lactic acid by a lactate dehydrogenase, and indole-3-acetaldehyde to indole-3-ethanol by an alcohol dehydrogenase, respectively. Theoretically, the bacteria without these dehydrogenases should produce the same level of IAA as wild type bacteria, since both pathways are for side reactions. There are no intermediates accumulated in the ccm mutants, which suggests that the reduced IAA production is not because of the lack of the functional enzymes involved in the IAA
biosynthetic pathway. The possibility of the presence of a different IAA biosynthetic pathway may explain the relationship between the ccm gene and IAA production. For example, if an indole-3-pyruvic pathway is operated through Tryptophan→indole-3-lactic acid to indole-3-acetaldehyde and then to the final product, IAA, it could explain the reduced IAA production in ccm mutants resulted from the non-functional lactate dehydrogenase. Intensive biochemical experiments must be followed to determine the role of cytochrome c in IAA biosynthesis.

Another possible explanation is the involvement of the cytochrome c in signaling. It has been shown that the eukaryotic cytochrome c can act as a signaling molecule to initiate apoptosis (Kluck et al., 1997; Yang et al., 1997). If cytochrome c is an important signaling factor controlling the induction of IAA biosynthesis, it could be possible that without functional cytochrome c bacteria cannot determine when IAA should be synthesized responding to various signals or to the state of the bacterial life cycle, and how much IAA needs to be produced. It has been shown that the level of IAA is very important to determine whether IAA will have beneficial influences or pathogenic effects. High concentrations of IAA can be detrimental to the plant, since it results in the inhibition of root growth and enlargement of plant cells causing plant tumors (Patten and Glick, 1996). Therefore, it is reasonable to think that IAA production is tightly regulated. As mentioned above, ccm mutants synthesize very little IAA, and no intermediates accumulate, suggesting that defects in cytochrome c act like the mutations in the genes involved in regulation of IAA biosynthesis.
The present findings indicated that the \textit{ccm} gene products are required for cytochrome \textit{c} biogenesis, as well as IAA biosynthesis in \textit{A. diazotrophicus}. Future work will include the characterization of the role of individual \textit{ccm} genes in IAA biosynthesis and respiratory chains. In addition, the biochemical study of the enzymes involved in the IAA biosynthetic pathway will tell us whether cytochrome \textit{c} is necessary for the function of these individual proteins. The role of IAA in sugarcane plant growth is currently under investigation using the \textit{ccm} mutants as IAA mutants, since all of the \textit{ccm} mutants have no growth defects in conditions tested and have the ability to fix nitrogen. Preliminary experiments indicate that the colonization and growth of the \textit{ccm} mutants inside sugarcane plants are similar to that of wild type \textit{A. diazotrophicus} (data not shown).

**Experimental procedures**

\textit{Bacterial strains, vectors, and growth conditions}

The bacterial strains and plasmids used are presented in Table 1. \textit{A. diazotrophicus} wild type PA15 ATCC49037 and \textit{ccm} mutants were maintained in either DYGS or LGIP medium (Reis, \textit{et al.}, 2000). \textit{E. coli} strains were grown at 37 °C in Luria-Bertani (LB) broth (Sambrook, \textit{et al.}, 1989). Antibiotics were added at the following concentrations (in \(\mu\text{g/ml}\)): ampicillin 500, kanamycin 200, and streptomycin 700. For the isolation of membranes, cells were grown aerobically at 30 °C in a 3 liter LGIP medium supplemented with 1.0 mM (NH4)\textsubscript{2}SO\textsubscript{4}.
Mutagenesis

Transposon mutants were generated by conjugation of strain PAI5 with E. coli S17-1 carrying the suicide plasmid pSUP1021, which contains a Tn5 transposon that confers Kanamycin resistance. Conjugations were performed on DYGS (pH 6) plates and incubated for 36 hours at 30 °C. After conjugation the cells were resuspended in LGI medium (Reis, et al., 2000), diluted and plated onto DYGS medium with kanamycin. Transconjugants were picked and stored as libraries in 96 well microtiter plates. After site-directed insertion mutagenesis using the Ω fragment (Streptomycin) recombinant plasmids were transformed into E. coli DH5α. The recombinant plasmids were electroporated into the chromosome of the PAI5 strain as described by Sevilla et al. (1998).

Tn5 screening

Tn5 exconjugants were grown in minimal LGI medium supplemented with tryptophan (100 µg/ml) as an IAA precursor in 96 well microtiter plates, and after 4 days of incubation, the amount of IAA produced by each exconjugants was determined using Salkowsky reagent (Fukuhara et al., 1994). IAA mutant candidates were further characterized by TLC and HPLC analysis.

Analytical methods

Preparation of membrane proteins, sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), staining for heme, spectral analysis of cytochromes,
dehydrogenase activities, and respiratory activities were performed as described by Flores-Encarnacion et al. (1999) with a few modifications. Briefly, cells in 3 liters of liquid culture were obtained after 36 hours of growth with shaking at 250 rpm. Cells were pelleted by centrifugation then washed twice with TCM (50 mM Tris-HCl (pH 7.4) containing 5 mM CaCl₂ and 5 mM MgCl₂) buffer. Membranes were isolated and protein concentrations were measured by a modification of the Lowry method (Dulley and Grieve, 1975). For spectra analysis, membranes (8 mg) were resuspended in 50 % (vol/vol) glycerol and analyzed in an SLM-Aminco DW 2000 spectrophotometer. Samples were reduced with a few grains of sodium dithionite in the presence or absence of KCN, and difference spectra at –77 ºC (77K) was recorded. c-type cytochrome spectra were measured after membranes were precipitated with 0.01 N HCl in acetone as described by Goodhew et al. (1986). Heme peroxidase staining was performed to detect c-type cytochrome bands. Membranes (3 mg) were washed with TCA (10% vol/vol), and separated by SDS-PAGE. SDS treatment removes noncovalently bound hemes, so that only c-type cytochrome is revealed after heme peroxidase staining. Protein blotting and detection were performed with the Amersham enhanced chemiluminescence Western blotting detection reagents (Miranda-Rios et al., 1997).

Oxidase activities were determined with various substrates at the final concentration of: 3 mM NADH, 10 mM glucose, 50 mM succinate, 10 mM ethanol, 10 mM acetaldehyde, 10 mM ascorbate plus 2 mM TMPD (N,N,N′,N′-tetramethyl-p-phenylenediamine, 10 mM ascorbate plus 1.5 mM THQ (2,3,5,6-tetrachloro-1,4-benzeneiol). The reactions were initiated with 0.1 mg of membrane protein and
measured polarographically with a Clark oxygen electrode in 2 ml of 50 mM potassium phosphate buffer (pH 7.4 or 6.0) at 30 °C. Dehydrogenase activities were measured spectrophotometrically with either potassium ferricyanide or TMPD as the alternative electron acceptors, as described by Flores-Encarnacion et al. (1999).

**DNA manipulations, sequencing and analysis**

General DNA manipulations were as described by Sambrook et al. (1989). The DNA region flanking the Tn5 integration was sequenced. The primer 5’-CGTTCAGGACGCTAC-3’, complementary to bases 17-34 within the Tn5 inverted repeat (Auerswald et al., 1981). All sequencing was performed with an ABI automated sequencer (model 373A) by using a PRISM Ready Reaction Dye Deoxy terminator cycle sequencing kit (Perkin-Elmer). DNA sequences were identified by using the BLAST server of the National Center for Biotechnology Information accessed over the Internet (Altschul et al., 1990). Computer-assisted sequence analysis was performed using programs of the University of Wisconsin package version 10.0, Genetics Computer Group software (GCG) (Devereux et al., 1984).

