NITROGEN FIXATION, HYDROGEN OXIDATION, AND NICKEL UTILIZATION BY *PSEUDOMONAS SACCHAROPHILA*

by

Wilfredo L. Barraquio

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

© Wilfredo L. Barraquio
November 1989

Department of Microbiology
Macdonald College of McGill University
Montreal, Quebec

To the memory of my parents

To my dear Virginia and Anne

ABSTRACT

Ph.D. Wilfredo L. Barraquio Microbiology

NITROGEN FIXATION, HYDROGEN OXIDATION, AND NICKEL UTILIZATION BY *PSEUDOMONAS SACCHAROPHILA*

Pseudomonas saccharophila could fix N_2 under microaerobic conditions, heterotrophically and chemolithotrophically. Uptake hydrogenase activity under heterotrophic conditions had no effect on the O_2 sensitivity of nitrogenase. H_2 induced whereas sucrose and O_2 repressed hydrogenase synthesis. Sucrose and O_2 did not inhibit hydrogenase activity. Hydrogenase and urease were located in the membrane and soluble fractions, respectively. Nickel stimulated growth, hydrogenase expression, and nitrogenase activity under N-limited chemolithotrophic conditions. Hydrogenase synthesis specifically required nickel and its repression by O_2 was alleviated by increasing the nickel concentration. Incorporated $^{63}Ni^{2+}$ was about 3 times higher in the soluble than in the membrane fraction. The short-term uptake of nickel was energy-independent and had an apparent K_m of 31.7 uM and V_{max} of 3.8 nmol Ni^{2+} (mg protein)$^{-1}$ min^{-1}.

A counting method for heterotrophic and chemolithotrophic N_2-fixing H_2-oxidizing bacteria was developed. The white bean rhizosphere soil showed relatively high numbers of these bacteria.

RESUME

Ph.D. Wilfredo L. Barraquio Microbiologie

LA FIXATION DE L'AZOTE, L'OXYDATION DE L'HYDROGENE, ET L'UTILISATION DU NICKEL PAR *PSEUDOMONAS SACCHAROPHILA*

Pseudomonas saccharophila a pu fixer le N_2 sous des conditions microaérobiques, hétérotrophiquement et chémolithotrophiquement. L'activité d'absorption de l'hydrogénase sous des conditions hétérotrophiques n'avait aucun effet sur la sensibilité à l'O_2 de la nitrogénase. H_2 a induit, tandis que le sucrose et l'O_2 ont réprimé, la synthèse de l'hydrogénase. Le sucrose et l'O_2 n'ont pas inhibé l'activité de l'hydrogénase. L'hydrogénase et l'uréase furent localisées dans la membrane et dans les fractions solubles, respectivement. Le nickel a stimulé la croissance, l'expression de l'hydrogénase, et l'activité de la nitrogénase sous des conditions chémolithotrophiques limitées en N. La synthèse de l'hydrogénase a requis spécifiquement du nickel, et sa répression par l'O_2 fut soulagée en augmentant la concentration de nickel. L'incorporation du $^{63}Ni^{2+}$ fut environ trois fois plus élevée dans la fraction soluble que dans la fraction de la

membrane. L'absorption du nickel à court-terme était indépendante de l'énergie et avait un K_m apparent de 31.7 uM et un V_{max} de 3.8 nmol Ni^{2+} (mg protein)$^{-1}$min^{-1}.

Une méthode de décompte des bactéries fixant le N_2 et oxydant le H_2 de façon hétérotrophique et chémolithotrophique fut développé. Le sol provenant de la rhizosphère des haricots blancs démontrait une population relativement élevée de ces bactéries.

ACKNOWLEDGMENTS

The author expresses his deep gratitude to Dr. R. Knowles for his patience, guidance, and interest in this study and in the preparation of this thesis. Dr. Knowles gave the author the opportunity to join his laboratory through an NSERC grant.

Sincere thanks go to the other members of my committee, Drs. J. Ingram and Y.-K. Chan for the initial preparation and other discussions concerning the conduct of the thesis.

I would like to thank the students and staff (academic and non-academic) of the Department of Microbiology for providing help and a very pleasant environment during the period of study. Discussion with Drs. R. A. Macleod and K. H. Tibelius regarding the nickel uptake experiment and the ribulose bisphosphate carboxylase assay, respectively, is highly appreciated. The help provided in particular, by D. Tito, N. Morin, A. Dumont, B. Courneyer, and C. Garcia is highly appreciated. I am grateful to C. Chauret for the French translation of the abstract.

The initial part of the study ($^{15}N_2$ incorporation) was done at the Soil Microbiology Department, International Rice Research Institute, Los Baños, Laguna, Philippines and the help provided by B. C. Padre, Jr. and Dr. I. Watanabe is greatly appreciated.

I wish to thank Dr. D. Smith and C. Hamel for allowing the author to take samples from their experimental plots, D. McQueen for providing the Lake St. George sediment samples, and all the donors of the test organisms.

The author was supported by a McGill University Graduate Fellowship (Dalbir Bindra Fellowship) for the period 1985-87.

CLAIM OF CONTRIBUTION TO KNOWLEDGE*

1. The ability of *Pseudomonas saccharophila* ATCC 15946 to fix N_2 was demonstrated unequivocally by C_2H_2 reduction and $^{15}N_2$ incorporation. The organism could fix N_2 under microaerobic conditions, heterotrophically and chemolithotrophically. This is the first report of an approved species of *Pseudomonas*, a genus otherwise known to contain no diazotrophs, that has the ability to fix N_2.
2. *P. saccharophila* showed an active uptake hydrogenase under both N_2-fixing heterotrophic and N-limited chemolithotrophic conditions. The former property suggests that the organism is capable of recycling the H_2 evolved during N_2 fixation. The ability of the organism to grow and fix N_2 under N-limited chemolithotrophic conditions suggests that the H_2-uptake system provides reducing power and energy for fixation of N_2 and CO_2.
3. Under heterotrophic conditions, uptake hydrogenase activity and exogenous H_2 had no effect on the O_2 sensitivity of nitrogenase activity.
4. Synthesis of hydrogenase was induced by H_2 and repressed by high concentrations of sucrose and O_2. Other utilizable organic substrates repressed synthesis and non-utilizable ones did not.

5. Uptake hydrogenase activity was not affected by high concentrations of sucrose and O_2.

6. Hydrogenase and urease activities were found mainly in the membrane and soluble fractions, respectively, from cells incubated under N-limited chemolithotrophic conditions. This is the first report of urease activity in *Pseudomonas saccharophila*.

7. Growth, hydrogenase expression, and nitrogenase activity was stimulated by nickel under N-limited chemolithotrophic conditions.

8. Nickel was specific in stimulating expression of hydrogenase activity because other divalent metals such as Co^{2+}, Zn^{2+}, Mn^{2+}, and Mg^{2+} were not as effective.

9. Nickel was required for the synthesis of hydrogenase.

10. Increasing the concentration of nickel under N-limited chemolithotrophic conditions alleviated the repressing effect of O_2 on the expression of hydrogenase activity.

11. *P. saccharophila* progressively incorporated $^{63}Ni^{2+}$ under N-limited chemolithotrophic conditions. The soluble fraction had about 3 times higher incorporated $^{63}Ni^{2+}$ than the membrane preparation. The relatively high percentage of radiolabel in the soluble fraction is very likely due to the presence of urease, a known nickel enzyme.

12. The short-term nickel-uptake system of *Pseudomonas saccharophila* was independent of the presence of preformed hydrogenase and hydrogenase-derepression conditions.

13. The nickel-uptake system followed Michaelis-Menten kinetics with an apparent K_m of 31.7 uM and a V_{max} of 3.8 nmol Ni (mg protein)$^{-1}$min^{-1}.

14. The nickel-uptake system of *P. saccharophila* was energy-independent.

15. The nickel-uptake system was relatively specific for nickel and was not coupled to the energy-dependent Mg^{2+}-transport system.

16. An enumeration method to accommodate both heterotrophic and chemolithotrophic free-living aerobic N_2-fixing, H_2-oxidizing bacteria was developed. It employed C_2H_2 reduction and tritium ($H^{\bar{3}}H$)-uptake techniques and a semisolid heterotrophic N-limited medium that allowed the expression of both nitrogenase and hydrogenase activities. By this method, *P. stutzeri* JM 300, another approved species of *Pseudomonas*, was found positive for both activities. Among the natural samples tested, the rhizosphere soil from white bean showed a relatively high population of N_2-fixing H_2-oxidizing bacteria. These results and those of other workers suggest that the rhizosphere is a good niche for H_2 transformation.

*Most of the results presented in this thesis have been published or submitted for publication as follows:

BARRAQUIO, W. L., PADRE, B. C., JR., WATANABE, I., and KNOWLES, R. 1986. Nitrogen fixation by *Pseudomonas saccharophila* Doudoroff ATCC 15946. J. Gen. Microbiol. **132**: 237-241.

BARRAQUIO, W. L., and KNOWLES, R. 1988. Effect of hydrogen, sucrose, and oxygen on uptake hydrogenase in nitrogen-fixing and ammonium-grown *Pseudomonas saccharophila* ATCC 15946. J. Gen. Microbiol. **134**: 893-901.

BARRAQUIO, W. L., DUMONT, A., and KNOWLES, R. 1988. Enumeration of free-living aerobic N_2-fixing H_2-oxidizing bacteria by using a heterotrophic semisolid medium and most-probable-number technique. Appl. Environ. Microbiol. **54**: 1313-1317.

BARRAQUIO, W. L., and KNOWLES, R. 1989. Beneficial effects of nickel on *Pseudomonas saccharophila* under nitrogen-limited chemolithotrophic conditions. Appl. Environ. Microbiol. **55**: 3197-3201.

BARRAQUIO, W. L., GARCIA, C., and KNOWLES, R. 1989. Energy-independent nickel uptake by *Pseudomonas saccharophila*. Arch. Microbiol. (submitted)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. GENERAL INTRODUCTION

The ultimate aim of many studies is to increase agricultural productivity through biological nitrogen fixation. The dwindling fuel resources, the environmental problems due to chemical fertilizers, and the increasing cost of producing ammonium fertilizer industrially are some of the reasons why it is imperative to turn to biologically fixed nitrogen. Biological nitrogen fixation is the nitrogenase-catalyzed conversion of atmospheric nitrogen to ammonia. The process is restricted to prokaryotes and is carried out under aerobic, microaerobic , and anaerobic conditions (Postgate 1982). N_2-fixing heterotrophs and autotrophs may occur in the free-living state, or in associative or symbiotic relationships with eukaryotes (usually plants) (Gallon and Chaplin 1987). The contribution of these groups to the nitrogen economy of ecosystems varies with the symbiotic N_2-fixers being the most agriculturally important. The other groups, however, may be important especially in ecosystems that have a high C:N ratio and that are undisturbed (Knowles 1977; Postgate 1982) and probably in habitats in which there are high concentrations of H_2 and CO_2 and relatively low O_2 tension (Watanabe *et al.* 1982; Dugnani *et al.* 1986; Barraquio *et al.* 1988; Conrad 1988).

Aside from the fact that the nitrogenase reaction is an energy-demanding process, it is obligatorily linked to

H_2 evolution which consumes about 25% of the total electron flux through nitrogenase (Adams *et al.* 1981). Most diazotrophs possess H_2-uptake systems which are capable of recycling the H_2 evolved during N_2 fixation (Bowien and Schlegel 1981; Eisbrenner and Evans 1983). H_2 uptake is linked to the electron transport chain which generates energy and/or reducing power and consumes O_2 (Dixon 1972; Adams *et al.* 1981; Bowien and Schlegel 1981). The presence of the H_2-uptake system should therefore increase the efficiency of N_2 fixation. Enhancing H_2 uptake would apparently have a favorable effect on the efficiency of N_2 fixation. Nickel was shown to stimulate expression of hydrogenase activity in some organisms (Friedrich *et al.* 1981; Takakuwa and Wall 1981; Colbeau and Vignais 1983; Nakamura *et al.* 1985; Stults *et al.* 1986; Doyle and Arp 1988).

The pseudomonads are amongst the most ubiquitous organisms known (see Section 2.1). They are metabolically versatile but no legitimate member was reported to fix N_2 (Palleroni 1984). In 1983, in the course of testing several *Pseudomonas*-like rice root isolates and known species of *Pseudomonas* for nitrogenase activity, only *P. saccharophila* ATCC 15946 among the known species was found positive (Barraquio and Watanabe, unpublished). *P. saccharophila* is a well-characterized facultative chemolithotroph (Palleroni 1984) isolated from the mud of a stagnant pond (Doudoroff 1940).

The objectives of this thesis are as follows:

(1) to confirm the N_2-fixing ability of *P. saccharophila* and to examine some factors that affect its nitrogenase activity

(2) to determine the presence and examine the regulation of the H_2-uptake system of *Pseudomonas saccharophila*

(3) to examine the role of nickel in the H_2-uptake system of *P. saccharophila*

(4) to develop a counting method that would accommodate both heterotrophic and chemolithotrophic N_2-fixing H_2-oxidizing bacteria

2. LITERATURE REVIEW

2.1. The genus *Pseudomonas*

2.1.1. *Taxonomic status*

The genus *Pseudomonas* is one of the most complex groups of Gram-negative bacteria (Palleroni 1984) apparently due to incomplete generic definition (De Vos *et al.* 1989). Extensive phenotypic characterization, polar flagellation, and DNA base composition were originally used for classification of strains (Palleroni *et al.* 1970). This classification scheme led to accumulation of a large collection of strains of incomplete description but which are aerobic, polarly flagellated, Gram-negative, rod-shaped bacteria. Palleroni *et al.* (1973) proposed 5 ribosomal ribonucleic acid homology groups based on competitive rRNA hybridization studies. Within each rRNA cluster, there was nearly 100% rRNA similarity (Palleroni *et al.* 1973) but the levels of DNA similarity between species were low (Palleroni *et al.* 1972). De Vos and De Ley (1983) using DNA:rRNA hybridization method showed that the five rRNA groups are remotely interrelated and cannot be maintained in a single genus. They suggested that most *Pseudomonas* species are located on three rRNA branches: (I) *P. fluorescens* branch that includes *P. aeruginosa* and *P. stutzeri* and other pigment-producing species, (II) *P. acidovorans* group which contains most hydrogen-oxidizing pseudomonads including *P. saccharophila*, and (III) *P. solanacearum* group which includes some plant pathogenic

pseudomonads. Only *P. fluorescens* rRNA branch was considered the true pseudomonads and all other species were generically misnamed (De Vos and De Ley 1983). Recently, a new genus *Comamonas* (De Vos *et al.* 1985; Tamaoka *et al.* 1987) was proposed to accommodate certain species of the rRNA homology group III of Palleroni's classification. According to the Woese classification (Woese *et al.* 1984; Woese 1987) which is based on phylogenetic relationships, the species of *Pseudomonas* are spread over the alpha to gamma subdivision of purple bacteria, now named *Proteobacteria* (Stackebrandt *et al.* 1988). De Vos *et al.* (1989) continued their phylogenetic studies on unclassified species of saprophytic *Pseudomonas* and found that two-thirds of the named *Pseudomonas* species have been misclassified and are distributed over at least seven genera all through the *Proteobacteria*. Willems *et al.* (1989) proposed a new generic name, *Hydrogenophaga*, for hydrogen-oxidizing species of *Pseudomonas* (*P. acidovorans* rRNA complex) which includes *P. flava*, *P. palleroni*, *P. pseudoflava*, *P. carboxydoflava*, and *P. taeniospiralis*. In the following discussion, I will follow the classification proposed by Palleroni (1984) until the phylogenetic studies on *Pseudomonas* are completed and all the proposed names of species and genera are approved and published by the International Journal of Systematic Bacteriology.