**Acknowledgements**

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References


Fig. 1. Physical and restriction map of *A. diazotrophicus ccm* operon and *ccm* mutants.

(A) The 5.6 kb *A. diazotrophicus* genomic fragment harboring the *ccm* operon with restriction sites indicated. B, *BamHI*; Bg, *BgIi*; E, *EcoRI*; K, *KpnI*; P, *PstI*; Sm, *SmaI*; Sp, *Sphi*; St, *StuI*. (B) A series of *ccm* mutants in which *ccm* genes were disrupted by deletion or insertion of Streptomycin (Ω) or Kanamycine (KIXX) cassettes.

Fig. 2. Alignments of proteins of *A. diazotrophicus* involved in cytochrome c maturation. Comparison of the deduced amino acid sequence of CcmC (a), CcmE (b), CcmF (c), and truncated CcmG (d) gene products of *A. diazotrophicus*. Completely and partially conserved residues are in bold letters. The active site that is relatively well conserved is shown by a horizontal line. Strongly predicted membrane-spanning α-helices are indicated by double lines above the sequences. The orientation of these helices with respect to the inner (i.e. cytoplasmic) and outer (i.e. periplasmic) faces of the cytoplasmic membrane are indicated by the letters *i* and *o* at the helix ends. The putative anchor (MA) of CcmE (b) is indicated by a single dotted line, and the conserved motifs are by overlining. The totally conserved histidine residue is indicated with an asterisk.

*Escherichia coli, Psudomonas fluorescens, Bradyrhizobium japonicum, Paracoccus denitrificans, Rhodobacter capsulatus.*

Fig. 3. Comparison of the bacterial cytochrome c maturation operon. Organisms: (A) *Bradyrhizobium japonicum*; (B) *Escherichia coli*; (C) *Acetobacter diazotrophicus*. 
Dotted segments of the *ccm* operon of *A. diazotrophicus* indicate that the sequence has not been completed.

Fig. 4. Difference spectra at 77K for c-type cytochromes associated with membranes of *A. diazotrophicus* PA15 and its *ccm* mutants (MAd10, Mad20, Mad21, and Mad22). (A) Reduced plus KCN minus oxidized spectra. Samples were reduced with dithionite in the presence of 1.0 mM KCN, and oxidized with air. (B) Dithionite reduced minus persulfate-oxidized spectra of acidic acetone treated membranes. 800 µg of protein were used in each samples.

Fig. 5. Membrane bound c-type cytochromes of *A. diazotrophicus* PA15 and its *ccm* mutants detected by haem staining after SDS-PAGE. Each lanes contain membrane protein samples. Lane a, PA15 (200 µg), lane b to e, *ccm* mutants, MAd10, MAd20, MAd21, and MAd22 (1000 µg each), lane f, horse heart cytochrome c (10 µg).

Fig. 6. Growth of the *A. diazotrophicus* and *ccm* mutant in a rich and minimal media. Cells pregrown in glucose minimal medium were inoculated into either DYGS rich or LGI minimal medium, and cell density was monitored by Klett machine. → PA15; , MAd 10; ← , MAd20; → , MAd21; ← , MAd22.
Fig. 1 Physical and restriction map of *A. diazotrophicus ccm* operon and *ccm* mutants.
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<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
</tr>
<tr>
<td>A. diazotrophicus</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
</tr>
<tr>
<td>R. capsulatus</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
<td>MA TIKQRRTIIGG SLAVLALAVLVA</td>
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</table>
Fig. 2. Alignments of proteins of *A. diazotrophicus* involved in cytochrome *c* maturation. Comparison of the deduced amino acid sequence of CcmC (a), CcmE (b), CcmF (c), and truncated CcmG (d) gene products of *A. diazotrophicus*. Completely and partially conserved residues are in bold letters. The active site that is relatively well conserved is shown by a horizontal line. Strongly predicted membrane-spanning α-helices are indicated by double lines above the sequences. The orientation of these helices with respect to the inner (i.e. cytoplasmic) and outer (i.e. periplasmic) faces of the cytoplasmic membrane are indicated by the letters *i* and *o* at the helix ends. The putative anchor (MA) of CcmE (b) is indicated by a single dotted line, and the conserved motifs are by overlining. The totally conserved histidine residue is indicated with an asterisk. *Escherichia coli, Pseudomonas fluorescens, Bradyrhizobium japonicum, Paracoccus denitrificans, Rhodobacter capsulatus.*
Fig. 3. Comparison of the bacterial cytochrome c maturation operon.
Fig. 4. Difference spectra at 77K for c-type cytochromes associated with membranes of *A. diazotrophicus* PA15 and its *ccm* mutants (MAd10, Mad20, Mad21, and Mad22).
Fig. 5. Membrane-bound c-type cytochromes of *A. diazotrophicus* PA15 and its *ccm* mutants detected by haem staining after SDS-PAGE.
Fig. 6. Growth of the *A. diazotrophicus* and *ccm* mutant in a rich and minimal media.
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristic(s)</th>
<th>Reference(s) or source</th>
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<tr>
<td><strong>A. diazotrophicus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI5</td>
<td>Wild-type strain</td>
<td>This work</td>
</tr>
<tr>
<td>MAd10</td>
<td>Spontaneous IAA mutant of PAI5,</td>
<td>This work</td>
</tr>
<tr>
<td>MAd20</td>
<td>PAI5 derivative, ΔccmCEF :: Ω (Sm(^{r}))</td>
<td>This work</td>
</tr>
<tr>
<td>MAd21</td>
<td>PAI5 derivative, ΔccmCE :: Ω (Sm(^{r}))</td>
<td>This work</td>
</tr>
<tr>
<td>MAd22</td>
<td>PAI5 derivative, ccmC :: Ω (Sm(^{r}))</td>
<td>This work</td>
</tr>
<tr>
<td>MAd23</td>
<td>PAI5 derivative, ccmG :: Km(^{f})</td>
<td>This work</td>
</tr>
<tr>
<td>MAd24</td>
<td>MAd10 containing pSL12</td>
<td>This work</td>
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<td><strong>E. coli</strong></td>
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<tr>
<td>DH5α</td>
<td>F-, recA1, endA1, hsdR17, supE44, thi-1, gyrA96, relA1, Δ(argF-lacZYA),</td>
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</tr>
<tr>
<td>S17-1</td>
<td>II69, Φ801lacZ, ΔM15, thi pro hsdR hsdM(^{r}) recA rpsL RP4-2 (Tc(^{r})::Mu) (Km(^{f})::Tn7)</td>
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<td><strong>Plasmids</strong></td>
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<td>Stratagene</td>
</tr>
<tr>
<td>pTZ19</td>
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<td>IncP1, Tc(^{r}), cos(^{r}), rlx(^{r})</td>
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<td>pSUP10-21</td>
<td>pLAFR3 containing the Km(^{f}) :: Tn5</td>
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<tr>
<td>pUC4-KIXX</td>
<td>Km(^{f}) cassette</td>
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<td>pH45Ω</td>
<td>Ap(^{f}) Sp(^{f}), pH45 carrying Sp(^{f}/Ap(^{f})</td>
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<td>pSL10</td>
<td>cosmid containing ccm operon</td>
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<tr>
<td>pSL11</td>
<td>pBS :: 12-kb EcoRI fragment from pSL10</td>
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</tr>
<tr>
<td>Description</td>
<td>Details</td>
<td>References</td>
</tr>
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<td>--------------------------------------------------------------------------</td>
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<td>pSLeh5.6</td>
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<td>pSL21</td>
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</tr>
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<td>pSL22</td>
<td>Ω (Sm') cassette inserted into pSL12 at Stul site</td>
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<td>pSL23</td>
<td>Km' cassette inserted into pSL12 at KpnI site</td>
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Table 2. Similarity between the *A. diazotrophicus* *ccm* and the *B. japonicum* *cyc* gene products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein size (Kdal)</th>
<th>Similarity to <em>E. coli ccm</em> gene products</th>
<th>Similarity to <em>B. japonicum cyc</em> gene products</th>
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<tr>
<td></td>
<td></td>
<td>Identity</td>
<td>similarity</td>
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<tr>
<td>ccmC/cycZ</td>
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<td>65</td>
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<tr>
<td>ccmG/cycY</td>
<td>20.0</td>
<td>41</td>
<td>58</td>
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</table>

a. Genes are shown as *E. coli/B. japonicum*.

b. Identity and similarity is based on the Blast search result and shown as % value.
Table 3. Oxidase activities associated with membranes of *A. diazotrophicus*

<table>
<thead>
<tr>
<th>Strains</th>
<th>PA15</th>
<th>MAd10</th>
<th>MAd20</th>
<th>MAd21</th>
<th>MAd22</th>
</tr>
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<tbody>
<tr>
<td>Glucose</td>
<td>2689</td>
<td>2292</td>
<td>1968</td>
<td>1771</td>
<td>2136</td>
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<tr>
<td>Ethanol</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>1258</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NADH</td>
<td>1995</td>
<td>2520</td>
<td>2160</td>
<td>2428</td>
<td>2208</td>
</tr>
<tr>
<td>Succinate</td>
<td>87</td>
<td>276</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lactate</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>TMPD-ascorbate</td>
<td>141</td>
<td>246</td>
<td>194</td>
<td>286</td>
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<td>THQ-ascorbate</td>
<td>750</td>
<td>444</td>
<td>703</td>
<td>468</td>
<td>350</td>
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</table>

*Activities represent the results of three independent experiments with a variation of less than 15%. The activities are displayed as the specific activities per milligram of membrane protein per minute. Activities are measured at pH 6.0 for glucose, acetaldehyde, ethanol, lactate, TMPD-ascorbate, and THQ-ascorbate, and at pH 7.4 for NADH and Succinate.*

*Oxidase activities were determined with different final concentrations of substrates: 10 mM glucose, ethanol, acetaldehyde and lactate; 3 mM NADH; 50 mM succinate; 10 mM ascorbate plus 2 mM TMPD and 10 mM ascorbate plus 1.5 mM THQ*
Table 4. Dehydrogenase activities associated with membranes of *A. diazotrophicus*

<table>
<thead>
<tr>
<th>Strains</th>
<th>Dehydrogenase Activities (nmol min$^{-1}$ mg$^{-1}$)</th>
<th>Ferricyanide (K$^+$)</th>
<th>TMPD$^+$</th>
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<tbody>
<tr>
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<td>MAd10</td>
</tr>
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<td>Ethanol</td>
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<tr>
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<td>1264</td>
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<td>Lactate</td>
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<td>Succinate</td>
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<td>Lactate</td>
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<td>30</td>
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"Activities represent the results of three independent experiments with a variation of less than 15%. The activities are displayed as the specific activities per milligram of membrane protein per minute. Activities are measured at pH 6.0 for glucose, acetaldehyde, ethanol, and lactate, and at pH 7.4 for NADH and Succinate.

Dehydrogenase activities were determined spectrophotometrically with potassium ferricyanide (K\(^+\)) or TMPQ\(^+\) as the electron acceptor. The assay mixture contained 0.1 M potassium phosphate buffer, 1.0 mM test substrate, 1 mM potassium cyanide or TMPQ, and 0.03 mg of membrane protein."
Table 5. IAA production by Ccm' mutants of *A. diazotrophicus* in a liquid medium

<table>
<thead>
<tr>
<th>Indole compounds</th>
<th>ILA</th>
<th>ICA</th>
<th>IAA</th>
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<tr>
<td>Pal5</td>
<td>1.79</td>
<td>0.97</td>
<td>6.8</td>
</tr>
<tr>
<td>MAd10</td>
<td>0.074</td>
<td>0.02</td>
<td>0.4</td>
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<td>MAd20</td>
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<td>0.02</td>
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<td>MAd21</td>
<td>0.09</td>
<td>0.05</td>
<td>0.48</td>
</tr>
<tr>
<td>MAd22</td>
<td>0.05</td>
<td>0.03</td>
<td>0.76</td>
</tr>
<tr>
<td>MAd23</td>
<td>1.5</td>
<td>1.1</td>
<td>6.3</td>
</tr>
<tr>
<td>MAd24</td>
<td>1.27</td>
<td>0.78</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*Data represent the means of the three experimental determinations, after 96 h culture.*
APPENDIX C. SUNHEE LEE, ELIZABETH PIERSON, AND CHRISTINA KENNEDY. 2001. CHARACTERIZATION OF INDOLE-3-ACETIC ACID PRODUCTION IN ACETOBACTER DIAZOTROPHICUS AND ASSESSMENT OF ITS ROLE IN SUGARCANE GROWTH ENHANCEMENT.
Characterization of indole-3-acetic acid production in *Acetobacter diazotrophicus* and assessment of its role in sugarcane growth enhancement.

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ABSTRACT

The endophytic nitrogen-fixing bacterium, Acetobacter diazotrophicus, and its beneficial association with sugarcane (Saccharum spp.) provides a model system to examine monocot/diazotroph symbiosis. Recent results from our laboratory, using mutant strains of A. diazotrophicus that are unable to fix nitrogen, have shown that there are two beneficial effects of A. diazotrophicus on sugarcane: one probably dependent on nitrogen-fixation and the other nitrogen fixation-independent. We hypothesized that indole-3-acetic acid (IAA), a growth promoting substance produced by A. diazotrophicus, may be the factor responsible for nitrogen fixation-independent plant growth enhancement. This study evaluated whether nitrogen fixation and/or Indole-3-acetic acid (IAA) production are important for the ability of A. diazotrophicus to stimulate sugarcane growth. IAA production relies on the presence of tryptophan as a precursor, age of the culture, and N availability and supply of fixed N. Sterile sugarcane inoculation experiments were performed using wild type along with Nif\(^{-}\), Iaa\(^{-}\), and Nif\(^{-}\)/Iaa\(^{-}\) mutants of A. diazotrophicus. Results of plant inoculation experiments demonstrated significant growth differences between plants inoculated with wild type of A. diazotrophicus and uninoculated plants, regardless of N availability, which confirms that A. diazotrophicus stimulates plant growth. Under N-limiting conditions, plants inoculated with Nif\(^{-}\), Iaa\(^{-}\), or Nif\(^{-}\)/Iaa\(^{-}\) mutants were similar in height and biomass to uninoculated plants, suggesting both nitrogen fixation and IAA biosynthesis are important factors for improving sugarcane growth by A. diazotrophicus. Under N-sufficient conditions, plants
inoculated with Iaa' mutants had significantly reduced height and biomass compared with plants inoculated with the wild type, implying that the effect of IAA production is more evident under high N conditions.