2.1.2. *Nutritional versatility*

The members of the genus *Pseudomonas* are aerobic,

having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor; in some cases nitrate can be used as alternate electron acceptor (Palleroni 1984). Most species can grow in mineral media with ammonium ions or nitrate and a single organic compound as the sole carbon and energy source (Stanier *et al.* 1966). They are chemoorganotrophs but some species can also grow under autotrophic conditions using CO and/or H_2 as energy sources. Carbon and energy sources range from simple to complex or unusual organic substrates. For example, pseudomonads are capable of degrading xenobiotics (Busse *et al.* 1989); 2-chlorobenzoate, a key intermediate in the degradative pathway of many chlorinated aromatics, toluene, *o*-, *m*-, and *p*-toluate, *m*- and *p*-xylene, and other aromatic compounds can be utilized (Worsey and Williams 1975; Palleroni 1984; Engelberts *et al.* 1989; Inoue and Horiuchi 1989; Sylvestre *et al.* 1989). Pseudomonads can carry out assimilatory and dissimilatory nitrate reduction (Palleroni 1984). They are considered the most commonly isolated denitrifying bacteria from both soils and aquatic sediments and may represent the most active denitrifiers in natural environments (Knowles 1982b). Despite their nutritional versatility, the ability of pseudomonads to fix nitrogen had long been doubted (Hill and Postgate 1969; Postgate 1980; Meganathan 1979) thus Palleroni (1984) assumed that none of the *Pseudomonas* species was a legitimate nitrogen fixer. Among the putative N_2-fixing *Pseudomonas* strains

shown in Table 1, *Pseudomonas saccharophila* can be considered the first approved species; some species failed to fix N_2 upon reexamination; some were not classified as *Pseudomonas*; and some may need reclassification or renaming. Undoubtedly, pseudomonads have *nif* genes and can accommodate and express *nif* genes from other sources.

2.1.3. *Ecological distribution*

Perhaps the catabolic versatility of *Pseudomonas* contributes to their ubiquity in nature. They can be found in various habitats - in terrestrial and aquatic (marine and freshwater) niches, in humans, animals, plants, foods, etc. (Stolp and Gadkari 1981; Palleroni 1984). *Pseudomonas* is considered as one of the predominant organisms in soil and in marine habitats (Quigley and Colwell 1968; Paul and Clark 1989) and in the rhizosphere in which they are capable of fixing N_2, producing growth-promoting substances and siderophores, and being antagonistic to soil-borne pathogens (Prabha *et al.* 1978; Barraquio *et al.* 1983; Kleeberger *et al.* 1983; Haahtela *et al.* 1983b; Thomas-Bauzon *et al.* 1983; Burr and Ceaser 1984; Rahman *et al.* 1986; Lifshitz *et al.* 1986; De Weger *et al.* 1986; Krotzky and Werner 1987; Schippers *et al.* 1987; Lalande *et al.* 1989). Geographically, pseudomonads can be found in the tropical, temperate, arctic, and antarctic regions (Haahtela *et al.* 1983b; Palleroni 1984; Lifshitz *et al.* 1986; Shivaji *et al.* 1989).

TABLE 1. List of putative and non-putative N_2-fixing *Pseudomonas*

Species	Species in BMSB[a]	Remarks	Reference[b]
P. methanica	-	not confirmed	1
P. azotocolligans 9391	-	did not reduce C_2H_2, mixed culture	2
P. azotogensis V & NCIB 9277	-	not *Pseudomonas*	3
Pseudomonas sp.	-	not confirmed	4
P. methanitrificans	-	contaminated by *Methylosinus*	5
P. ambigua	-	FTN[c]	6
P. glathei	+	did not reduce C_2H_2	7
P. fluorescens	+	genetically constructed	8
P. diazotrophicus	-	FTN	9
Pseudomonas sp. DC	-	FTN	10
P. paucimobilis 5AJ	+	FTN	11
Pseudomonas sp. 4B	-	FTN	12
P. saccharophila ATCC 15946	+	approved strain	13
P. diminuta	+	FTN	14
P. vesicularis	+	FTN	14
Pseudomonas sp.	-	FTN	15
P. stutzeri CMT.9.A	+	FTN	16
P. putida MT 20-3	+	genetically constructed	17
P. stutzeri JM 300	+	approved strain	18
P. pseudoflava	+	FTN	19

[a]Bergey's Manual of Systematic Bacteriology (1984) (see genus *Pseudomonas* by Palleroni (1984)).

[b]1, Sohngen 1906 (Dalton, 1980a); 2, Anderson (1955), Hill & Postgate (1969); 3, Voets & Debacher (1956), Paul & Newton (1961), De Ley & Park (1966), Hill & Postgate (1969), Prabha *et al.* (1978); 4, Proctor & Wilson (1958); 5, Davis *et al.* (1964); 6, Golovacheva & Kalininskaya (1968); 7, Zolg & Ottow (1975); 8, Mergeay & Gerits (1978); 9, Barraquio *et al.* (1983), Watanabe *et al.* (1987); 10, Haahtela *et al.* (1983a & b); 11, Thomas-Bauzon *et al.* (1982), Bally *et al* (1983); 12, Chan *et al.* (1986); 13, Barraquio *et al.* (1986); 14, Rahman *et al.* (1986); 15, Lifshitz *et al.* (1986); 16, Krotzky & Werner (1987); 17, Postgate & Kent (1987); 18, Barraquio *et al.* (1988); 19, Jenni *et al.* (1989).

[c]Further tests needed are rRNA homology and other genetic and biochemical tests.

1.1.4. *Pseudomonas saccharophila*

P. saccharophila is a Gram-negative, polarly flagellated non-pigmented rod which grows very well on sucrose and other sugars and organic acids, is capable of autotrophic growth with H_2 as energy source, does not denitrify but reduces nitrate to nitrite, hydrolyzes starch, accumulates poly B-hydroxybutyrate, and has a % mol G + C of 68.9 (Palleroni 1984). Glucose, fructose, mannose, and D-arabinose are utilized only after mutation. Its ability to fix nitrogen was demonstrated only recently (Barraquio *et al.* 1986). It was isolated by autotrophic enrichment in 1940 from the mud taken from a stagnant pool (Doudoroff 1940). So far only one strain (strain ATCC 15946) is known; a similar strain was isolated by Palleroni (1980) but differed in some phenotypic properties and DNA sequence; some isolates from surface sediment of freshwater lakes assigned to *P. saccharophila* (Schink and Zeikus 1984) need further characterization. Taxonomically, it belongs to the rRNA homology group III in which other H_2-oxidizing pseudomonads (*P. facilis*, *P. flava*, *P. pseudoflava*, and *P. palleroni*) and non-oxidizing ones (*P. acidovorans*, *P. testosteroni*, and *P. delafieldii*) are included (Palleroni 1984). *P. saccharophila* shows many phenotypic similarities to the members of this rRNA group but exhibits relatively low levels of DNA similarity (Ralston *et al.* 1972; Johnson and Palleroni 1989).

P. saccharophila has been the object of many studies

of pathways and enzymes of heterotrophic and autotrophic metabolism (Doudoroff 1940; Bernstein 1944; Entner and Doudoroff 1952; Palleroni 1956; Palleroni and Doudoroff 1956; Markowits *et al.* 1956; Young and Klein 1967; Donawa *et al.* 1971; Ishaque *et al.* 1971a & b, 1973; Donawa *et al.* 1973; Aleem *et al.* 1979). Most hexoses and related compounds are catabolized by the Entner Doudoroff pathway which was discovered in this organism (Entner and Doudoroff 1952). Of particular interest to this thesis are some aspects of energy generation during growth under autotrophic as well as heterotrophic conditions (Donawa *et al.* 1971; Ishaque *et al.* 1971a & b; Ishaque *et al.* 1973; Aleem *et al.* 1979). Under heterotrophic growth conditions, electron flow from NADH to O_2 is mediated by a complete electron transport chain with three energy conservation sites whereas only phosphorylation (proton translocation) sites I and II are involved in the anaerobic (NO_3^- as electron acceptor) oxidation of NADH. Electrons from succinate and ascorbate enter at cyt *b* and cyt *c* levels, respectively, with two and one energy conservation sites. Anaerobic oxidation of succinate yields a very low P/NO_3^- ratio, thus is poorly coupled to site II. Under autotrophic growth conditions, only the first two proton translocation sites are functional and the third one is inoperative. Electrons from H_2 enter at the cyt *b* level and H_2 oxidation and coupled phosphorylation appear to involve only coupling site II under aerobic or anaerobic conditions.

Cytochrome a_3 was detected but the third site lacks cyt *a* and was not found functional in energy generation. Cytochrome *o* appears to act as the terminal oxidase both for NO_3^-- and O_2-grown cells. Bone (1960) reported the presence of two H_2-activating enzymes in *Pseudomonas saccharophila*, one in the soluble fraction (NAD^+-dependent hydrogen dehydrogenase) and one in the particulate fraction (NAD^+-independent hydrogenase). However, Podzuweit *et al.* (1983) and the present author practically did not find hydrogenase activity in the soluble fraction. Thus, *P. saccharophila* has to carry out energy-requiring reverse electron transfer for NADH formation under chemolithotrophic conditions (Gottschalk 1986).

2.2. Nitrogen fixation by free-living aerobic heterotrophs

Biological dinitrogen fixation is the nitrogenase-catalyzed reduction of dinitrogen to ammonia according to the reaction

$$N_2 + 8(H^+ + e^-) + 16MgATP \longrightarrow 2NH_3 + H_2 + 16(MgADP + Pi)$$

The first report of the ability of free-living aerobic heterotrophic bacteria such as *Azotobacter chroococcum* and *Azomonas agilis* to fix N_2 was made my Beijerinck in 1901 (Burris 1988). In the strict sense, free-living aerobic heterotrophic N_2-fixing bacteria are those that fix N_2 aerobically and not in any association, loose or intimate, with another micro- or macroorganism with the carbon and energy supplied by an organic substrate. Some of them are plant-associated and some are not.

2.2.1. *Significance, ecological distribution, and generic diversity*

Since most ecosystems are organic carbon and energy limited, it is generally agreed that free-living aerobic heterotrophic N_2 fixers contribute little to the fertility of agricultural systems (Knowles 1977; Sprent 1979; Postgate 1982). Only in ecosystems where the C:N ratio is high could their contribution to the nitrogen economy be of significant value (Postgate 1982). However, they could help maintain fertility in undisturbed ecosystems and in non-intensive agricultural systems. Other benefits could be the growth-promoting effects on plants and their role in the degradation of recalcitrant substances (Postgate 1982). Their beneficial role in the rhizosphere has been claimed but the exact mechanism has been difficult to understand due to the complexity of the rhizosphere system. The amounts of N_2 fixed by this group of N_2 fixers under natural conditions are only estimates due to methodological problems in quantifying N_2 fixation.

Free-living aerobic heterotrophic diazotrophs are ecologically diverse. They can be found in soil, root zone (terrestrial and aquatic), fresh water, freshwater sediment, marine water, marine sediment, phylloplane (terrestrial and aquatic), and animals (Knowles 1977; Guerinot and Patriquin 1981). Important ecological niches in which significant amounts of N_2 could be fixed are the rhizosphere and unamended forest and agricultural soils

(Knowles 1977). Environmental factors that affect the natural occurrence of diazotrophs and their N_2-fixing activities include carbon and energy supply, oxygen tension, combined nitrogen, pH, water content, and availability of trace elements such as iron and molybdenum (Mulder and Brotonegoro 1974; Postgate 1974; Knowles 1977; Jensen 1979). Geographically, they occur in tropical, temperate, and arctic regions (Knowles 1977; Haahtela 1985; Lifshitz *et al.* 1986).

Free-living aerobic heterotrophic diazotrophs are generically diverse. At the time of this writing, a total of 22 unrelated genera are reported to have the ability to fix N_2 heterotrophically under aerobic and microaerobic conditions (Table 2). All species in some genera are capable of fixing N_2 but in most cases only a single or few species of a certain genus are diazotrophs. Our knowledge of the range of aerobic and other types of diazotrophs has increased rapidly in the last few years due to the use of the acetylene reduction technique and semi-enrichment techniques that involve the inclusion of a certain organic carbon source or mixture of carbon sources plus some vitamins or yeast extract in a nitrogen-deficient semisolid medium (Rennie 1981; Knowles 1982a; Dobereiner 1988). New N_2-fixing heterotrophs will undoubtedly be discovered in the future for it is often a matter of employing the proper isolation techniques. The use of *nifHDK* (nitrogenase structural genes) hybridization method is an excellent

TABLE 2. List of genera of free-living aerobic heterotrophic N_2-fixing bacteria[a]

Condition when fixing N_2	Genus[b]
Aerobic	*Azotobacter* (6), *Azomonas* (3), *Azotococcus* (1), *Beijerinckia* (4), *Derxia* (1)[c], *Lignobacter* (1)
Microaerobic	*Azospirillum* (4)[c], *Aquaspirillum* (4), *Campylobacter* (1), *Arthrobacter* (1), *Pseudomonas* (9)[c & d], *Alcaligenes* (2)[c], *Xanthobacter* (2)c, *Herbaspirillum* (1), *Acetobacter* (1)
Microaerobic to anaerobic[e]	*Klebsiella* (4), *Enterobacter* (2), *Citrobacter* (2), *Erwinia* (1), *Escherichia* (2)[f], *Bacillus* (4), *Vibrio* (1)

[a]Compiled from Knowles (1977), Sprent (1979), Sallinoja-Salonen *et al.* (1979), Dalton (1980a), Postgate (1981), Guerinot and Patriquin (1981), Malik and Schlegel (1981), Guerinot *et al.* (1982), Postgate (1982), McClung *et al.* (1983), Bazylinski and Blakemore (1983), Ladha *et al.* (1983), You *et al.* (1983), Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1984), Seldin *et al.* (1984), Baldani *et al.* (1986), Gallon and Chaplin (1987), Pedrosa (1988), Gillis *et al.* (1989), and all the references listed in Table 1.

[b]Numerals in parentheses are the number of diazotrophic species reported.

[c]Some members have chemolithotrophic capabilities.

[d]Two species are genetically constructed.

[e]Some members tested are capable of fixing N_2 in semisolid medium suggesting that probably all of them can tolerate low oxygen tension when fixing N_2.

[f] One species is genetically constructed.

way of screening for N_2-fixing bacteria which otherwise could be missed by the acetylene reduction test (Postgate *et al.* 1986; Possot *et al.* 1986; Oakley and Murrell 1988). Very recently, Jenni *et al.* (1989) showed the presence of *nif* genes in *Pseudomonas pseudoflava* strain NEU 2252, a strain which did not reduce C_2H_2 to C_2H_4.