INTRODUCTION

The endophytic bacterium, *Acetobacter diazotrophicus*, is considered a strong candidate for BNF in sugarcane (Boddey, 1995; Urquiaga et al., 1992). Strains of *A. diazotrophicus* have been isolated from macerates of surface-sterilized roots, stems, and leaves of sugarcane plants grown around the world (Baldani et al., 1997; Bellone et al., 1997; Cavalcante and Dobereiner, 1988; Dong et al., 1994; Li and MacRae, 1991). This bacterium has also been occasionally isolated from other plants such as banana, pineapple, tea, sweet potato, Cameroon grass, and coffee, strengthening the notion that its association with plants is widespread and beneficial (Bellone et al., 1997; Dong et al., 1995; Gillis et al., 1989; Jiminez-Salgado et al., 1997; Li and MacRae, 1991).

Results of $^{15}$N isotope dilution experiments indicated that up to 80% of the total N of sugarcane (equivalent to 200 kg N/ha/year) could be derived from atmospheric N$_2$ with biological nitrogen fixation (BNF) (Boddey et al., 1991). The correlation of the existence of *A. diazotrophicus* at high numbers inside sugarcane grown for decades with no N fertilizer, and fixed nitrogen input by BNF suggested that *A. diazotrophicus* might contribute significant fixed N for sugarcane growth (Boddey et al., 1991; Cavalcante and Dobereiner, 1988). Indirect evidence of BNF significance was obtained by co-cultivation
of *A. diazotrophicus* and acid-tolerant yeast, in which about 50 % of the bacterially fixed N was transferred to the yeast cells (Cohjo et al., 1993). In addition, recent plant experiments in our laboratory demonstrated that plants inoculated with wild type *A. diazotrophicus* strain PAIS have greater biomass and N content as compared to uninoculated plants, or Nif' mutants under N limiting conditions. However, this effect differs in the case of N sufficient conditions: plants inoculated with wild type or Nif' mutants are similar in growth compared to uninoculated plants (Sevilla et al., 1998). These data suggested that an additional factor or factors are involved in stimulation of sugarcane growth.

Bacterial phytohormone synthesis has often been proposed as one factor responsible for plant growth promotion in several plant-microbe interactions (Barbieri et al., 1986; Brown, 1974; Holland, 1997; Jacobson et al., 1994). In both symbiotic and beneficial plant-associating bacteria, several different pytohormones such as auxins (indole-3-acetic acid; IAA), cytokinines, gibberellins, and ethylene, can act to enhance various stages of plant growth. For example, cultures of *Azospirillum brasilense* contain all three types of plant growth promoting substances (Bottini et al., 1989; Reynders and Vlassak, 1979; Tien et al., 1979). The quantitively most important phytohormone produced by *A. brasilense* and other microorganisms such as *Rhizobium meliloti* is IAA, and the beneficial effects on plant growth are well-documented (Arshad and Frankenberger, 1998; Chanway, 1997; Glick, 1995; Kapulnik, 1991; Lazarovits and Nowak, 1997). Mutant strains of the *A. brasilense* that synthesized very low levels of IAA, when compared with the wild type strain, no longer promoted the formation of
lateral roots of wheat seedlings (Barbieri et al., 1986; Barbieri and Galli, 1994).

Although the role of bacterially produced IAA in the initiation and development of root nodules on legumes is still controversial, many researchers support the idea that nod-inducers (flavonoids) could control nodule morphogenesis, in combination with phytohormones, such as IAA, released by rhizobia (Bertrand et al., 1996; Fukuhara et al., 1994; Hirsch et al., 1989; Prinsen et al., 1991).

IAA is synthesized by two primary biosynthetic pathways; the TRP-dependent pathway in which TRP is used as a precursor of IAA, and the TRP-independent pathway, in which the precursor is not known. IAA biosynthesis without TRP has been shown previously in plants, but appears to be rare in bacteria (Baldi et al., 1991; Normanly and , 1999; Wright et al., 1991). The pathways for conversion of tryptophan (TRP) to indole-3-acetic acid (IAA) can occur by different routes, which involve deamination, decarboxylation, and/or hydrolysis reactions (Fig. 1). In higher plants and most microorganisms, the indole-3-pyruvic acid (IpyA) pathway is the main route for IAA synthesis, whereas other pathways operate in certain species. The formation of IpyA from TRP is catalyzed by aminotransferases, followed by spontaneous or enzymatic decarboxylation to indole-3-acetaldehyde (IAld), which is then oxidized by an IAld oxidase to IAA. As a side reaction, IpyA may be reduced to indole-3-lactic acid (ILA) by lactate dehydrogenase. Indole-3-ethanol (also known as tryptophol; TOL) is the product of a side reaction from IAld. This reaction is carried out by an alcohol dehydrogenase. Other bacterial IAA biosynthetic pathways include the indole-3-acetamide pathway, the
tryptamine pathway, and indole-3-acetonitrile pathway (Frankenberger and Arshad, 1995; Kawaguchi and Syono, 1996; Patten and Glick, 1996).

Two types of plant growth promoting substances were detected in the supernatant of *A. diazotrophicus* cultures (auxins and gibberellins) (Bastian et al., 1998; Fuentes-Ramirez et al., 1993). The amount of other IAA produced by *A. diazotrophicus* is comparable to that of IAA-producing diazotrophs (Atzoron et al., 1988; Crozier et al., 1988; Gonzalez-Lopez et al., 1986). Moreover, the wild type strain PA15 used in previous sugarcane plant experiments is one of the highest IAA producing *A. diazotrophicus* strains. Quantitatively, the most important pytohormone produced by this bacterium is IAA. Therefore, it is reasonable to hypothesize that an alternative explanation for the observed plant growth stimulation by *A. diazotrophicus* could be IAA production. In the current study, IAA production under various conditions was investigated in order to study possible relationships between IAA production, nitrogen fixation, and plant growth enhancement. Mutant strains of *A. diazotrophicus* were constructed that produced significantly reduced amounts of IAA (Iaa'), or not to fix nitrogen (Nif'), or neither (Nif'/Iaa'), and used in sterile sugarcane inoculation experiments to determine whether IAA production and/or nitrogen fixation is important for the ability of *A. diazotrophicus* to stimulate plant growth.

**RESULTS**

*Generation of a Nif' mutant and Nif'/Iaa' double mutant*
A combination of techniques, such as a southern hybridization, PCR, complementation, and random Tn5 mutagenesis were used in an attempt to isolate IAA' mutants of *A. diazotrophicus*. Unfortunately, only one Tn5 mutant, MAd10 was identified that produced about 6% of the amount of IAA synthesized and excreted by the wild type strain. As described in Appendix 2, this mutant carries a lesion in the cytochrome c biogenesis gene. Another *ccmC* mutant, MAd22, carrying a *ccmC::Ω* (Streptomycin) mutation was constructed. This was the strain chosen for use in sugarcane inoculation studies (Lee et al., 2001). Insertion of a Chloramphenicol resistant marker in the middle of the nifD region of wild type and *CcM* resulted in a Nif- and a Nif'/Iaa' double mutant (Fig. 1). The phenotypes of these mutants were confirmed by an acetylene reduction assay for nitrogen fixation and HPLC for IAA production (data not shown). Nif- and Nif'/Iaa' mutants were unable to grow on N-free media and failed to reduce acetylene to ethylene. Iaa' and Nif'/Iaa' mutants produced only 6% of indole-3-acetic acid in the liquid culture compared with wild type (Table 4). These mutants had a similar growth in both rich and minimal medium, and no phenotypic difference was evident.

**Production of IAA by PA15 and MAd23**

The production of indole-3-acetic acid and related indoles was investigated by TLC and HPLC. The HPLC elution profiles of acidic ethyl acetate extracts contained five major peaks: two early-eluting unknown substances; indole-3-lactic acid (ILA); indole-3-acetic acid (IAA); indole-3-carboxylic acid (ICA) (Fig. 2). Chromatography by
TLC of ethyl acetate extracts from *A. diazotrophicus* cultures showed indole-3-lactic acid, indole-3-acetic acid, and indole-3-ethanol (Tryptophol-TOL), in addition to IAA and ILA, but no ICA could be detected by this method (data not shown). ILA and TOL are the intermediates of indole-3-pyruvic acid (IpyA) pathway. ICA is a decarboxylated inactive form of IAA catabolite. Quantification of the indole derivatives was performed based on the peak area of HPLC chromatogram, and yields of 5-7 µg/ml of IAA were present in the stationary culture medium depending on the culture conditions. Mutant MAd23 produced only a small amount of IAA (0.41 µg/ml).

**Effect of tryptophan concentration, culture age, and culture conditions on IAA production in PA15**

IAA production increased with increasing concentrations of tryptophan (TRP) from 1 to 100 µg/ml in 4 day old culture medium (Table 2). The amounts of IAA formed in the culture were 0.22, 1.85, and 6.7 µg/ml at 1, 10, and 100 µg/ml TRP, respectively. No indole-3-acetic acid was detectable when no TRP was supplemented. Greater amounts of ILA and ICA were accumulated as the TRP concentration was increased. However, at a concentration of 1000 µg/ml TRP bacteria did not grow at all, probably due to the toxic effect of a high concentration of TRP in the medium.

The production of ICA, ILA, and IAA by *A. diazotrophicus* growing in N-sufficient and aerobic conditions is shown in Fig. 3. IAA biosynthesis was proportional to the bacterial population in the medium. ICA production also increased with time, but
less rapidly than IAA. ILA accumulation was consistent regardless of the age of the culture.

The effect of ammonium and oxygen on IAA and its derivatives was examined with 4-day old cultures supplemented with 100 µg/ml concentration of TRP (Table 3). Results indicated that IAA production is generally higher in cultures grown with 10 mM ammonium than with 1 mM ammonium, whereas ILA and ICA production is more dependent on air. Conversion of TRP to IAA was similar under both aerobic (shaking culture) and microaerobic conditions (static culture) regardless of ammonium availability. Addition of 10 mM ammonium increased indole-3-acetic acid production by a factor of 1.7 under both aerobic and microaerobic conditions (Table 3). Production of ILA was stimulated under microaerobic conditions by a factor of 14.3 at low ammonium (1 mM (NH₄)₂SO₄) and of 2.2 at high ammonium (10 mM (NH₄)₂SO₄). In the presence of high ammonium ILA production was increased by a factor of 4.7 under aerobic condition, but was not altered under microaerobic conditions. ICA concentration increased under aerobic conditions by a factor of 6.8 and of 6.7, at the low ammonium, and high ammonium conditions, respectively. Higher ammonium concentrations did not change ICA production.

Effects of wild type and mutants as inocula in plant growth experiments

The effects of *A. diazotrophicus* on sugarcane growth were evaluated under N-limiting and N-sufficient conditions by measuring shoot and root dry weight and plant height, 30 days after inoculation. Regardless of nitrogen (N) availability, sugarcane
plants inoculated with wild type PA15 were always significantly taller and heavier than uninoculated plants (Fig. 4). The benefits of inoculation with any mutant to plant growth depended on nitrogen availability. Under nitrogen-limited conditions, plants inoculated with wild type had greater shoot and root mass than plants inoculated with all mutant strains. For example, in the first experiment under N-limiting conditions, plant height and root dry weight of wild-type inoculated plants were significantly greater than plants inoculated with all mutants, but shoot biomass of plants inoculated with the Nif'\!/laa' double mutant was similar to that of wild type. Typically, growth of plants inoculated with mutants was similar to that of uninoculated plants. In high nitrogen conditions, plants inoculated with the wild type, the Nif' mutant, and the Nif'/laa' mutant resulted in greater biomass and height. Plants inoculated with the Iaa' mutant were always smaller than plants inoculated with other mutants and typically not significantly different in height than uninoculated plants. In this experiment, the responses of plant growth from the addition of nitrogen resulted in significantly greater plant height and shoot dry weight compared to plants in nitrogen-limited conditions. However unlike aboveground production, root dry weight of plants was not significantly different between nitrogen conditions.

In the second experiment, the effects of inoculation with mutant strains were less apparent. For example, under both nitrogen-limited and -nonlimited conditions, plants inoculated with each mutant were smaller than plants inoculated with wild type, with one exception. The height of plants inoculated with the Nif' mutant were comparable to that of wild type under N-limiting conditions.
Colonization of sugarcane

Colonization of sugarcane was determined by measuring colony-forming units (CFU) 30 days after inoculation. Results demonstrated that both wild type and mutants colonized the sugarcane with similar efficiency (Table 4). *A. diazotrophicus* was not recovered from un-inoculated plants. Bacteria isolated from plants inoculated with different mutants retained antibiotic resistances: Nif⁻, chloromphenicol; Iaa⁺, Streptomycin; Nif⁺/Iaa⁺, chloromphenicol and Streptomycin. Acetylene reduction assays confirmed the inability of the Nif⁻ and Nif⁺/Iaa⁺ strains to fix dinitrogen (data not shown). No difference was observed in the growth and morphology of bacteria re-isolated from inoculated plants compared to the original bacterial inoculum. Moreover, *A. diazotrophicus* was not isolated from the rhizosphere of the sugarcane.

**Discussion**

Improved plant growth via plant-associated bacterial nitrogen-fixation have been reported in many plant-microbe interactions (James and Olivares, 1998; Reinhold-Hurek and Hurek, 1998; Sturz et al., 2000). One example of this beneficial symbiotic relationship is that between sugarcane and *A. diazotrophicus*. *A. diazotrophicus* may provide biologically fixed nitrogen as well as a significant amount of bacterial IAA. In this work we investigated the influence of these two factors on sugarcane growth using mutant strains that cannot fix nitrogen, produce IAA, or both.
Based on the presence of particular intermediates, it was hypothesized that IAA is synthesized through indole-3-pyruvic acid (IpyA) pathway, (Bastian et al., 1998; Fuentes-Ramirez et al., 1993). The synthesis of IAA via the IpyA pathway, the most common pathway present in both plant and microorganisms, has been proposed to occur also in several nonpathogenic plant-associated bacteria and in the tumorigenic A. tumefaciens, Pseudomonas syringae subsp. savastanoi, and Erwinia herbicola pv. gysophila (Patten and Glick, 1996). Our work also suggested that A. diazotrophicus produces IAA through the IpyA pathway. Two IpyA intermediates, ILA and TOL, were detected in the stationary phase liquid culture (Fig. 3). Other intermediates, indole-3-pyruvic acid (IpyA) and indole-3-acetaldehyde (IAld), were not detected with the solvent and condition used in this experiment probably due to inherent instability of IpyA and IAld (Ernstsen et al., 1987; Schneide and Wightman, 1974; Tien et al., 1979). However, the present study does not clarify whether ILA and TOL are connected to IAA metabolism of the bacterium. Metabolic studies with isotopically labeled substrates are necessary to confirm the IAA biosynthetic pathways of A. diazotrophicus. The presence of ICA on the HPLC chromatogram also suggested that IAA catabolism is associated with oxidative decarboxylation processes. This pathway has been reported only in Rhizobium phaseoli and Rhizobium trifolii (Badenoch-Jones et al., 1984; Ernstsen et al., 1987). The catabolism of IAA involves an indole-3-acetic acid → indole-3-methanol → indole-3-acetaldehyde → indole-3-carboxylic acid pathway (Frankenberger and Arshad, 1995; Reinecke and Bandurski, 1987; Sandberg et al., 1987). Decarboxylated forms of IAA such as ICA have been considered to be an important mean regulating the
endogenous IAA level of microorganisms (Jacob and Bopp, 1990; Normanly J, 1995).