2.2.2. *Chemolithotrophic N_2 fixation*

Organisms that use reduced inorganic compounds as source of energy and electrons and CO_2 as the major or sole source of carbon are called chemolithotrophs (Gottschalk 1986) or in the strict sense, chemolithoautotrophs or chemoautotrophs (Wood 1988). Dalton's definition (Dalton 1980b) states that a chemoautotroph is an organism that is able to obtain the bulk of its biosynthetic carbon from carbon dioxide or the metabolism of a one-carbon compound using either organic or inorganic compounds as energy source. If the organism can utilize N_2 as its sole nitrogen source then it is called a N_2-fixing chemolithotroph or chemoautotroph. Under Dalton's definition, the chemoautotrophic N_2-fixing organisms include the H_2-oxidizing *Xanthobacter autotrophicus* and *Pseudomonas saccharophila* (Berndt and Wolfe 1978; this thesis), the Fe-oxidizing *Thiobacillus ferrooxidans* (Mackintosh 1978), the CH_4-oxidizing *Methylosinus trichosporium* and *Methylococcus capsulatus* (Dalton and Whittenbury 1976; Whittenbury *et al.* 1970), and the CO-utilizing organisms (Ooyama 1976). This section of the thesis is concerned mainly with H_2-

dependent chemolithotrophy in which the nitrogen source is N_2, the energy and electron source is H_2, and the carbon source is CO_2. The process, therefore, involves at least 3 key enzymes: uptake hydrogenase, nitrogenase, and ribulose bisphosphate carboxylase (Bowien and Schlegel 1981; Gottschalk 1986). Since N_2 fixation and CO_2 fixation are both reducing power- and energy-demanding processes (Gottschalk 1986), the hydrogenase reaction should be able to provide these requirements in order for the organism to grow. Dalton (1980b) listed 3 reasons why the study of chemolithotrophic N_2-fixers (obligate and facultative) lagged much behind other free-living N_2-fixers: (a) they appeared late in the literature as authentic diazotrophs (the first 2 reports were by Ooyama (1971) and Gogotov and Schlegel (1974)), (b) their relative unimportance in agricultural terms, and (c) the difficulty of growing them to cell densities sufficient to obtain meaningful biochemical and physiological data. They could be of industrial importance, however, as biofilters in the waste processing industry (Dugnani *et al.* 1986). Chemolithotrophy by free-living aerobic heterotrophic diazotrophs can be a means of survival in ecosystems where there is low O_2 tension and high concentrations of H_2 and CO_2. It was found to be important for the survival of hydrogenase-positive *Bradyrhizobium japonicum* and indigenous soil bradyrhizobia (Lambert *et al.* 1985b; Viteri and Schmidt 1989). The importance of trace elements, particularly nickel, in

stimulating growth has been reported (Bartha and Ordal 1965; Repaske and Repaske 1976; see Section 2.4 and Chapter 5).

Of the 22 genera of free-living aerobic heterotrophic diazotrophs shown in Table 2, only 5 so far are known to contain members capable of chemolithotrophic N_2 fixation. They are as follows: *Azospirillum (lipoferum)*, *Pseudomonas (saccharophila, diazotrophicus)*, *Alcaligenes (latus)*, *Xanthobacter (autotrophicus)*, and *Derxia (gummosa)* (Wiegel and Schlegel 1976; Berndt and Wölfle 1978; Pedrosa *et al.* 1980; Malik and Schlegel 1981; Bowien and Schlegel 1981; Pinkwart *et al.* 1983; Gowda and Watanabe 1983; Ravi Shankar *et al.* 1986; Barraquio *et al.* 1986; Watanabe *et al.* 1987). Some aerobic diazotrophs have uptake hydrogenase but lack the ability to grow or fix N_2 chemolithotrophically. Examples are *Azospirillum brasilense*, *Azomonas agilis*, *Bacillus polymyxa*, *Beijerinckia indica*, *Klebsiella pneumoniae*, and *Azotobacter vinelandii* (Pedrosa *et al.* 1980; Bowien and Schlegel 1981; Gowda and Watanabe 1983; Wong and Maier 1985). This is why the usual method of isolating and enumerating N_2-fixing H_2-oxidizing bacteria using the N-deficient autotrophic medium with H_2, CO_2, O_2, and N_2 as balance gas (Wiegel and Schlegel 1976; De Bont and Leijten 1976; Aragno and Schlegel 1981; Knowles 1982a) is not applicable. Accordingly, a new method to accommodate both heterotrophic and chemolithotrophic N_2-fixing H_2-oxidizing bacteria was developed using a heterotrophic

semisolid medium that allows expression of both nitrogenase and uptake hydrogenase activities (Barraquio *et al.* 1988). This method is further discussed in Chapter 6.

2.2.3. *Kinds and properties of nitrogenases*

Three kinds of nitrogenases have been purified from *Azotobacter* species: molybdenum (Mo) and vanadium (V) nitrogenases found in both *A. vinelandii* and *A. chroococcum*, and iron (Fe) nitrogenase so far found only in *A. vinelandii* (Chisnell *et al.* 1988; Robson 1986; Bishop *et al.* 1988; Eady *et al.* 1988; Pau 1989; Premakumar *et al.* 1989). Vanadium nitrogenase seemed to be present in other diazotrophs (Dilworth *et al.* 1987; Chan *et al.* 1988; Kenntemisch *et al.* 1988). The three nitrogenases are similar in structure and function (Pau *et al.* 1988; Bishop *et al.* 1988). They consist of two metalloproteins: a 230-240 kDa protein (component 1) called dinitrogenase which contains the active site for substrate binding and is specifically reduced by the second protein, a 60-62 kDa dinitrogenase reductase (component 2) (Fig. 1). Component 1 is an $\alpha_2\beta_2$ tetramer with subunits of about 55 kDa and 59 kDa, respectively. It has 4 (4Fe-4S) clusters and 2 molecules of cofactors. Component 2 is a homodimer with subunits of about 30-32 kDa with a cluster of (4Fe-4S) bridged between the subunits. The nitrogenases differ in their cofactor centres: FeMoco for Mo-nitrogenase, FeVco for vanadium nitrogenase, and the putative FeFeco for Fe-nitrogenase (Fig. 1). V and Fe nitrogenases have a small

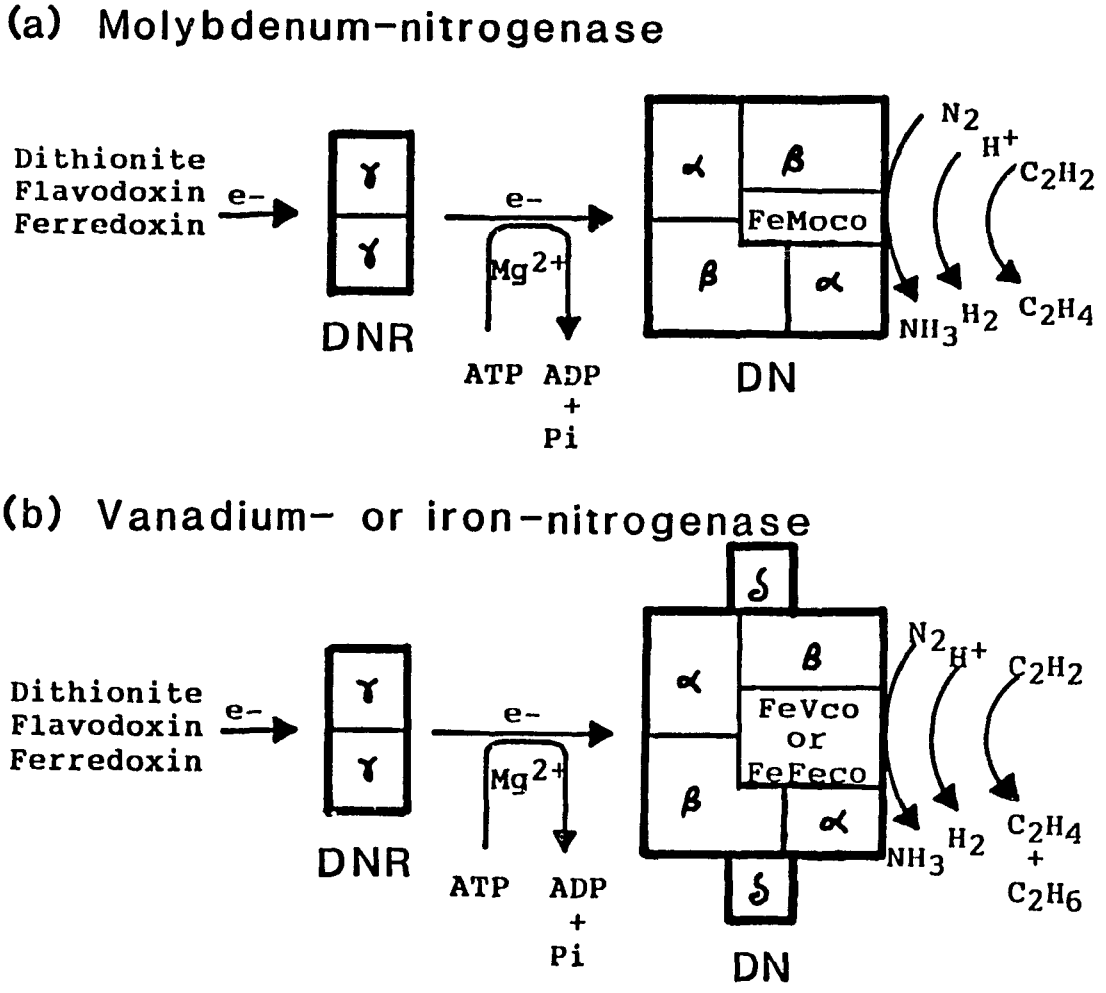


Fig.1. Components of the *Azotobacter* nitrogenases (slightly modified from Pau (1989)).DNR, dinitrogenase reductase; DN, dinitrogenase.

additional subunit in component 1 with molecular mass of 13.5 kDa and 15.3 kDa, respectively (Pau 1989). The function of this additional subunit is not known yet. All the nitrogenases can reduce acetylene to ethylene but the Mo-independent nitrogenases are relatively inefficient (Pau 1989; Bishop *et al.* 1988). V and Fe nitrogenases produce a small proportion of ethane (C_2H_6) and greater amounts of H_2 in the presence of C_2H_2 or N_2 (Bishop *et al.* 1988; Pau 1989), thus detection of C_2H_6 by gas chromatography (Dilworth *et al.* 1987) could become a routine procedure for measuring activity of Mo-independent nitrogenases.

2.2.4. *Requirements for the nitrogenase reaction*

The components of the nitrogenase reaction are quite similar for the three kinds of nitrogenases and for the Mo-nitrogenases from different N_2-fixing organisms. At least 6 different components are required for the nitrogenase reaction including 2 proteins, ATP, a divalent metal cation, a reductant and electron acceptor (Postgate 1982)(Fig. 1). The arbitrary sequence of steps in the nitrogenase reaction is as follows (Postgate 1982; Gallon and Chaplin 1987):

(a) Reduction of Component 2 (Fe protein). An artificial reductant of the Fe-protein is sodium dithionite while the natural reductants are flavodoxin and ferredoxin (Yates 1972; Yoch *et al.* 1969; Yoch 1974). Based on the studies of Lowe *et al* (1984), Gallon and Chaplin (1987) argued that the species that is actually reduced is the

complex of the oxidized form of the Fe-protein and MgADP (Fe-protein$_{ox}$ $(MgADP)_2$). Eight and twelve electrons are required for the Mo- and V-nitrogenase reactions, respectively (Gallon and Chaplin 1987).

(b) Activation of Component 2 by MgATP. It is not free ATP that activates the Fe-protein but $MgATP^{2-}$ (Thorneley and Willison 1974). $MgATP^{2-}$ forms a stable complex with Fe-protein (Thorneley and Eady 1973). There are two sites in the Fe-protein for $MgATP^{2-}$ binding. Sixteen ATP's are required for activation.

(c) Reduction of Component 1 (MoFe-, VFe-, and FeFe-protein) by Component 2. Component 1 is reduced by the MgATP-Fe protein complex based on Mössbauer spectroscopy data (Smith and Lang 1974). The transfer of electrons from Component 2 to Component 1 is rapid and irreversible and is preceded by hydrolysis of $MgATP^{2-}$. The electrons within Component 1 are then used for substrate reduction. The Component 2-Component 1 complex dissociates following electron transfer (Lowe *et al.* 1984).

(d) Reduction of substrate. Aside from N_2, nitrogenase catalyzes the reduction of C_2H_2, hydrogen ion (H^+), N_2O, cyanide, azide , alkyl cyanides, alkyl isocyanide, alkynes, allene, cyclopropene, diazirine (Gallon and Chaplin 1987). N_2 reduction which is intrinsically coupled to H_2 evolution takes place at the cofactor (FeMoco, FeVco, or FeFeco) site of Component 1. It is not clear whether N_2 binds to the metal of Component 1.

(e) Release of products. The products of nitrogenase reaction are NH_3, H_2, ADP, inorganic phosphate, and dissociated Component 1 and Component 2.

2.2.5. *Hydrogen evolution by nitrogenase*

As shown in the nitrogen reaction (Sect. 2.2), H_2 is obligatorily evolved as nitrogenase catalyzes the reduction of N_2 and protons (see also Section 2.3.4). Mo-independent nitrogenases evolved more H_2 (3 moles H_2 per mole of N_2 fixed as compared to 1 mole H_2 per mole of N_2 fixed in Mo-dependent nitrogenase) (Gallon and Chaplin 1987). The relationship between H_2 evolution and nitrogenase in aerobic N_2 fixers was first noticed by Hoch *et al.* (1957). Hydrogen evolution *in vitro* was demonstrated in nitrogenase preparations of *Azotobacter vinelandii* by Burns and Bulen (1965). Under an inert atmosphere of argon or helium, nitrogenases catalyzes solely the reduction of H^+ to H_2 (Simpson and Burris 1984). All substrates of nitrogenase are known to inhibit H_2 evolution to different extents. For example, very high pressures of N_2O or C_2H_2 block H_2 evolution completely, whereas very high pressures of N_2 block evolution of H_2 only partially (Simpson and Burris 1984). Under optimal conditions for N_2 fixation, 25% of the total electron flux and energy requirement of nitrogenase is allocated for formation of H_2 (Simpson 1987). *In vitro*, 100% electron flux to H_2 can be obtained even in the presence of N_2 by manipulating the reactants (Yates and Walker 1980). Yates (1988) stated that if the

reductant, ATP, or the reduced Fe protein are limited, the effective reductant (the reduced Fe protein : $2Mg^{2+}$ ATP complex) is limited resulting in more H_2 production. Stam *et al.* (1987) concluded from their calculations that the nitrogenase-catalyzed H_2 evolution has more influence on the efficiency of N_2 fixation than the presence or absence of a H_2-uptake system.

The mechanism of H_2 evolution is not yet understood. Chatt (1981) postulated that reducing equivalents supplied to the FeMo protein active site generate a trihydride of bound Mo, and that N_2 may displace two H atoms from the Mo trihydride to form a bound Mo-N_2 complex and one mole of H_2.

2.2.6. *Electron transport to nitrogenase*

Laane *et al* (1978) and Haaker *et al* (1974) claimed that the production of reducing equivalents for nitrogenase is the rate-limiting step in aerobic N_2-fixation. NAD(P)H_2 which is generated from the pentose phosphate cycle is generally accepted as the reductant for ferredoxin and flavodoxin (Gallon and Chaplin 1987). The reductants for nitrogenase are ferredoxin and flavodoxin (Yoch 1979). Proton motive force, particularly the membrane potential component, was reported to be involved in the provision of reductant in *Azotobacter* (Haaker *et al.* 1980).

2.2.7. *Regulation of nitrogenase*

Nitrogenase activity *in vivo* is regulated by O_2, NH_4^+, H_2, ATP availability, covalent modification, and

availability of reducing equivalents (Eady 1981). Synthesis of nitrogenase is regulated by O_2, NH_4^+, amino acids, nitrate or nitrite, molybdenum, cyclic nucleotides, nitrogen starvation, and carbamoyl phosphate. Of these factors, O_2 and NH_4^+ are the most studied. The following discussion will be limited to these two factors. Factors that regulate nitrogenase have been reviewed (Brill 1979; Robson and Postgate 1980; Eady 1981; Postgate 1982; Gallon and Chaplin 1987; Yates 1988).