Unlike bacteria, the catabolism of IAA in higher plants is thought to involve primarily non-decarboxylative pathways (Basu and Ghosh, 1998; Ernsten et al., 1987; Ghosh and Basu, 1999; Tsurumin and Wada, 1990; Tuominen et al., 1994).

To understand the factors influencing IAA production of A. diazotrophicus, IAA and indole derivatives in the liquid culture at different conditions were examined. Addition of TRP as a precursor strongly stimulated IAA production, as shown in many bacteria (Table 2). When TRP was not supplemented to the culture, no IAA was detected, which suggests that no TRP-independent pathway exists in A. diazotrophicus. However, TRP-independent IAA production could occur below the detection limit of approximately 300-500 pg. A well-known example of closely related plant-associated bacteria, Azospirillum brasilense synthesizes 90% of IAA through Trp-independent pathway (Prinsen et al., 1993).

In culture, the concentration of IAA was low during the logarithmic growth phase and increased rapidly with the beginning of the stationary phase (Fig. 4), confirming the characteristics of IAA as a secondary product. ICA production also steadily increased as IAA accumulates, but ILA synthesis did not. The effect of NH₄⁺ and oxygen on IAA biosynthesis was more complicated than that of Trp (Table 3). IAA production increased approximately 1.7 fold at the high ammonium concentration (10 mM (NH₄)₂SO₄); however, ILA and ICA production was slightly reduced with the same concentration of ammonium. Different effects of ammonium on IAA production were reported in many other plant-associated bacteria, including Rhizobium spp. and A. brasilense. In these
organisms, increased IAA production in low ammonium concentration (usually 1 mM ammonium) was explained as being similar to the natural setting. In the microaerobic conditions, ICA production was significantly reduced, whereas ILA production was increased and IAA production was consistent regardless of oxygen concentration in the culture. The differences in ICA and ILA production might be explained by the fact that the enzyme (peroxidase) converting indole-3-aldehyde to ICA requires oxygen (Arshad, 1998; Egebo et al., 1991; Sembdner et al., 1980), but lactate dehydrogenase responsible for making ILA has a higher activity under anaerobic conditions (Higuchi et al., 2000).

To test whether bacterial IAA biosynthesis and/or nitrogen fixation inside of sugarcane, very different from in vitro conditions, could affect sugarcane growth, sterile plant inoculation experiments were performed with wild type, Nif−, Iaa+, Nif+/Iaa−, and no inoculum control (Fig. 4). Inoculation of sugarcane with A. diazotrophicus consistently resulted in improved growth of plants across experiments, although differences were not always significant in all parameters measured. Regardless of nitrogen availability, plants inoculated with strain PAI5 were significantly taller and heavier than un-inoculated plants, demonstrating the beneficial effects of inoculation with wild type A. diazotrophicus and confirming the results of Sevilla et al., (2001). In the first experiment, under N-limiting conditions, plants inoculated with Nif− mutants or Iaa− mutants of A. diazotrophicus had similar height and dry weights compared with uninoculated plants, suggesting both nitrogen fixation and IAA biosynthesis are important factors influencing sugarcane growth in the low N condition. Plants inoculated with the Iaa− strain were smallest under low N conditions. It is possible that A. diazotrophicus utilizes a greater portion of its
metabolic energy to fix nitrogen under low N conditions, and thereby has fewer resources for IAA biosynthesis. When IAA production was examined in the liquid culture supplemented with either 1 mM (NH₄)₂SO₄ or 10 mM (NH₄)₂SO₄, approximately a two-fold higher amount of IAA was synthesized at high N concentrations when compared with IAA production at low concentrations of N. When N is sufficiently available plants inoculated with Nif" mutants capable of producing IAA had grown about as well as plants inoculated with wild type. In contrast, plants inoculated with Iaa" mutants are comparable to uninoculated plants, suggesting that IAA biosynthesis by A. *diazotrophicus* could be important regardless of N availability, and the effect is more pronounced under high N conditions.

A second set of experiments also demonstrated that N fixation and IAA biosynthesis by A. *diazotrophicus* are important factors improving sugarcane growth. However, unlike the first experiment, the effect of inoculation of plants with mutant strains were minimal; plants inoculated with Nif" , Iaa", and Nif"/Iaa" mutants were always significantly different from wild type inoculated plants, and were similar to plants with no inoculum. Since the number of mutants and wild type inside sugarcane were similar based on the CFU (Table 4), it is not clear what caused the different plant responses by the same mutants between two sets of experiments. One possibility is that in the second experiment the N provided was not utilized as effectively as in the first experiment. In this case, the plants inoculated with the wild type strain might receive the benefit of extra N fixed by bacteria, but plants with Nif" , and Nif"/Iaa" mutants might not obtain enough N to perform other biosynthetic processes such as IAA production.
Different responses to the excess N in two sets of plant experiments were also observed by Sevilla et al (2001). However, when plant response to N was investigated by comparing the differences in size with uninoculated plants, it was evident that in both sets of experiments, plants response similarly to added N, averaging 1.5 fold higher in all three parameters with N. Another possibility to explain the behavior of the mutants in the second experiment stems from the differences in two sets of tissue-cultured sugarcane. It is known that some variations can occur among plants from tissue culture.

It is evident that both N fixation and IAA biosynthesis are essential for improving sugarcane growth by *A. diazotrophicus*. However, it is not clear why a synergistic effect of these two factors was not observed in plants inoculated with the Nif^{-}/Iaa^{-} mutant. One explanation is the possibility that the Iaa^{-} strain reverted to Iaa^{+} phenotype after inoculation. This is particularly interesting since all three growth parameters of plants inoculated with the double mutant were always similar to those of plants inoculated with a Nif^{+} mutant, regardless of the N availability. Although the antibiotic resistant markers used to generate the mutant (chloromphenicol and streptomycin) were still present (based on the growth on antibiotics media) (Table 4), it is possible that the Nif^{-}/Iaa^{-} mutant phenotype is ‘leaky’ inside sugarcane, e.g. the double mutant synthesizes higher amounts of IAA than the Iaa^{-} mutant. It is possible that if both beneficial factors are lost, the plant may favorably select bacteria that can provide one, or both, beneficial factors. Therefore, *A. diazotrophicus* may develop ways to prevent loss from sugarcane by reverting the Iaa^{-} phenotype, either by turning on another pathway that is normally silent.
or by manipulating the regulation of IAA. Such reversion could lead to higher than expected IAA production in the double mutant and could explain the lack of synergistic effects by the Nif'/Iaa' mutant in our experiment. Further work is necessary to test this hypothesis.