2.2.7.1. *Oxygen*

Oxygen is responsible for reversible inhibition of nitrogenase activity *in vivo* and causes irreversible damage to nitrogenase. Not only both nitrogenase proteins but also electron carriers are sensitive to O_2 (Gallon and Chaplin 1987). Component 2 (Fe-protein) is more rapidly inactivated compared with Component 1. The mechanisms of O_2 inactivation of nitrogenase are not yet well understood. In most bacterial systems studied, the toxicity of O_2 is attributed to the production of superoxide radicals ($O_2^{\cdot -}$) (Youngman 1984). More reactive O_2 species such as perhydroxy and hydroxyl radicals can also be produced from superoxide and cause a lot of oxidative damage (Robson and Postgate 1980; Gallon and Chaplin 1987). Aerobic diazotrophs have several protection mechanisms against O_2 damage: respiratory and conformational protection, H_2-linked O_2 uptake, involvement of superoxide dismutase, catalase, and peroxidase, extracellular gum production, and

presence of carotenoids (Robson and Postgate 1980; Dingler and Oelze 1987; Gallon and Chaplin 1987; Pedrosa 1988; Yates 1988). The most studied mechanisms are respiratory and conformational protection in azotobacters. In respiratory protection, the organism can adjust its respiratory rate in response to increased O_2 concentration (Robson and Postgate 1980). At high O_2 tension, azotobacters can exhibit remarkably high rates of respiration. This is because electrons flow through an alternative branch of the respiratory chain which is not associated with the energy coupling site thus increasing the rate of electron flow to O_2 (Yates 1988).

Nitrogenase activity in azotobacters "switch off" and "switch on" in response to sudden increases and decreases in aeration (Robson and Postgate 1980). In the "switch off" state, nitrogenase is conformationally protected. A protein which forms an O_2-stable complex with nitrogenase proteins in the presence of Mg^{2+} ions was found in *Azotobacter chroococcum* and *Azotobacter vinelandii* (Haaker and Veeger 1977; Robson 1979). Veeger *et al* (1980) reported that both nitrogenase components in *Azotobacter vinelandii* could partially be protected against O_2 damage by associating separately with the protein but the two proteins differed in amounts of Mg^{2+} required. On the other hand, Kuhla and Oelze (1988) demonstrated that the "switch off" behavior depends entirely on the rate of supply of the energy and carbon source rather than on the rate of total oxygen

consumption by the cells. Conformational protection was also observed in *Xanthobacter flavus* (Yates 1977), *Derxia gummosa* (Hill 1971), and *Azospirillum brasilense* (Hartmann and Burris 1987).

Oxygen represses nitrogenase synthesis in a number of diazotrophs. The most understood system concerning O_2 regulation of nitrogenase synthesis at the molecular level is that of *Klebsiella pneumoniae*. Repression of nitrogenase synthesis by O_2 at the level of transcription and the *nifL* gene product is involved (Hill 1988). The current model of *nif* regulation in *Klebsiella pneumoniae* is shown in Fig. 2. At 60 uM, O_2 inhibits derepression of *nifLA*; at 0.1 uM, O_2 inhibits derepression of other *nif* operons via the *nifL* product; and at 6 uM, O_2 inhibits nitrogenase activity which, through N deprivation, indirectly prevents nitrogenase synthesis.

2.2.7.3. *Ammonium*

The short-term inhibition of nitrogenase activity by NH_4^+ (NH_4^+-switch off) was observed in *Azotobacter* species (Eady 1981), *Azospirillum* species (Hartmann *et al.* 1986), and *Herbaspirillum seropedicae* (Fu and Burris 1989) but not in *Klebsiella pneumoniae* (Tubb and Postgate 1973). The inhibition is reversible, the recovery time depending on the amount of NH_4^+ added. Different mechanisms of NH_4^+ inhibition seem to operate in different diazotrophs. In some N_2-fixing bacteria, the inhibition involves covalent modification of the Fe protein by ADP-ribosylation of a

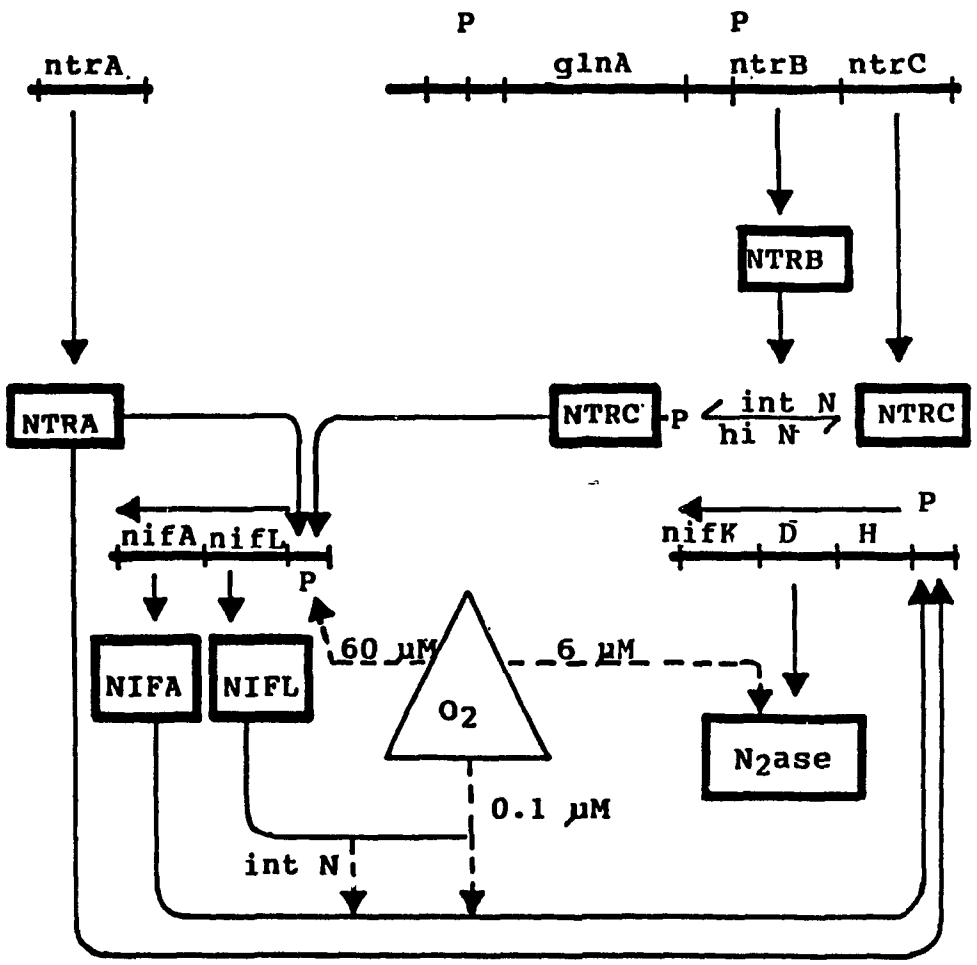


Fig. 2. The current model for *nif* regulation in *Klebsiella pneumoniae* (Hill 1988). The two-tier regulation by high (hi) and intermediate (int) levels of fixed N (N) are shown, together with 3 apparent interactions of O_2.

specific arginine residue (Pope *et al.* 1985; Hartmann *et al.* 1986). In *Azotobacter vinelandii*, the following factors affect inhibition of nitrogenase activity by NH_4^+ (Juglist and Haaker 1984): (a) dissolved oxygen concentration - when cells are incubated with low concentrations of oxygen, nitrogenase activity is low and ammonia inhibits strongly, but with more oxygen, nitrogenase activity is high and ammonia inhibits weakly, (b) pH - at a low pH, NH_4^+ inhibits more strongly than at higher pH and (c) respiration rate - when cells are grown with excess oxygen the respiration rate is high and inhibition is small, but with oxygen-limited conditions, respiration rate is low and inhibition is strong. Laane *et al.* (1980) showed that ammonium specifically switches off the flow of reducing equivalents to nitrogenase by lowering the membrane potential.

Nitrogenase synthesis is repressed by NH_4^+ in all free-living diazotrophs. Like O_2 regulation of nitrogenase synthesis, repression by NH_4^+ occurs at the transcriptional level in *Klebsiella pneumoniae* (Hill 1988; Cannon *et al.* 1985). It involves the *nifL* gene product which responds quickly to the presence of excess fixed nitrogen and apparently inactivates the *nifA* protein thus preventing further transcription of *nif* genes (Fig. 2). It also involves the gene products of nitrogen regulation (*ntr*) genes *A* and *C* which act on the promoter region of *nifLA* operon depending on the level of fixed N. The product of gene *ntrC* is subject to regulation by the product of *ntrB*.

The *ntrA* gene product functions as the σ-factor of the RNA polymerase required for initiation of transcription at promoters of *ntr* genes and *nif* genes (Cannon *et al.* 1985).

2.2.8. *Nitrogenase genes*

The *nif* genes of *Klebsiella pneumoniae* are the most studied among diazotrophs. Its *nif* regulon occurs as a single cluster of 21 genes extending over 23 kb next to the histidine operon in the chromosome (Cannon *et al* 1985; Merrick 1988; Dixon 1988). The *nif* genes and their products and function of the products are shown in Fig. 3 and Table 3. The structural genes are *nifHDK* and these genes are said to be highly conserved in diazotrophs that possess Mo-nitrogenase. Mutation in any of these genes renders the organism incapable of acetylene reduction (nitrogen fixation). Newly discovered genes are *nifC*, *T*, *Z*, and *W* and these together with *nifY*,*X*, *U*, and *S*, have as yet no known function. *nifV* was suggested to be involved in FeMoco synthesis but recently it was reported to encode for homocitrate synthase (Hoover *et al.* 1987). The relation of homocitrate synthase to FeMoco synthesis is not known.

Relatively fewer studies have been done on the genetics of nitrogenase in *Azospirillum* and *Azotobacter* compared with those on *Klebsiella*. However, the data available show some evidence that the *nif* gene organization in *Azotobacter* and *Azospirillum* differ from that of *Klebsiella* in some significant aspects (Cannon *et al.* 1985; Kennedy and Toukdarian 1987; Merrick 1988; Elmerich *et al.*

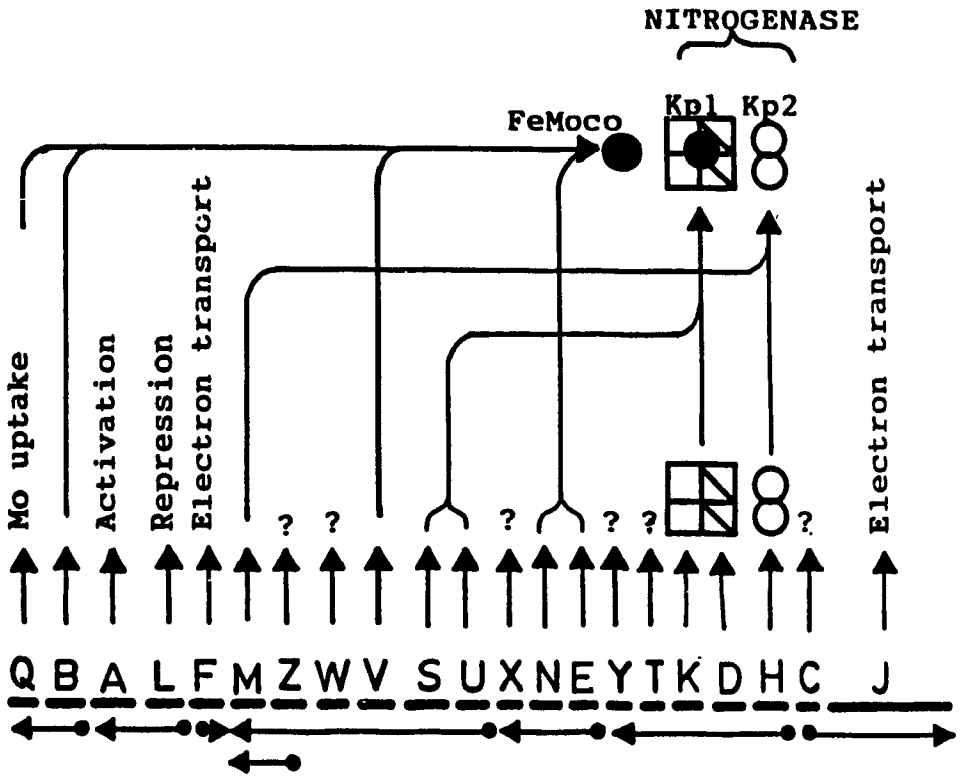


Fig. 3. Organization of *Klebsiella pneumoniae nif* genes. Vertical columns indicate gene functions and horizontal arrows indicate gene transcripts (Cannon *et al* 1985; Merrick 1988; Dixon 1988).

TABLE 3. Mo-nitrogenase genes and their products in *Klebsiella pneumoniae*[a]

nif gene	Molecular mass (kDa)	Function
Q	ND[b]	Mo uptake
B	49	FeMoco synthesis
A	57	Transcription activation
L	45	Transcription repression
F	19	Flavodoxin subunit
M	28	Fe-protein processing
Z	ND	Unknown
W	ND	Unknown
V	42	FeMoco synthesis
S	45	MoFe-protein processing
U	25	MoFe-protein processing
X	18	Unknown
N	50	FeMoco synthesis
E	40	FeMoco synthesis
T	ND	Unknown
Y	24	Unknown
K	60	MoFe-protein ß-subunit
D	56	MoFe-protein a-subunit
H	35	Fe-protein subunit
C	ND	Unknown
J	120	Pyruvate-flavodoxin oxidoreductase subunit

[a]After Cannon *et al* 1985; Gallon and Chaplin 1987; Merrick 1988; Dixon 1988.

[b]Not determined.

1988; Dixon 1988). For example (a) presence of *nifABC* genes in *Azospirillum* and *Azotobacter*, (b) occurrence of 5 open-reading-frames: 2 between *nifY* and *nifE*, 2 between *nifV* and *nifW*, and one upstream of *nifU* in *Azotobacter*, (c) presence of larger spacer regions between *nifX* and *nifU* and between *nifM* and *nifF* in *Azotobacter*, and (d) absence of *nifJ* in *Azotobacter*. Otherwise, the relative organization and regulation of the *nif* genes have considerable similarity in *Klebsiella* and *Azotobacter*.

2.3. Hydrogen oxidation by free-living aerobic heterotrophic diazotrophs

The ability to oxidize H_2 is a unique property of hydrogen (knallgas) bacteria and was first observed in 1905 (Schlegel 1976). The relationship between N_2 fixation and H_2 oxidation was first noted in 1941 by Phelps and Wilson in the nodules of *Pisum sativum* formed by *Rhizobium leguminosarum* strain 311. Hydrogenase activity of whole cells and cell-free extracts of *Azotobacter vinelandii* was observed to increase when the organism was fixing N_2 (Wilson *et al.* 1942; Lee *et al.* 1942; Lee and Wilson 1943; Green and Wilson 1953). Dixon (1967, 1968, 1972) showed that the H_2 produced during N_2 fixation by pea nodules was utilized by an H_2 oxidation system. H_2 evolution was observed in N_2-fixing *Azotobacter chroococcum* in the presence of CO and C_2H_2, inhibitors of uptake hydrogenase (Smith *et al.* 1976).

The above observations led to more intensive studies

on the H_2-recycling system of aerobic diazotrophs, both symbiotic and free-living. Many data are available on the different aspects of H_2 oxidation in non-diazotrophic hydrogen bacteria (e.g. *Alcaligenes eutrophus* and *Paracoccous denitrificans*) (Schlegel and Eberhardt 1972; Probst 1980; Bowien and Schlegel 1981; Adams *et al.* 1981; Vignais *et al.* 1981; Schlegel 1989; Friedrich 1989) and in diazotrophs (e.g. *Bradyrhizobium japonicum* and *Rhodobacter capsulatus*) (Adams *et al.* 1981; Eisbrenner and Evans 1983; Vignais *et al.* 1985; Maier 1986; Evans *et al.* 1987; Stam *et al.* 1987; O'Brian and Maier 1988 and 1989). This section will focus on the hydrogen-oxidation system of *Azotobacter* and other aerobic free-living diazotrophs and reference to other systems will be made when necessary. This subject was recently reviewed by Yates (1988) and Pedrosa (1988).

2.3.1. *Chemolithotrophic and heterotrophic hydrogen oxidation*

Chemolithotrophy is defined in Section 2.2.2. Bacteria that have the ability to oxidize H_2 with O_2 as the electron acceptor (NO_3^- may serve as an alternate electron acceptor for some) may be arbitrarily classified according to nutritional capabilities. Those that can utilize H_2 (a) only under autotrophic conditions are chemolithotrophic (or obligate) H_2 oxidizers; (b) heterotrophically and autotrophically are facultative chemolithotrophic H_2-oxidizers; and (c) only under heterotrophic conditions are heterotrophic H_2 oxidizers. The ability to oxidize H_2 and

grow chemolithotrophically is dependent on the ability to synthesize CO_2-fixing enzymes (Bowien and Schlegel 1981). So far there are only two reports of obligate chemolithotrophic non-diazotrophic H_2 oxidizers (Kawasumi *et al.* 1980; Nishihara *et al.* 1989). Most hydrogen bacteria are facultative chemolithotrophic H_2 oxidizers (Aragno and Schlegel 1981; Bowien and Schlegel 1981). *Pseudomonas saccharophila*, *Xanthobacter autotrophicus*, and *Derxia gummosa* are examples of N_2-fixing facultative chemolithotrophic H_2 oxidizers (Berndt and Wölfe 1978; Pedrosa *et al.* 1980; Ravi Shankar *et al.* 1986; Barraquio *et al.* 1986; Barraquio and Knowles 1989). *Azotobacter vinelandii*, *Azospirillum brasilense*, *Azomonas agilis*, *Bacillus polymyxa*, *Beijerinckia indica*, and *Klebsiella pneumoniae* are N_2-fixing heterotrophic H_2 oxidizers which do not have the ability to grow chemolithotrophically (Pedrosa *et al.* 1980; Bowien and Schlegel 1981; Malik and Schlegel 1981; Gowda and Watanabe 1983; Wong and Maier 1985). Some hydrogen bacteria have mixotrophic capabilities (Bowien and Schlegel 1981). *Azotobacter vinelandii* was reported to have mixotrophic capability in which H_2 serves as the energy source for uptake of mannose which is eventually utilized (Wong and Maier 1985; Maier and Prosser 1988).

2.3.2. *Benefits of hydrogen oxidation for diazotrophs*

Fig. 4 shows some of the apparent advantages of the presence of uptake hydrogenase in aerobic diazotrophs. Theoretically, nitrogen fixation efficiency will increase

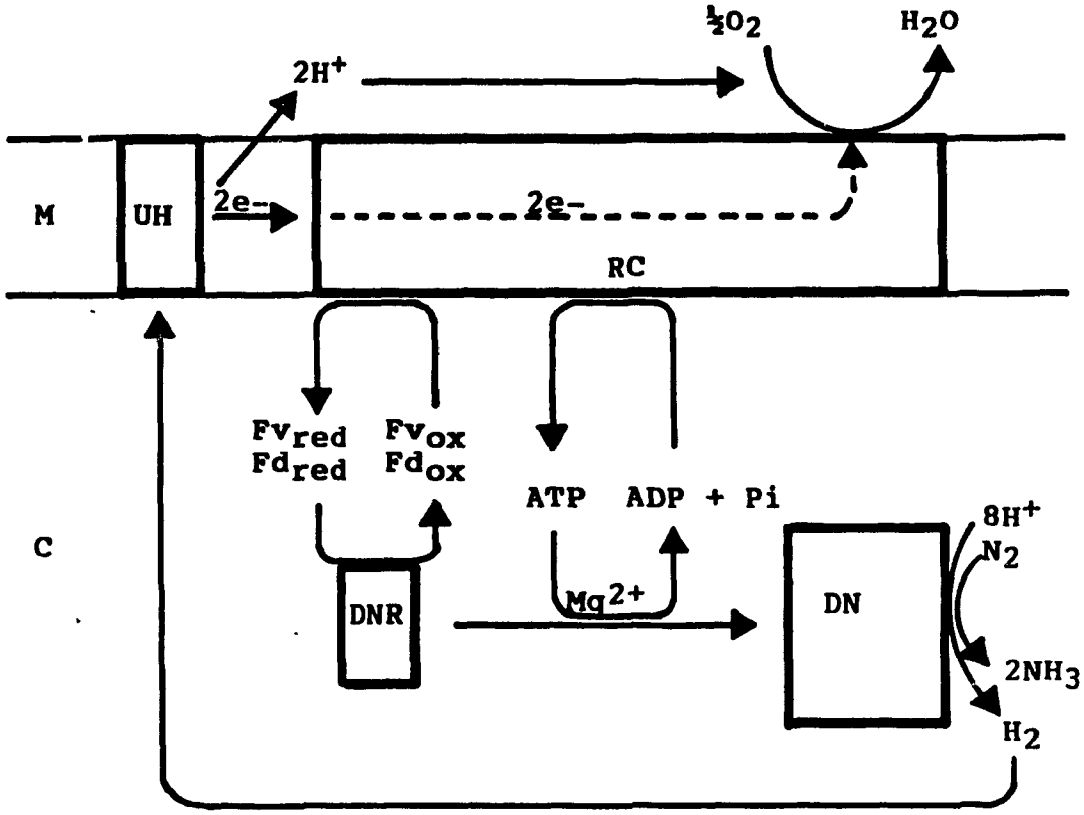


Fig. 4. Scheme for nitrogenase-hydrogenase relationship in aerobic diazotrophs (modified from Adams *et al.* 1981). DNR, dinitrogenase reductase; DN, dinitrogenase; UH, uptake hydrogenase; RC, respiratory chain; Fd, ferredoxin (oxidized or reduced); Fv, flavodoxin (oxidized or reduced); C, cytoplasm; M, membrane.

in the presence of an active H_2-recycling system. In *Azotobacter chroococcum*, H_2 oxidation is beneficial for growth under sucrose-limited N_2-fixing chemostat culture (Aguilar *et al.* 1985; Yates and Campbell 1989). The proposed benefits to diazotrophs of recycling H_2 are as follows (Dixon 1972; Robson and Postgate 1980):

(a) Protecting nitrogenase from O_2 inactivation

H_2 oxidation via hydrogenase is coupled to the electron transport chain with O_2 as the electron sink thus keeping O_2 away from the vicinity of nitrogenase. The experimental test for this is the shifting of the optimum nitrogenase activity from lower to higher O_2 tension (Robson and Postgate 1980). This phenomenon was observed in *A. chroococcum* under carbon-limited conditions (Walker and Yates 1978), *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bacteroids (Emerich *et al.* 1979; Ruiz-Argueso *et al.* 1979; Nelson and Salminen 1982), and *Rhodobacter capsulatus* (Meyer *et al.* 1978) but not in *Azospirillum brasilense* (Pedrosa *et al.* 1982). *In vivo* experimental data on legumes do not support this mechanism (Minchin *et al.* 1985).

(b) Generation of ATP and /or reducing power for nitrogenase reaction

The electrons generated by the uptake hydrogenase reaction pass through the electron transport chain and produce ATP (by oxidative phosphorylation) and reducing power (Adams *et al.* 1981; see Section 2.3.3). This ATP may

be utilized for the nitrogenase reaction. Walker and Yates (1978) reported utilization of H_2 as hydrogen donor but no H_2-supported ATP generation in *Azotobacter chroococcum*. However, Laane *et al.* (1979) speculated that H_2 recycling only recovers ATP and is less coupled to generation of reducing power in *Azotobacter vinelandii*. Both ATP and reducing power were provided by H_2 via hydrogenase in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bacteroids (Ruiz-Argüeso *et al.* 1979; Emerich *et al.* 1979; Nelson and Salminen 1982; Salminen and Nelson 1984). The ability of *Xanthobacter autotrophicus*, *Derxia gummosa*, *Pseudomonas* strain H8 (*P. diazotrophicus*), and *Pseudomonas saccharophila* to grow under N_2-fixing chemolithotrophic conditions suggests that H_2 via hydrogenase and the electron transport chain generates reducing power and ATP not only for N_2 fixation but also for CO_2 fixation (Berndt and Wölfe 1978; Ravi Shankar *et al.* 1986; Barraquio *et al.* 1986; Barraquio and Knowles 1989; see also Section 2.2.2).

(c) Protection of nitrogenase from H_2 inhibition

The nitrogenase reaction evolves H_2 which ironically, is a competitive inhibitor of N_2 reduction (Hwang *et al.* 1973). This proposed protective mechanism is considered by Mortenson (1978) as a prime function of hydrogenase. Berndt and Wölfe (1978) showed evidence of this protective mechanism of hydrogenase in *Xanthobacter autotrophicus* but only when H_2 was added in the headspace suggesting that the concentration of H_2 evolved by nitrogenase is not

inhibitory. In *Azotobacter*, Walker and Yates (1978) suggested that the intracellular H_2 concentration would not increase sufficiently to inhibit N_2 reduction. On the other hand, Dixon *et al.* (1981) concluded that without hydrogenase to recycle the evolved H_2, the H_2 concentration in pea and lupine nodules could inhibit nitrogenase activity.

2.3.3. *Hydrogen oxidation and the respiratory chain*

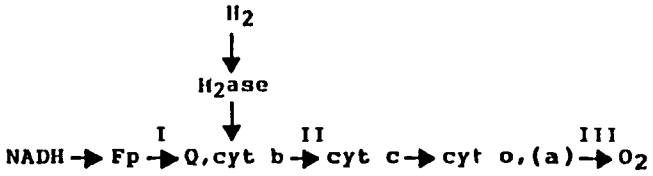
Many studies on the H_2-oxidation-related electron transport chain have been done on diazotrophs (e. g. *Bradyrhizobium japonicum* and *Rhodobacter capsulatus*) and hydrogen bacteria (e.g. *Alcaligenes eutrophus* and *Paracoccus denitrificans*) and several excellent reviews on the subject are available (Bowien and Schlegel 1981; Probst 1980; Vignais *et al.* 1981, 1985; Eisbrenner and Evans 1983; Maier 1986; Evans *et al.* 1987; O'Brian and Maier 1988, 1989). Relatively few studies, however, have been done on free-living aerobic heterotrophic diazotrophs (Donawa *et al.* 1971; Ishaque *et al.* 1971a & b, 1973; Laane *et al.* 1979; Wong and Maier 1984; Podzuweit and Arp 1987). It is generally accepted that all diazotrophs so far studied have a membrane-bound hydrogenase which couples H_2 to the electron transport chain. However, the question at what site in the respiratory chain the electrons from H_2 enter is not yet resolved. There are suggestions that the entry site is at the ubiquinone level in some H_2-oxidizing diazotrophs (Wong and Maier 1984; Vignais *et al.* 1985;

Maier 1986) and hydrogen bacteria (Probst 1980).

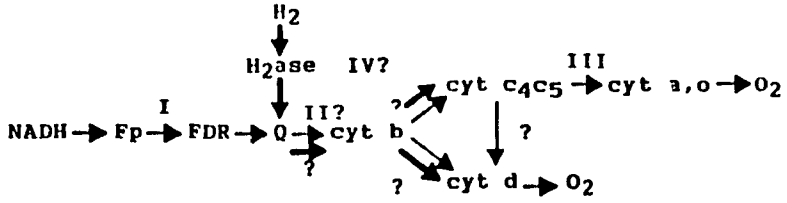
Fig. 5 shows the respiratory chain of *Pseudomonas saccharophila*, *Azotobacter vinelandii*, and *Bradyrhizobium japonicum*. In autotrophically grown cells of *P. saccharophila* the electrons from H_2 were reported to enter at cyt *b* bypassing NADH dehydrogenase (Donawa *et al.* 1971; Ishaque *et al.* 1971a & b, 1973; Aleem *et al.* 1979) (Fig 5a). Because Site I specific inhibitors (rotenone and atebrin) did not eliminate phosphorylation linked to H_2 but did eliminate the NADH associated ATP generation, it was concluded that only coupling Site II (between cyt *b* and cyt *c*) was involved in H_2 oxidation. It is surprising that Site III was found to be non-functional. Donawa *et al.* (1971) suspected however, the existence of a phosphorylation (proton translocation) site between H_2 and cyt *b* to account for the ATP formed. The absence of soluble NAD-dependent hydrogenase in *P. saccharophila* (Podzuweit *et al.* 1983; this thesis) suggests that the organism growing under chemolithotrophic conditions has to generate NADH by reverse electron flow (Gottschalk 1986).

In *A. vinelandii*, cytochromes *b*, *c*, and *d* with cyt *d* as the terminal oxidase were found to be involved in H_2 oxidation (Laane *et al.* 1979; Wong and Maier 1984) (Fig. 5b). Cytochrome *o* was also found but did not seem to be involved in H_2 oxidation. The pathway to cyt *d* is not proton translocating. The specific sequence shown in Fig. 5b is hypothetical. Laane *et al.* (1979) proposed a fourth

(a) Pseudomonas saccharophila



(b) Azotobacter vinelandii



(c) Bradyrhizobium japonicum

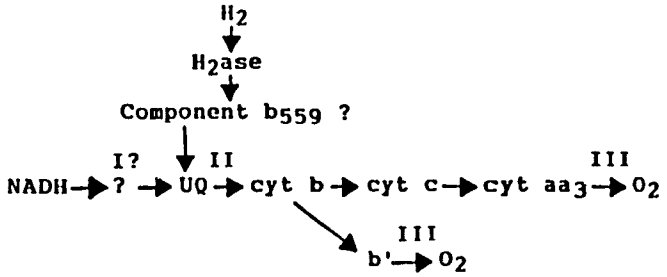


Fig. 5. Hydrogen-oxidation-coupled electron transport chain in (a) *Pseudomonas saccharophila* (Donawa *et al.* 1971; Ishaque *et al.* 1971; Ishaque *et al.* 1973); (b) *Azotobacter vinelandii* (Laane *et al.* 1979; Wong and Maier 1984); and (c) *Bradyrhizobium japonicum* (Eisbrenner and Evans 1982; Eisbrenner *et al.* 1982; O'Brian and Maier 1982, 1985a & b; Maier 1986). Roman numerals represent proton-translocating or energy coupling sites. Fp, flavoprotein; FDR, flavodoxin reductase; Q or UQ, quinone or ubiquinone; H_2ase, hydrogenase; question marks indicate hypothetical electron carriers or pathways.

coupling site at the level of hydrogenase as the site of entry of electrons from H_2. They suggested that hydrogenase in the proper orientation within the membrane can act as a proton pump. Wong and Maier (1984) suggested the involvement of ubiquinone as an electron carrier from H_2. Flavoproteins were found not to be involved in the *P. saccharophila* and *A. vinelandii* electron transport chains indicating that the flow of electrons in H_2 oxidation is not the same as that in NADH oxidation.

In *Bradyrhizobium japonicum* (Fig 5c), electrons from H_2 enter at the ubiquinone level (O'Brian and Maier 1985b). However, Eisbrenner and Evans (1982) and Eisbrenner *et al.* (1982) suggested the presence of component-559 similar to *b*-type cytochrome as the electron carrier from H_2 to ubiquinone. However, O'Brian and Maier (1985a & b; 1988) provided evidence against the existence of component-559.

It appears from Fig. 5 that at most 2 energy coupling sites are involved in H_2 oxidation as compared to NADH oxidation which is generally accepted to have 3 (Jones 1982). Stam *et al.* (1984) concluded from their chemostat studies in *Rhizobium* ORS 571 that H_2 oxidation is less energy yielding than the oxidation of endogenous substrates.

2.3.4. *Types, localization and properties of hydrogenase*

Hydrogenase, the class of enzymes that activate H_2, was discovered about 60 years ago by Stephenson and Stickland (1931). The types of hydrogenases according to

metal components are Fe, NiFe, and NiFeSe (Teixeira *et al.* 1987; Moura *et al.* 1988a; Fauque *et al.* 1988). The three types of hydrogenases according to the reaction they catalyze can be found in diazotrophs (aerobic and anaerobic) (Yates and Robson 1985):

(a) reversible hydrogenase

This enzyme catalyzes the reaction in the direction of H_2 evolution (reaction 1). The reaction is sensitive to inhibition by C_2H_2 (Smith *et al.* 1976). It is found in

$$2H^+ + 2e^- \longrightarrow H_2 \qquad (1)$$

facultative and obligate anaerobes, diazotrophic as well as non-diazotrophic. The physiological role of this hydrogenase in both groups of organisms is to dispose of excess reductant (Adams *et al.* 1981).