We obtained IAA' mutants by Tn5 random mutagenesis and the phenotype was derived from the defect in a gene involved in cytochrome c biogenesis (ccm genes). While the Iaa' mutant used in this study was not directly derived from a mutation in a gene involved directly in IAA biosynthesis or the regulation, it is apparent that the plant responses were not the results of pleiotrophic effects resulting from the lack of c-type cytochromes, based on several lines of evidence. First, it has been shown that there is no difference in colonization of these mutants inside plants, and there are no growth defects in both rich and minimal media (Lee et al., 2001). Second, although c-type cytochromes are the major components of the respiration system in most microorganisms (Kerfeld and Krogmann, 1998; Thony-Meyer, 1997), the respiration chains of A. diazotrophicus do not contain the c-type cytochromes. Finally, a preliminary experiment indicated that when plants were inoculated with a CcmG'/Iaa' mutant that is not able to make c-type cytochromes, but synthesize the same amount of IAA as wild type, the height of these plants are comparable to wild type inoculated plants (data not shown). These evidences suggested that the Iaa' and Nif'/Iaa' mutants (actually both are also Ccm') were not due to the pleiotrophic effect of a non-functional cytochrome c.

In summary, this work showed that IAA biosynthesis is dependent on the presence of tryptophan as a precursor, age of the culture, and N availability in cell
cultures. Using mutant strains in plant inoculation experiments, it was also demonstrated that *A. diazotrophicus* could stimulate plant growth regardless of N availability as evidenced by the significant growth difference between plants inoculated with wild type and uninoculated plants. Typically, under N-limiting conditions plants inoculated with Nif\(^{-}\) or Nif\(^{-}/\)Iaa\(^{-}\) mutants were similar in height and biomass to uninoculated plants, suggesting nitrogen fixation by *A. diazotrophicus* is important in stimulating sugarcane growth. In addition, plants inoculated with Iaa\(^{-}\) mutants are always comparable to uninoculated plants regardless of N availability, which indicated that IAA biosynthesis is another major bacterial factor influencing sugarcane growth, and the influence of this compound is more evident in N-sufficient conditions. However, the role of IAA produced by *A. diazotrophicus* in sugarcane growth should be further studied in the future with genuine Iaa\(^{-}\) mutants specifically defective in the ability to synthesize this compound.

**MATERIALS AND METHODS**

**Plant material and bacterial growth**

Sterile sugarcane plants of cultivar SP70-1143 were obtained by meristem tissue culture and micropropagated (Sevilla et al., 1998). All the bacterial strains used in these experiments are listed in Table 1. *A. diazotrophicus* strains were grown in DYGS, LGIP, or LGI media at 30 °C (Cavalcante and Dobereiner, 1988). *E. coli* strains were cultured in LB medium. The bacterial inocula were prepared and inoculated into sugarcane as
described by Sevilla et al. (1998). Antibiotics added at the following concentrations (in μg/ml): Ampicillin 100, Streptomycin 700, and Chloramphenicol 700.

Construction of mutant strains

The isolation and characterization of ccm genes involved in indole-3-acetic acid production were described elsewhere (Lee et al., 2001). The IAA– strain was generated by inserting a Ω cassette (Streptomycin) in the ccmC gene. A Nif– and Nif–/Iaa– double mutant strain was constructed by inserting the Chloramphenicol (Cm) cassette (uidA-cat) to the nifD gene fragment. Transconjugants in which the nifD::uidA-cat replaced nifD were isolated in a background of wild type and Iaa– strains (Fig. 2). The abilities of strains to fix nitrogen or produce indole-3-acetic acid were tested by the acetylene reduction assay and HPLC chromatography. In addition, the growth of both wild type and mutant strains in minimal and rich media were examined.

HPLC analysis

Extraction and HPLC of A. diazotrophicus cultures for IAA were performed according to Costacurta et al. (1994) with slight modifications. Bacterial cultures (20 ml) were made cell-free by centrifugations at 3000 xg, and supernatants were extracted three times with ethyl acetate after adjusting pH to 2.8. 5-15 ul aliquots of the filtered extracts were injected into an Alltech, type Econosphere C185U column (250 x 4.6 mm) equipped with a differential ultraviolet detector absorbing at 280 nm. The isocratic solvent used
for a reverse phase chromatography was acetonitrile: glacial acetic acid (1 %) in water (10:90). Flow rate was adjusted to 1 ml/min. Peak retention times were compared with those of authentic IAA standards and quantified by comparison of peak areas.

**Acetylene reduction assay**

Acetylene reduction assays were performed by injecting 0.5 ml of freshly generated acetylene into the vials containing bacteria grown in semisolid agar (0.16 %). The vials were sealed with a rubber stopper to prevent gas leakage. After 2 h at 30 °C, 0.5 ml of gas samples were monitored for the presence of ethylene. A Shimadzu Gas Chromatograph fitted with a 50 cm Poropak column and a hydrogen ionization detector was used to detect ethylene.

**Inoculation of sugarcane plants and assessment of colonization**

Two independent experiments were performed with 22 plant replicates in each treatment (a total of 320 plants were tested for each experiment). The plant death rate was less than 5 % in every experiment. Inoculation was performed using the method of Sevilla et al. (1998) with some modifications. Plants were separated by tearing into single individual plantlets of similar root mass. The plants with uniform height and root mass were chosen as a block for each replicate and each plantlet was placed in baby food jars containing 40 ml of half strength MS medium with ammonium nitrate. 200 μl of bacteria (approximately 10^7 cells) was added to the medium and 200 ul of LG1 medium was added as a no inoculum control. Plants were placed on the shelves at 28 C with a 12
hr light and dark diurnal cycle for ten days in order to give the inoculum time to colonize the sugarcane.

After ten days, plantlets were transferred to conical containers containing sterile silica sand (grain #20). The plants were grown in a growth chamber at 28 °C with 12 hr light and dark cycles and watered 3 times a day. Every week 20 mls half strength of MS medium with N, or without N, were provided for each plant. The growth effect of the inoculation was determined 30 days after planting in a growth chamber by measuring the plant height, and root and shoot dry weights. Bacterial colonization was monitored 25 days after planting by plating 1 g of macerated plant material on LGIP medium (10 % sucrose, pH 4.5) with or without antibiotics (Streptomycin for Iaa\(^{+}\); Chloramphenicol for Nif\(^{-}\); Streptomycin and Chloramphenicol for Nif\(^{-}\)/Iaa\(^{+}\)). Plating was performed using a Autoplate 4000, Automated Spiral Plater. Colonies were located on the plates by a CASBA \(^{4}\) scanner and each colony was analyzed for the plate count (cfu/ml) by CIA-BEN software (Spiral Biotech, Inc., Bethesda, MD). Bacteria isolated from the inoculated plants were tested for their ability to grow in N-free media and to reduce acetylene to ethylene.

**Statistical analysis**

Statistical analysis was performed using the PROC GLM procedure of the SAS (Statistical Analysis Systems, SAS Institute, Cary, NC) computer program. Data were transformed using the log function before the statistical analysis and each experiment was
analyzed separately. Mean separations were determined by Waller-Duncan \( k \) ratio \( t \) test (K ratio=100).

**ACKNOWLEDGMENTS**

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Fig. 1. Pathways of IAA biosynthesis. —► represents pathways present in both bacteria and plant. —► is a proposed tryptophan-independent biosynthetic pathway. --► shows bypass pathways.

Fig. 2. Mutagenesis to construct Nif\(^{-}\), Iaa\(^{-}\), and Nif\(^{-}\)/Iaa\(^{-}\) mutant strains. The location of cat::uidA and \( \Omega \) (Sm) cassette and their direction of insertion are shown by triangles and arrows, respectively. Boxed arrows show direction of nifHDK and ccmCDFG.