(b) unidirectional uptake type of hydrogenase

This enzyme catalyzes the reaction in the direction of proton and electron separation (reaction 2). It is

$$H_2 \longrightarrow 2H^+ + 2e^- \qquad (2)$$

sensitive to inhibition by CO, C_2H_2, NO_2^-, and NO (Smith *et al.* 1976; Tibelius and Knowles 1984; Hyman and Arp 1987). It is present in aerobic N_2-fixing bacteria as well as non-diazotrophic hydrogen bacteria. The physiological role of uptake hydrogenase in N_2-fixing bacteria is to recycle the

H_2 produced by the nitrogenase reaction. In hydrogen bacteria, there are two types of uptake hydrogenase according to its localization in the cell: membrane-bound (NAD-independent) and soluble (NAD-dependent) (Adams *et al.* 1981). It is generally accepted that all aerobic diazotrophs have membrane-bound hydrogenase. Wang and Watt (1984) reported uptake hydrogenase activity associated with the purified and crystallized MoFe protein (dinitrogenase) of *Azotobacter vinelandii*. Chen *et al.* (1986) reported *Anabaena* strains with nitrogenase-dependent H_2-uptake activity in the dark. Further studies are needed to verify these interesting reports.

(c) ATP-dependent H_2 evolution

Nitrogenase acts as a hydrogenase by catalyzing che the ATP-dependent H_2 evolution (see also Section 2.2.5) (reaction 3). This reaction is insensitive to inhibition by

$$ATP + 2(H^+ + e^-) \longrightarrow ADP + Pi + H_2 \qquad (3)$$

CO (Smith *et al.* 1976).

Hydrogenases from diazotrophs can be found in the membrane and/or periplasm, and/or cytoplasm (Bowien and Schlegel 1981; Moura *et al.* 1988b) and in some cases they can exist in multiple forms (Moura *et al.* 1988a). Except for the hydrogenases of *Rhodobacter capsulatus* and *Desulfovibrio desulfuricans* which have only one subunit (Colbeau *et al.* 1983; Moura *et al.* 1988b), all hydrogenases

have 2 subunits with molecular mass of approximately 60 and 30 kDa (Bowien and Schlegel 1981; Moura *et al.* 1988b). Many hydrogenases contain nickel, all have iron in the form of iron-sulfur centres, and some have selenium (Teixeira *et al.* 1987; Fauque *et al.* 1988; Moura *et al* 1988b; Boursier *et al.* 1988). Cammack *et al.* (1988) noted that nickel-containing hydrogenases are more tolerant to O_2 than those without nickel. It also appears from the review of Evans *et al.* (1987) that nickel content is correlated with stability to O_2. Antisera against hydrogenase from *Bradyrhizobium japonicum* cross-react with purified membrane-bound hydrogenase of (in decreasing order of cross-reactivity): *B. japonicum* - *Alcaligenes latus* - *Alcaligenes eutrophus* - *Azotobacter vinelandii* (Arp *et al.* 1985).

The membrane-bound hydrogenase of *A. vinelandii* has 0.68 mol Ni and 6.6 mol Fe per mole of enzyme, a specific activity of 124 umol H_2 oxidized (mg protein)$^{-1}$ min^{-1} with methylene blue as electron acceptor, a *Km* of 0.86 uM, an isoelectric point of 6.1, a pH optimum of 6 to 8.5 for activity, and a half-life of 20 min under O_2 (Kow and Burris 1984; Seefeldt and Arp 1986). Other aerobic heterotrophic diazotrophs such as *A. latus*, *Pseudomonas saccharophila*, *Azospirillum lipoferum*, *Azospirillum brasilense*, *Xanthobacter autotrophicus*, *Derxia gummosa*, and *Azotobacter chroococcum* have membrane-bound hydrogenases which might contain nickel based on their nickel requirement for chemolithotrophic growth and/or hydrogenase synthesis

(Tabillion *et al.* 1980; Bowien and Schlegel 1981; Partridge and Yates 1982; Pedrosa and Yates 1983; Podzuweit *et al.* 1983; Nakamura *et al.* 1985; Doyle and Arp 1987; Barraquio and Knowles 1989; see also Section 2.4.2). All these hydrogenases await purification and characterization.

2.3.5. *Regulation of hydrogenase*

Generally, synthesis of hydrogenase in aerobic diazotrophs (including free-living rhizobia) and hydrogen bacteria is regulated by O_2, H_2, carbon substrates, nitrogen sources, and nickel. DNA topology was suggested to regulate hydrogenase synthesis (O'Brian and Maier 1989) based on the repression of hydrogenase expression when DNA gyrase inhibitors were added to the derepression medium (Novak and Maier 1987). Nothing is known about the regulation of hydrogenase at the molecular level.

2.3.5.1. *Oxygen*

Oxygen represses hydrogenase synthesis in *Aquaspirillum autotrophicum*, *Bradyrhizobium japonicum*, *Azospirillum brasilense*, *Pseudomonas saccharophila*, and *Alcaligenes latus* (Aragno and Schlegel 1978; Maier *et al.* 1979; Pedrosa *et al.* 1982; Tibelius and Knowles 1983; Fu and Knowles 1986; Doyle and Arp 1987; Barraquio and Knowles 1988; Van Berkum 1987). Mutants of *B. japonicum* and *Alcaligenes eutrophus* which expressed hydrogenase activity at relatively high O_2 tension were isolated (Merberg *et al.* 1983; Cangelosi and Wheelis 1984) indicating that O_2 regulation of hydrogenase is genetically linked.

Hydrogenase activities of *Azospirillum brasilense* and *Azospirillum lipoferum* are sensitive to O_2 (Pedrosa *et al.* 1982; Tibelius and Knowles 1983; Fu and Knowles 1986, 1989) whereas those of *Aquaspirillum autotrophicum*, *Alcaligenes eutrophus*, *Azospirillum amazonense*, *Bradyrhizobium japonicum* and its bacteroids, and *Pseudomonas saccharophila* are not (Aragno and Schlegel 1978; Maier *et al.* 1979; Ruiz-Argüeso *et al.* 1979; Wilde and Schlegel 1982; Fu and Knowles 1986; Barraquio and Knowles 1988).

2.3.5.2. *Hydrogen*

H_2 induces synthesis of hydrogenase in *Xanthobacter autotrophicus* (Berndt and Wölfe 1978), *A. autotrophicum* (Aragno and Schlegel 1978), *B. japonicum* (Maier *et al.* 1979), *Paracoccus denitrificans* strain Stanier 381 (Nokhal and Schlegel 1980), *Azotobacter chroococcum* (Partridge *et al.* 1980), *A. eutrophus* H16 (Friedrich *et al.* 1981), *Rhodobacter capsulatus* (Colbeau and Vignais 1983), *Alcaligenes hydrogenophilus* (Friedrich *et al.* 1984), *Rhizobium* ORS 571 (De Vries *et al.* 1984) *Alcaligenes latus* (Doyle and Arp 1987), *P. saccharophila* (Barraquio and Knowles 1988), and *Azotobacter vinelandii* (Prosser *et al.* 1988). In *A. eutrophus*, however, H_2 appeared not to be a true inducer because hydrogenase activity in glycerol, a poor substrate for the organism, was higher than that under autotrophic conditions (Friedrich *et al.* 1981). Graham *et al.* (1984) concluded that H_2 either exogenously added or nitrogenase-produced is not needed for derepression of

hydrogenase in N_2-fixing cultures of *Bradyrhizobium japonicum*. Hydrogenases of *Azospirillum brasilense* (Tibelius and Knowles 1983) and most strains of *Paracoccus denitrificans* (Nokhal and Schlegel 1980) are not inducible.

2.3.5.3. *Organic substrates*

High concentrations of organic substrates repress synthesis of hydrogenase in *B. japonicum* (Maier *et al.* 1979), *Pseudomonas saccharophila* (Barraquio and Knowles 1988), *Azotobacter chroococcum* (Partridge *et al.* 1980), *Alcaligenes latus* (Doyle and Arp 1988), and in cowpea rhizobia (Thimmaiah *et al.* 1986). In *B. japonicum*, cyclic AMP reversed hydrogenase repression by malate suggesting a catabolite repression phenomenon (Lim and Shanmugam 1979; McGetrick *et al.* 1985). However, Graham *et al.* (1984) obtained high levels of both nitrogenase and hydrogenase activity in a carbon-containing medium. Van Berkum (1987) and Van Berkum and Maier (1988) found no repression of hydrogenase by carbon substrates in *B. japonicum*. They concluded that acidification and O_2 limitation were the factors responsible for repression. Merberg *et al.* (1983) suggested that a common element is involved in the regulation of hydrogenase by O_2 and carbon substrates. In *Alcaligenes eutrophus* and *A. chroococcum*, cAMP had no effect on hydrogenase expression (Friedrich 1982; Yates and Partridge 1984). In *A. eutrophus*, Friedrich (1982) concluded that the redox state of the cell rather than the organic substrate *per se* regulates hydrogenase synthesis.

This finding was in agreement with that of Yates and Partridge (1984) who observed a close correlation between the NAD/NADH ratio and hydrogenase activity in a continuous culture of sucrose-limited *Azotobacter chroococcum*.

2.3.5.4. *Nitrogen sources*

Higher hydrogenase activity from N_2-fixing cells and cell-free extracts than from ammonium-grown cells of *Azotobacter* was observed by Lee *et al.* (1942), Lee and Wilson (1943), and Green and Wilson (1953). It was later confirmed by Partridge *et al.* (1980) and Prosser *et al.* (1988) in *A. chroococcum* and *Azotobacter vinelandii*, respectively. In *Xanthobacter autotrophicus*, hydrogenase activity was higher with N_2 as nitrogen source than with NH_4Cl (Berndt and Wölfe 1978). Cowpea rhizobia hydrogenase was also shown to be repressed by fixed N sources (Thimmaiah *et al.* 1986). NH_4^+ and NO_3^- repressed synthesis of the inducible hydrogenase of *Methylosinus trichosporium* (Chen and Yoch 1987). However, nitrogenase and hydrogenase were independently synthesized in *Bradyrhizobium japonicum* (Maier *et al.* 1979; Lepo *et al.* 1980), *Rhodobacter capsulatus* (Colbeau *et al.* 1980), *Anabaena cylindrica* (Lambert and Smith 1981), *A. chroococcum* (Partridge *et al.* 1980), *Alcaligenes latus* (Doyle and Arp 1987), *Pseudomonas saccharophila* (Barraquio and Knowles 1988), and *A. vinelandii* (Prosser *et al.* 1988). In these organisms, hydrogenase was expressed in NH_4^+-containing medium in the presence of H_2 suggesting that NH_4^+ *per se* does not inhibit

hydrogenase activity. The H_2 evolved during N_2 fixation is most likely responsible for stimulating hydrogenase expression (Colbeau and Vignais 1983; Barraquio and Knowles 1988; Prosser *et al.* 1988). Berndt and Wolfe (1978) suggested that the induction of membrane-bound hydrogenase might be due to a higher degree of energization of the membrane which takes place under N_2-fixing conditions. *Azospirillum brasilense* and *Azotobacter chroococcum* could synthesize hydrogenase in NH_4^+-containing medium with no H_2 present (Tibelius and Knowles 1983; Partridge *et al.* 1980).

2.3.5.5. *Nickel*

Nickel was shown to be involved in the synthesis of hydrogenases in *Alcaligenes eutrophus*, *Xanthobacter autotrophicus*, *Rhodobacter capsulatus*,*Bradyrhizobium japonicum*, *Alcaligenes latus*, and *Pseudomonas saccharophila*, (Friedrich *et al.* 1981; Takakuwa and Wall 1981; Colbeau and Vignais 1983; Nakamura *et al.* 1985; Stults *et al.* 1986; Doyle and Arp 1988; Barraquio and Knowles 1989). Stults *et al.* (1986) claimed that nickel regulation of *B. japonicum* hydrogenase occurs at the transcriptional level. However, Doyle and Arp (1988) argued that nickel might be involved in the stabilization of newly formed apohydrogenase. Nickel is only required for activity in *Nocardia opaca* 1b (Schneider *et al.* 1984) and *Methylosinus trichosporium* OB3b (Chen and Yoch 1987). Nickel was suggested to bring the subunit dimers of *N. opaca* hydrogenase to association or to prevent dissociation (Schneider *et al.* 1984). The role of nickel in

hydrogenase is further discussed in Chapter 6.

2.3.6. *Hydrogen oxidation genes*

Information on the genetics of hydrogen oxidation in *Bradyrhizobium japonicum*, *Azotobacter chroococcum*, and in *Alcaligenes eutrophus* has been obtained only recently. Recent reviews (Maier 1986; Evans *et al.* 1987; Kennedy *et al.* 1987; O'Brian and Maier 1988, 1989; Yates 1988; Friedrich 1989) covered this subject. The following discussion will review the progress of the studies on the hydrogen uptake (*hup*) genes of *A. chroococcum* as well as *B. japonicum*, the latter being the model system and the source of a *hup*-specific DNA probe. *hup* and *hox* genes are synonymous, the former quite often used for diazotrophs and the latter for non-diazotrophic hydrogen bacteria.

The *hup* genes of *B. japonicum* and *A. chroococcum* are most probably in the chromosome (Cantrell *et al.* 1983; Robson *et al.* 1984) but of *Rhizobium leguminosarum* and *Alcaligenes eutrophus* H16 on a plasmid (Brewin *et al.* 1980; Friedrich *et al.* 1986). In *B. japonicum*, cosmids containing *hup* genes were isolated from gene banks of DNA of a Hup^+ strain (Cantrell *et al.* 1983). A cosmid pHU1 was then isolated and was shown to contain around 15 kb of hydrogenase-specific DNA organized into at least two transcriptional units (Haugland *et al.* 1984). Hom *et al.* (1985) isolated a recombinant cosmid that apparently contains a gene involved in both nitrogenase and hydrogenase activities and with a restriction pattern

similar to that of pHU1. A cosmid (pHU52) with restriction map similar to that of pHU1 but containing an extra 5.5-kb *Eco*R1 fragment was then isolated (Lambert *et al.* 1985a). Unlike pHU1, pHU52 was found to confer hydrogen uptake activity and ability to grow chemolithotrophically on Hup^- *Bradyrhizobium japonicum* mutants, free-living Hup^- field isolates of *B. japonicum*, and other rhizobia. These observations suggest that all the essential Hup determinants are present in pHU52 (Lambert *et al.* 1985a). Subsequently, pHU52 was found to confer on Hup^- strains the ability to synthesize the 60- and 30-kDa subunits of the uptake hydrogenase, indicating that the genes for hydrogenase synthesis are present in pHU52. Further evidence for the presence of hydrogenase structural genes in pHU52 was obtained when 2 subclones of pHU52, 5.9- and 2.9-kb fragments generated by plasmid expression vector pMZ545 in *Escherichia coli* "maxi cells", directed the synthesis of 60- and 30-kDa proteins (Zuber *et al.* 1986). These proteins cross-reacted with antibodies of the respective hydrogenase subunits. However, the cross reaction of the 30-kDa protein from *E. coli* with the 30-kDa hydrogenase was later found non-specific and the gene or genes encoding for the two subunits was found only in the 5.9-kb fragment in pHU52 (Sayavedra-Soto *et al.* 1988).

In *Azotobacter chroococcum*, Yates and Robson (1985) isolated Hup^- mutants which were classified phenotypically into 4 types: A - no H_2 uptake or H_2-evolving activity; B -

no H_2 uptake but with low level of H_2-evolving activity; C - low but significant levels of both activities; and D - high hydrogenase activities but the enzyme was in the cytoplasm. Plasmid pHU1 from *Bradyrhizobium japonicum* restored hydrogenase activity only to type A and C mutants (Yates and Robson 1985; Tibelius and Yates 1989). Tibelius *et al.* (1987) showed by DNA hybridization that the genomic DNA of *Azotobacter chroococcum* contained sequences homologous to *B. japonicum hup* genes carried on pHU1. They further isolated recombinant cosmids (pACD101 and pACD102) which hybridized to a restriction fragment from pHU1. A 15-kb region in these pACD cosmids was shown to correspond to the amount of *hup*-specific DNA in pHU1. Subcloned fragments (pKHT22, pKHT30, pKHT32, pKHT40) from pACD102 complemented mutant types A, B, and C but not D. Specific oligonucleotide probes were constructed based on the N-terminal amino acid sequences of the subunit proteins of *Azotobacter vinelandii* hydrogenase (Ford *et al.* 1988). A 3.6-kb *Sal*I fragment of the pKHT30 hybridized to both oligonucleotide probes and eventually the precise location of the region encoding for the small and large subunits was identified.

2.4. Nickel utilization by diazotrophs

Nickel was known only for its toxicity (Babich and Stozky 1983) until Bartha and Ordal (1965) demonstrated that nickel was required for chemolithotrophic growth of *Alcaligenes eutrophus*. Before 1965 however, nickel was observed to stimulate growth of certain plants (Ankel-Fuchs

and Thauer 1988). Subsequently, nickel was shown to be a component of at least four enzymes found in microorganisms and plants (Welch 1981; Hausinger 1987). Now nickel is well recognized as an essential element in prokaryotes and eukaryotes (Hausinger 1987; Mobley and Hausinger 1988; Ankel-Fuchs and Thauer 1988). In some N_2-fixing bacteria, nickel plays an important role not only in hydrogen (via hydrogenase) but also in nitrogen metabolism (via urease).

2.4.1. *Nickel and nitrogen fixation*

Nickel has probably no direct role in N_2 fixation. Its beneficial effects observed in some nitrogen-fixing systems are presumably in increasing the efficiency of N_2 fixation through the stimulation of the H_2-uptake system. The effects could possibly be significant upon addition of nickel to ecosystems that are nickel-deficient, carbon-limited, with high fluxes of H_2 and CO_2, and low O_2 tension. Bertrand (1974) and Bertrand and DeWolff (1973) reported the stimulatory effect of nickel on soil nitrogenase activity and on yield of soybean plants, respectively. J. J. Slater and D. G. Capone (unpublished, cited by Duxbury 1985) observed that, in general, 1000 ppm of Hg, Pb, Zn, Cr, Mo, and Cd all caused inhibition of C_2H_2 reduction activity in saltmarsh sediments, whereas Ni caused a stimulation at concentrations of 100 and 1000 ppm. In pure cultures, addition of nickel to heterotrophic semisolid cultures of *Azospirillum brasilense* Sp7 did not have any effect on diazotrophic growth (Pedrosa and Yates

1983). Pederson *et al.* (1986) found that the vigorous oxyhydrogen reaction in the presence of nickel did not confer any significant additional protection of nitrogenase either under aerobic conditions or at elevated O_2 level for *Anabaena cylindrica* and *Mastigocladus laminosus*. In *Anabaena inaequalis*, photosynthesis and acetylene reduction were completely inhibited by 170 and 340 uM Ni, respectively (Stratton and Corke 1979), both relatively high concentrations. The exponential growth rate of N_2-fixing *A. cylindrica* was found to be unaffected by omission of nickel from the growth medium (Daday *et al.* 1985). However, nickel was recently shown to facilitate the transition of *A. cylindrica* from a metabolic state in which nitrogenase activity is repressed to one of active nitrogen fixation (Daday *et al.* 1988) suggesting that the involvement of nickel is not via hydrogenase.

2.4.2. *Nickel for growth and enzyme synthesis*

Generally, nickel is required for growth by N_2-fixing organisms that have the ability to grow chemolithotrophically (Table 4). Examples are facultative chemolithotrophs and photoautotrophs. Nickel is also involved in the synthesis of hydrogenases, urease, carbon monoxide dehydrogenase and factor F_{430} (Table 4; see also Section 2.3.5.5.). It is not involved in hydrogenase synthesis but in activity in *Methylosinus trichosporium* OB3b (Chen and Yoch 1987) and *Nocardia opaca* 1b (Schneider *et al.* 1984).

TABLE 4. Diazotrophs that require nickel

Organism	Nickel for[a]	Reference[b]
Heterotrophs		
Alcaligenes latus	HS,HC(0.54)[c]	1
Azospirillum brasilense	HS	2
Azospirillum lipoferum	HS	2
Azotobacter chroococcum	HS	3
Azotobacter vinelandii	HC(0.7)	4
Bradyrhizobium japonicum	G,HS,HC(0.6)	5
Clostridium pasteurianum	CMDS,CMDC	6
Derxia gummosa	HS	2
Desulfovibrio desulfuricans	HC(1.0)	7
Desulfovibrio gigas	HS,HC(1.0)	7
Desulfovibrio salexigens	HC(1.0)	7
Pseudomonas saccharophila	G,HS,US?	8
Xanthobacter autotrophicus	G,HS,US?	9
Phototrophs		
Anabaena cylindrica	HS	10
Anabaena variabilis	G,HS	11
Chromatium vinosum	HS,HC(1.0)	12
Mastigocladus laminosus	HS	13
Oscillatoria sp.	G	14
Rhodobacter capsulatus	G,HS.HC(0.2),US?	15
Rhodospirillum rubrum	HC,CMDA	16
Thiocapsa roseopercina	HS,HC(1.0)	17
Methane-oxidizer		
Methylosinus trichosporium	HA	18
Archaeobacterium		
Methanosarcina barkeri	HC	19

[a]G, growth; HS, hydrogenase synthesis; HA, hydrogenase activity; HC, hydrogenase component; CMDS, carbon monoxide dehydrogenase synthesis; CMDC, carbon monoxide dehydrogenase component; CMDA, carbon monoxide dehydrogenase activity; US, urease synthesis; question marks indicate further clarification is required.

[b]1, Pinkwart *et al.* (1983); Doyle and Arp (1988); 2, Pedrosa and Yates (1983); 3. Partridge and Yates (1982); 4, Seefeldt and Arp (1986); 5, Klucas *et al.* (1983); Stults *et al.* (1984); Harker *et al.* (1984); Stults *et al.* (1986); 6, Diekert *et al.* (1979); Drake (1982); 7, LeGall *et al.* (1982); Rieder *et al.* (1984); Teixeira *et al.* (1986); Moura *et al.* (1988b); 8, Barraquio and Knowles (1989); this thesis; 9, Tabillion *et al.* (1980); Nakamura *et al.* (1985); 10, Daday and Smith (1983) Pederson *et al.* (1986); 11, Almon and Boger (1984); 12, Albracht *et al.* (1982); Van der Zwaan *et al.* (1985); 13, Pederson *et al.* (1986); 14, Van Baalen and O'Donnell (1978); 15, Takakuwa and Wall (1981); Colbeau and Vignais (1983); Takakuwa (1987); 16, Adams and Hall (1979); Bonam *et al.* 1988; 17, Gogotov (1986); 18, Chen and Yoch (1987); 19, Fauque *et al.* (1984).

[c]Numerals in parentheses indicate gram atom per mole of hydrogenase.

2.4.3. *Nickel as a component of hydrogenases and other enzymes*

Nickel is a component of most hydrogenases, urease, factor F_{430}, and carbon monoxide dehydrogenase (Table 4; see also Hausinger 1987; Ankel-Fuchs and Thauer 1988). Hydrogenases that do not contain nickel are those of *Clostridium pasteurianum* and *Desulfovibrio vulgaris* (Adams and Mortenson 1984; Huynh *et al.* 1984). Nickel content in hydrogenase ranges from about 0.5 to 1.0 units (Table 4).

Studies have been and are being done to elucidate the involvement of nickel in H_2 activation by hydrogenase using the enzyme of *Desulfovibrio gigas* as a model system (Moura *et al.* 1988a & b; Cammack *et al.* 1988; Fauque *et al.* 1988). Electron paramagnetic resonance (EPR) and Mossbauer spectroscopy have been very useful tools in understanding the metal environment in hydrogenase. Nickel exists in 4 oxidation states: Ni(III), Ni(II), Ni(I), and Ni(0) with the first 3 as the most interesting for biological systems (Fauque *et al.* 1988). EPR spectra have been recorded of *D. gigas* hydrogenase in various states of activation: (a) unready state - correlated with the presence of signal Ni-A and the enzyme is considered in inactive high oxidation state and incorrect conformation, (b) ready state - with signal Ni-B and the enzyme is in inactive higher oxidation state but correct conformation, and (c) active state - with signal Ni-C and the enzyme is in its reduced form and correct Ni-B conformation (Cammack *et al.* 1988). Signals

corresponding to Ni-A and Ni-B represent oxidation state Ni(III). Ni-C species is most likely reduced to Ni(II) by H_2 (Scott *et al.* 1984). A Ni(III) hydride complex was suspected to be an intermediate (Teixeira *et al.* 1985).

2.4.4. *Nickel uptake*

Uptake of nickel is the prerequisite for its utilization. The N_2-fixing organisms shown in Table 4 must in one way or another take up nickel from the environment. Relatively few studies have been done on nickel uptake by either diazotrophs or non-diazotrophs (see reviews by Kaltwasser and Frings 1980; Hausinger 1987; Drake 1988). Nickel transport by microorganisms may be classified according to dependence on energy (chemical or electrochemical) (Table 5). Energy-dependent nickel transport may further be classified according to the presence or absence of a Mg^{2+}-transport system to which nickel uptake is coupled. With regard to the energy-independent uptake system, only those of *Azotobacter chroococcum* (Partridge and Yates 1982), *Bradyrhizobium japonicum* (Stults *et al.* 1987), and *Methanothrix concilii* (Baudet *et al.* 1988) are known. The uptake system of *Pseudomonas saccharophila* may also be of this type (Chapter 5). Transport of nickel exhibits Michaelis-Menten kinetics. The kinetic parameters of the known nickel-uptake systems are shown in Table 6. The affinity of the uptake system towards nickel seems to be higher in prokaryotes than eukaryotes. The kinetic values differ greatly among

TABLE 5. Mechanisms of nickel uptake by microorganisms

Mechanism	Organism
Energy-dependent	
a. Mg^{2+}-transport system	*Saccharomyces cerevisiae*[1], *Escherichia coli*[2,3]
	Enterobacter aerogenes[3]
	Bacillus megaterium[3]
	Neurospora crassa[4]
	Rhodobacter capsulatus[5]
	Alcaligenes eutrophus[6]
	Clostridium pasteurianum[7]
b. Mg^{2+}-independent transport system	
i. Phosphate-stimulated	*Phaeodactylum tricornutum*[8]
ii. Citrate-complex	*Bacillus subtilis*[9]
iii. High affinity, highly specific	*Alcaligenes eutrophus*[6,10]
	Clostridium thermoaceticum[11]
	Methanobacterium bryantii[12]
	Acetogenium kivui[13]
iv. carrier-facilitated	*Anabaena cylindrica*[14]
Energy-independent	*Azotobacter chroococcum*[15]
	Bradyrhizobium japonicum[16]
	Methanothrix concilii[17]
	Pseudomonas saccharophila[18]

(1) Fuhrmann and Rothstein (1968); (2) Jasper and Silver (1977); (3) Webb (1970); (4) Mohan *et al.* (1984); (5) Takakuwa (1987); (6) Lohmeyer and Friedrich 1987; (7) Bryson and Drake (1988); (8) Skaar *et al.* 1974; (9) Willecke *et al.* (1973); (10) Tabillion and Kaltwasser (1977); (11) Lundie *et al.* (1988); (12) Jarell and Sprott (1982); (13) Yang *et al.* (1989); (14) Campbell and Smith (1986); (15) Partridge and Yates (1982); (16) Stults *et al.* (1987); (17) Baudet *et al.* (1988); (18) this thesis.

TABLE 6. Summary of kinetic parameters for nickel-uptake systems in microorganisms[a]

Organism	K_m (uM)	V_{max} (pmol Ni min^{-1} mg dry wt^{-1})
Saccharomyces cerevisiae	500	0.5
Neurospora crassa	290	15
Methanobacterium bryantii	3.1	24
Anabaena cylindrica	0.017	0.4
Bradyrhizobium japonicum	26-50	2723-13146[b]
Clostridium thermoaceticum	3.2	400
Alcaligenes eutrophus	17	2015[c]
Rhodobacter capsulatus	5.5	330
Methanothrix concilii	91	23000
Acetogenium kivui	2.3	670
Clostridium pasteurianum	85	1400
Pseudomonas saccharophila	31.7	2470

[a]References are those shown in Table 5.

[b]Assuming 10^8 cells per mL = 0.1065 mg dry wt.

[c]Assuming 1 mg protein = 1.5385 mg dry wt.

bacteria possibly indicating involvement of different mechanisms of uptake.

Not much is known about the genetics of the nickel-uptake system. Yates and Robson (1985) observed that Hup^- mutants of *Azotobacter chroococcum* absorbed $^{63}Ni^{2+}$ as readily as the parent strain. Stults *et al.* (1987) reported however, that a Hup^c (hydrogenase constitutive) mutant of *Bradyrhizobium japonicum* accumulated almost 10-fold more $^{63}Ni^{2+}$ than the wild type strain. Eberz *et al.* (1989) reported that nickel-deficient (Nic^-) mutants of *Alcaligenes eutrophus* carried defined deletions in the hydrogenase gene cluster of the pHG megaplasmid. They showed that nickel deficiency correlated with a low level of the nickel-containing hydrogenase activity, a slow rate of nickel transport, and reduced activity of urease. The Nic^+ phenotype was restored by a cloned DNA sequence (*hoxN*, a part of the hydrogenase gene cluster) of a megaplasmid pHG1 DNA library of *A. eutrophus*.

3. HETEROTROPHIC AND CHEMOLITHOTROPHIC NITROGEN FIXATION BY *PSEUDOMONAS SACCHAROPHILA*

3.1. Introduction

Despite their metabolic versatility (heterotrophic and autotrophic) and wide distribution in nature, the ability of members of the genus *Pseudomonas* to fix N_2 had long been doubted. This view no longer holds because of the recent discovery of authentic species of *Pseudomonas* capable of N_2 fixation (see Table 1, Section 2.1).

In the course of identifying the species of N_2-fixing *Pseudomonas*-like organisms from wetland rice roots (Barraquio and Watanabe 1981; Barraquio *et al.* 1983), the following known reference cultures were used and also tested for N_2-fixing (acetylene reduction) activity in semisolid glucose + yeast extract medium (Watanabe and Barraquio 1979): *Pseudomonas saccharophila* ATCC 15946, *Pseudomonas facilis* ATCC 17695, *Pseudomonas ruhlandii* (*Alcaligenes ruhlandii*) DSM 653, *Pseudomonas flava* DSM 619, *Pseudomonas pseudoflava* DSM 1034, and *Pseudomonas palleroni* ATCC 17724. Of these cultures, only *P. saccharophila* was found to reduce acetylene (Barraquio and Watanabe, unpublished).

This study was conducted to demonstrate the ability of *P. saccharophila* to fix N_2 heterotrophically and chemolithotrophically and examine some factors that affect its nitrogenase activity.