Abbreviations for restriction sites: B, BamHI; Bg, BglII; C, ClaI; E, EcoRI; P, PstI; S, SmaI, St, Stul

Fig. 3. Reversed-phase HPLC chromatogram of the culture fluid of \( A. \) diazotrophicus wild type PA15 (a) and an Iaa\(^{-}\) mutant MAd22 (b) after 4 days of growth. The cultures were grown in minimal medium (LGI) supplemented with 100 \( \mu \)g/ml Trp.

Figure 4. Growth curve (OD\(_{670}\)) and IAA, ILA, and ICA production in the liquid culture during growth of \( A. \) diazotrophicus PA15 in 10 mM (NH\(_4\))\(_2\)SO\(_4\) supplemented with 100 \( \mu \)g/ml Trp.

Figure 5. Mean plant biomass and height of sugarcane plants inoculated with \( A. \) diazotrophicus wild type strain PA15 and mutant strains, 30 days after inoculation. Number of plants analyzed for experiment 1, without N and with N, respectively: PA15, 18 and 17; Nif\(^{-}\) mutant, 17 and 16; Iaa\(^{-}\) mutant, 18 and 14; Nif\(^{-}\)/Iaa\(^{-}\), 17 and 18;
uninuated, 15 and 15. Number of plants for experiment 2, without N and with N, respectively: PA15, 19 and 19; Nif' mutant, 19 and 18; Iaa' mutant, 18 and 19; Nif'/Iaa', 19 and 18; uninoulated, 19 and 18. Data were analyzed for each experiment separately. Means with the same letter are not significantly different.
Fig. 1. Pathways of IAA biosynthesis.
Fig. 2. Mutagenesis to construct Nif⁻, Iaa⁻, and Nif⁻/Iaa⁻ mutant strains.
Fig. 3. Reversed-phase HPLC chromatogram of the culture fluid of *A. diazotrophicus*
Fig. 4. Growth curve (OD$_{670}$) and IAA, ILA, and ICA production in the liquid culture during growth of *A. diazotrophicus* PA15
Fig. 5. Mean plant biomass and height of sugarcane plants inoculated with *A. diazotrophicus* wild type strain PA15 and mutant strains.
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains or plasmids</th>
<th>Relevant characteristic(s)</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. diazotrophicus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA15 (ATCC 49837)^T</td>
<td>Wild-type strain</td>
<td>This work</td>
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<td>MA122</td>
<td>PA15 derivative, ccmC::Ω (Sm', Iaa')</td>
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<tr>
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<td>PA15 derivative, nifD::uidA-cat (Cm', Nif')</td>
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<td>PA15 derivative, ccmC::Ω, nifD::uidA-cat (Cm', Sm', Nif'/Iaa')</td>
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<td>DH5α</td>
<td>F-, recA1, endA1, hsdR17, supE44, thi-1, gyrA96, relA1, Δ(argF-lacZYA),</td>
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<td>S17-1</td>
<td>Φ801lacZ, ΔM15, thi pro hsdR hsdM' recA rpsL RP4-2 (Tc':Mu) (Km':Tn7)</td>
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Table 2. Effect of tryptophan concentration implied on the production of indole derivatives by *A. diazotrophicus*

<table>
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<tr>
<th>tryptophan concentration (µ g/ml)</th>
<th>ILA</th>
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<th>IAA</th>
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<tr>
<td>1</td>
<td>nd</td>
<td>nd</td>
<td>0.22</td>
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<tr>
<td>10</td>
<td>1.35</td>
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<tr>
<td>100</td>
<td>5.89</td>
<td>0.64</td>
<td>6.7</td>
</tr>
<tr>
<td>1000</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

aData show indole compound production by 4 day-old cultures of *A. diazotrophicus* growing in 10 mM (NH₂)SO₄ supplemented with different concentrations of L-tryptophan.

b_†_nd = not detected
Table 3. Effect of tryptophan, ammonium and oxygen on the production of indole compounds in *A. diazotrophicus* PA15

<table>
<thead>
<tr>
<th>Growth conditions&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Indole derivatives production (µg/ml/OD&lt;sub&gt;670&lt;/sub&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
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</table>

<sup>a</sup>Growth conditions refer to the (NH₄)₂SO₄ concentration (mg/ml), 100 µg/ml tryptophan supplement (Trp), and aeration. - indicates no Trp, and no shaking; + indicates Trp supplement and shaking at 250 rpm.

<sup>b</sup>IAA, ILA, and ICA production was analyzed by HPLC and IAA production was expressed as µg/ml per OD<sub>670</sub> because of the variation in culture density of different growth conditions.

<sup>nd = not detected.</sup>
Table 4. Number of bacteria re-isolated from sugarcane inoculated with different strains.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Number of cells/gram fresh weight(^a)</th>
<th>Growth in N- free media</th>
<th>Acetylene reduction</th>
<th>Antibiotics resistance(^c)</th>
<th>IAA production ((\mu g/ml))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-N(^b)</td>
<td>+N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No inoculum</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wild type (PAL5)</td>
<td>9.5 (\times 10^5)</td>
<td>4.9 (\times 10^6)</td>
<td>+</td>
<td>+</td>
<td>6.7</td>
</tr>
<tr>
<td>Nif(^-)</td>
<td>3.4 (\times 10^5)</td>
<td>1.2 (\times 10^6)</td>
<td>-</td>
<td>-</td>
<td>Cm</td>
</tr>
<tr>
<td>Iaa(^+)</td>
<td>2.3 (\times 10^5)</td>
<td>9.2 (\times 10^6)</td>
<td>+</td>
<td>+</td>
<td>Sm</td>
</tr>
<tr>
<td>Nif(^-)/Iaa(^+)</td>
<td>7.4 (\times 10^6)</td>
<td>6.5 (\times 10^6)</td>
<td>-</td>
<td>-</td>
<td>Cm/Sm</td>
</tr>
</tbody>
</table>

\(^a\) Means of two experiments for 25 days after inoculation. Bacterial colonization was monitored after macerating 1 g of plant materials. The number of bacteria was determined by colony forming units (CFU).

\(^b\) -N, bacteria grown without added N; +N, bacteria grown with added N.

\(^c\) Antibiotics resistance: Cm, Chloramphenicol; Sm, Streptomycin
Table 5. IAA production by Ccm mutants.

<table>
<thead>
<tr>
<th>Indole compounds</th>
<th>IIA</th>
<th>ICA</th>
<th>IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI5</td>
<td>1.79</td>
<td>0.97</td>
<td>6.8</td>
</tr>
<tr>
<td>MAd10</td>
<td>0.074</td>
<td>0.02</td>
<td>0.4</td>
</tr>
<tr>
<td>MAd20</td>
<td>0.06</td>
<td>0.02</td>
<td>0.53</td>
</tr>
<tr>
<td>MAd21</td>
<td>0.09</td>
<td>0.05</td>
<td>0.76</td>
</tr>
<tr>
<td>MAd22</td>
<td>0.58</td>
<td>0.03</td>
<td>0.42</td>
</tr>
<tr>
<td>MAd23</td>
<td>1.5</td>
<td>1.1</td>
<td>6.3</td>
</tr>
<tr>
<td>MAd24</td>
<td>1.17</td>
<td>0.78</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*IAA and other indole derivatives were measured by HPLC after growing wild type PAI5 and Ccm' mutant strains in liquid cultures, supplemented with 100 μg/ml of tryptophan. Peak retention times were compared with those of authentic IAA standards and quantified by comparison of peak areas.*