3.2 Materials and methods

3.2.1. *Organisms and cultivation conditions*

Pseudomonas saccharophila (ATCC 15946) used in the $^{15}N_2$ incorporation experiment (done at the Soil Microbiology Dept., International Rice Research Institute) was obtained from the American Type Culture Collection, Rockville, Md., USA. All other experiments with *P. saccharophila* were performed on a *P. saccharophila* strain carrying the same ATCC designation (15946) but which was a gift from Dr. Y. -K. Chan, Chemistry and Biology Research Institute, Agriculture Canada. *Pseudomonas diazotrophicus* strain H8 was a gift from Dr. I. Watanabe. Upon receipt of the cultures, single colony isolates were made and maintained on slants containing 0.1% Difco tryptic soy broth solidified with 1.5% (w/v) Difco Noble agar and on half-strength nutrient broth supplemented with 10% glycerol and kept at -80°C.

Unless otherwise stated, the inoculum was prepared in 10 mL sucrose + NH_4^+ + mineral salts + yeast extract medium (Section 3.2.2) contained in a 50-mL Erlenmeyer flask. The culture was grown for 20-24 h then transferred to 100 mL of the same medium contained in a 250-mL Erlenmeyer flask. The culture was incubated for another 10-12 h. Unless otherwise stated, all the incubations in this thesis were done at 30°C; shaking was at 250 rpm. The cells were harvested by centrifugation (10000 x g) at 4°C for 10 min, washed thrice with the test medium and then resuspended in 10-20 mL of

the same medium.

Open batch cultivation was carried out in a 1-litre flask containing 950 mL medium which was inoculated with 9.5 mL of the washed cells. The culture was continuously sparged at 450 and 200 mL min^{-1} for heterotrophic and chemolithotrophic cultivations, respectively, with a cotton-filtered gas mixture. The gas mixture for heterotrophic culture was 0.71 to 1 kPa O_2 in N_2 and for chemolithotrophic culture, 0.95 kPa O_2, 5.2 kPa H_2, 5.9 kPa CO_2, and balance N_2. Mixing of gases was done by using flowmeters. The culture was continuously stirred by a Teflon-coated magnetic bar. Culture samples were withdrawn from the flask through a Suba-seal-plugged bottom port using sterile 5- or 10-mL syringes.

In the closed batch cultivation, 125-mL side-arm flasks each containing 15 mL of medium were used. After inoculation, the flasks were plugged with rubber stoppers fitted with cut 5-mL plastic syringes containing a cotton filter. The syringes were stoppered with Suba-seals (W. R. Freeman and Co, Ltd., Barnsley, England). A headspace of 0.8 kPa O_2 and balance N_2 was used. The flasks were evacuated (15 min each time) and backfilled 3 times with N_2 and then evacuated one more time and backfilled with a gas mixture of 0.8 kPa O_2 and balance N_2. The flasks were incubated with shaking. Where desired, the flasks were directly used for acetylene reduction assay.

For semisolid cultures, 14-mL serum bottles each

containing 4 mL of semisolid medium were used. The cultures were incubated statically for 3 days. For C_2H_2 reduction assays, the foam plugs were replaced with butyl stoppers.

3.2.2. *Media*

The basal medium consisted of the following (g L^{-1}): KH_2PO_4, 4.54; $Na_2HPO_4.7H_2O$, 8.94; sequestrene NaFe (13% Fe) (Ciba Geigy Corp.), 0.062; $NaMoO_4.2H_2O$, 0.0059; $CaCl_2.2H_2O$, 0.20; $MgSO_4.7H_2O$, 0.20; and (mg L^{-1}), H_3BO_3, 0.15; $ZnSO_4.7H_2O$, 0.11; $CoSO_4.7H_2O$, 0.07; $CuSO_4.7H_2O$, 0.005; and $MnCl_2.4H_2O$, 0.004. The pH of the medium was 6.8. Yeast extract was supplied at 100 mg L^{-1}. Sucrose and NH_4Cl were both added at 1.0 g L^{-1}. For semisolid medium, Difco Noble agar (0.5 g L^{-1}) was added. The media and additions were prepared in glass-distilled water except in the $^{15}N_2$ and chemolithotrophic experiments for which deionized distilled water was used. The medium for the $^{15}N_2$ experiment was that of Watanabe and Barraquio (1979) except that the concentration of phosphates was 2.0 g L^{-1} KH_2PO_4 and 3.0 g L^{-1} K_2HPO_4 and trehalose (5.0 g L^{-1}) was used as the carbon source. The basal medium was later modified as follows (g L^{-1}): KH_2PO_4, 2.27; $Na_2HPO_4.7H_2O$, 4.47; and $CaCl_2.H_2O$, 0.02. This modified medium was used in the experiments done under Sections 3.3.3, 3.3.4, and 3.3.5 (only for Fig. 8), 3.3.6, and in the subsequent Chapters. For chemolithotrophic N_2-fixing culture, sucrose and NH_4Cl were not added but yeast extract was retained. Because of the presence of

yeast extract, the culture conditions are referred to as N-limited chemolithotrophic.

3.2.3. *$^{15}N_2$ incorporation*

Erlenmeyer flasks (50 mL) each containing 20 mL of trehalose + yeast extract semisolid medium were inoculated with 0.2 mL samples of a stationary phase culture (grown statically in the same medium at 30°C). The cotton plugs were then quickly replaced with rubber stoppers. After evacuation and flushing 5 times with Ar, a gas mixture of 30 kPa $^{15}N_2$ (99.89 atom %; Monsanto Research Corp., USA), 1 kPa O_2, and 69 kPa Ar was introduced. Another set of flasks was exposed to 30 kPa N_2/1 kPa O_2/69 kPa Ar. The flasks were incubated at 28 ± 1°C with reciprocal shaking (about 120 strokes per min) for 24 h.

The content of each flask was subjected to Kjeldahl digestion and total N was analyzed by steam distillation and potentiometric titration. The ^{15}N content in the samples was measured using an emission spectrometer (JASCO, N-150, Japan).

3.2.4. *Assays*

Nitrogenase (C_2H_2 reduction) assay of batch culture samples was done using 2-mL culture aliquots contained in 14-mL serum bottles. The serum bottles in this assay and in other assays were capped with silicone-reinforced butyl stoppers and then gas-tightened with aluminum caps. The assay conditions were as follows: for heterotrophic culture the headspace was 5 kPa C_2H_2, O_2 as indicated, and balance

He and the time was 1 h with shaking; for chemolithotrophic culture, the headspace was 1 or 5 kPa C_2H_2, 0.75 kPa O_2, 10 kPa H_2, and balance He and the time was 1 h unless otherwise stated. C_2H_2 reduction assay of the semisolid cultures was carried out by removing 5 kPa of the headspace (air) and replacing it with C_2H_2. The cultures were incubated statically for 24 h. Boiled cultures were included as controls. Gas samples (0.2 mL) from all the above assay vessels were taken by syringe. Ethylene (C_2H_4) formed was measured by FID gas chromatography as described by Tibelius and Knowles (1983).

Uptake hydrogenase assay was done by the O_2-dependent H^3H uptake method as described by Tibelius and Knowles (1983). Two-mL culture samples were transferred into 14-mL serum bottles and then H^3H (specific activity of 19.9 to 20.6 uCi mL^{-1} or 736 to 762 kBq mL^{-1}) was added to a final partial pressure of 3 kPa. Either O_2 (2 kPa or as indicated) or methylene blue (MB) at 5 mM were used as electron acceptors in this thesis (see Chapter 4). The MB (J. T. Baker Chemicals), dissolved in phosphate buffer (pH 6.8) was kept in serum bottles which were evacuated and backfilled thrice with N_2, and was added by N_2-flushed syringe into N_2-prefilled bottles containing the culture samples. This technique was later modified by having the sample bottles prefilled with MB and N_2 before the culture samples were injected. The assay bottles were incubated with shaking for 20-30 min (see Chapter 4 for the kinetics

of H_2 uptake). Boiled cells were used as control.

Ribulose bisphosphate carboxylase in whole cells permeabilized with toluene was determined by $^{14}CO_2$ incorporation into acid-stable products (Lepo *et al.* 1980; Tabita *et al.* 1978). The cells were prepared for assay by centrifuging and then resuspending in TEMB buffer pH 8.0 (McFadden *et al.* 1975). The cells were stored frozen until analysis. The cells were thawed, centrifuged and then resuspended in 100 mM Tris-HCl pH 8.0. The following reagents were mixed in 10 x 40 mm test tubes: 20 uL of 125 mM $MgCl_2.6H_2O$, 100 uL of 100 mM Tris-HCl pH 8.0, 80 uL of cell suspension, and 20 uL of 85.33 mM $NaH^{14}CO_3$ (specific activity, 2.0 mCi $mmol^{-1}$). The tubes were stoppered then 10 uL of toluene was injected. The contents were mixed thoroughly then the tubes were pre-incubated for 10 min at 30°C on a water bath shaker. Ribulose bisphosphate (20 uL of 12.5 mM) was injected. The reaction mixture was incubated for 5 min at 30°C then stopped by adding 150 uL of 4 N HCl. A 0.2-mL aliquot was evaporated to dryness in a scintillation vial, resuspended in 1 mL distilled water and then 10 mL of Beckman MP scintillation fluid was added. Two controls, one lacking RuBP and the other without cells, were used. Radioactivity was measured with a Beckman LS 7500 liquid scintillation counter.

3.2.5. *Analyses*

H_2 and O_2 were measured by gas chromatography as described by Chan *et al.* (1980). CO_2 was analyzed using the

same system as for O_2. Optical density was measured at 430 nm with a Bausch and Lomb Spectronic 20. Other spectrophotometric measurements were done using Beckman DU-7 spectrophotometer (Beckman Instruments Inc., California). Protein of washed cells was estimated by the Lowry method (Lowry *et al.* 1951) and later by the modified Lowry method (Markwell *et al.* 1978) using bovine serum albumin as the standard and sodium citrate in place of sodium tartrate (Eggstein and Kreutz 1955). Dissolved C_2H_4 was calculated using the appropriate Ostwald coefficients (Wilhelm *et al.* 1977).

3.2.6. *Chemicals and gases*

All the chemicals used were of reagent grade. All gases were of high purity and most of them were obtained from Linde (Union Carbide, Canada). Some O_2/N_2 mixtures were purchased from Liquid Carbonic Canada. $H^{-}H$ was from Gollob Analytical Service, Berkeley Heights, N. J. D-ribulose-1,5-bisphosphate (tetrasodium salt) was from Calbiochem. $NaH^{14}CO_3$ was obtained from ICN Biomedicals Canada.

3.3 **Results**

3.3.1. *$^{15}N_2$ incorporation*

The organism incorporated 3.74 to 8.87 atom % excess (6.29 $\pm$ 1.94 atom % excess, mean of 7 replicates $\pm$ SD) into cellular nitrogen. Excreted fixed nitrogen was not detected in the medium. The cells exposed to N_2 had a ^{15}N content of 0.503 $\pm$ 0.050 atom % (mean of 4 replicates $\pm$ SD).

3.3.2. *Effect of various carbon sources on growth and N_2 fixation*

The organism grew well on many sugars, organic acids and amino acids in the presence and absence of NH_4Cl (Table 7). Of the 30 carbon sources tested, 8 did not support any growth. Four amino acids supported good growth in the absence of NH_4Cl, suggesting that they served as sources of both carbon and nitrogen. In the absence of a carbon source, there was very little growth observed with and without yeast extract. With glucose, fructose, and cellobiose as carbon sources, there was slight growth with yeast extract but none with NH_4Cl. The carbon utilization pattern generally agreed with the data reported by Palleroni (1984).

The bacterium reduced C_2H_2 with many of the sugars and organic acids tested (Table 8). Sucrose and arabinose supported the highest rates. It is noteworthy that all the amino acids (L-alanine, L-aspartate, L-glutamate, and L-proline) that sustained growth in the absence of NH_4Cl did not support C_2H_2 reduction.

3.3.3. *Effect of yeast extract, casamino acids, and vitamins on growth and N_2 fixation*

Microaerophilic heterotrophic growth in a N-deficient medium was stimulated by addition of small amounts of yeast extract, casamino acids, and NH_4Cl but not by vitamins (Fig. 6). Nitrogenase activity was much higher in cultures supplemented with yeast extract and casamino acids than

TABLE 7. Growth of *Pseudomonas saccharophila* on various carbon sources[a]

Carbon source[b]	Optical density at 430 nm	
	+ NH_4Cl − yeast extract	− NH_4Cl + yeast extract
None	0.014 ± 0.00	0.007 ± 0.010
L-Arabinose	0.700 ± 0.046	0.433 ± 0.076
D-Xylose	0.058 ± 0.017	0.345 ± 0.020
D-Glucose	0[c]	0.312 ± 0.079
β-D-Fructose	0.004 ± 0.012	0.082 ± 0.009
D-Galactose	0.848 ± 0.027	0.501 ± 0.065
Sucrose	1.006 ± 0.098	0.557 ± 0.076
D(+) Trehalose	0.873 ± 0.08	0.617 ± 0.037
Maltose	0.697 ± 0.057	0.579 ± 0.050
β-D(+) Cellobiose	0.008 ± 0.005[c]	0.034 ± 0.021
Starch	0.749 ± 0.055	0.523 ± 0.032
Acetate	0.063 ± 0.008	0.264 ± 0.037
Succinate	0.539 ± 0.125	0.612 ± 0.030
Citrate	0.600 ± 0.072	0.544 ± 0.009
Fumarate	0.608 ± 0.110	0.557 ± 0.034
Pyruvate	0.419 ± 0.016	0.402 ± 0.021
L-Malate	0.607 ± 0.075	0.564 ± 0.013
D-Gluconate	1.006 ± 0.082	0.588 ± 0.012
D-Quimate	0.618 ± 0.059	0.454 ± 0.045
L-Alanine	0.157 ± 0.022	0.852 ± 0.032
L-Aspartate	0.117 ± 0.015	0.660 ± 0.026
L-Glutamate	0.677 ± 0.019	1.084 ± 0.019
L-Proline	0.046 ± 0.020	0.891 ± 0.010

[a]Growth of semisolid cultures after 3 days incubation is presented. The cultures were vortexed and then allowed to stand for about 3 min before OD determination. The organism did not grow with the following carbon sources: α-L-rhamnose, D-mannose, D(+)lactose, ethanol, mannitol, L(+) tartrate, DL-α-ε-diaminopimelate, and DL-phenylalanine. Data are averages of 3 replicates ± SD.

[b]All the carbon sources (1 g L^{-1}) were filter-sterilized (0.45 μm pore size, Millipore HA filter). The acids were supplied as their sodium salts.

[c]A small amount of growth was observed if a larger inoculum was used.

TABLE 8. C_2H_2 reduction by *Pseudomonas saccharophila* with various carbon sources[a]

Carbon source[b]	nmol C_2H_4 (mg protein)$^{-1}$ h^{-1}
L-Arabinose	82 ± 12
D-Xylose	11 ± 2
D-Glucose	8 ± 1
D-Galactose	10 ± 2
Sucrose	95 ± 4
D(+) Trehalose	13 ± 2
Maltose	20 ± 0.6
Starch	25 ± 3
Acetate	47 ± 2
Succinate	33 ± 2
Citrate	15 ± 2
Fumarate	17 ± 1
Pyruvate	6 ± 5
L-Malate	13 ± 0.6
D-Gluconate	50 ± 5
D-Quimate	12 ± 0.6

[a]Three-day old semisolid cultures were used in the assay. Specific activities were calculated between 6 and 8 h or between 7 and 9 h of incubation. The following carbon sources did not support C_2H_2 reduction: β-D-fructose, α-L-rhamnose, D-mannose, D(+) lactose, β-D(+) cellobiose, ethanol, D-mannitol, L(+) tartrate (Na), DL-α-ε-diaminopimelate (Na), L-alanine, L-aspartate (Na), L-glutamate (Na), L-proline, and DL-phenylalanine. Data are averages of 3 replicates ± SEM.

[b]All the carbon sources (1 g L^{-1}) were filter-sterilized (0.45 μm pore size, Millipore HA filter). The acids were supplied as their sodium salts.

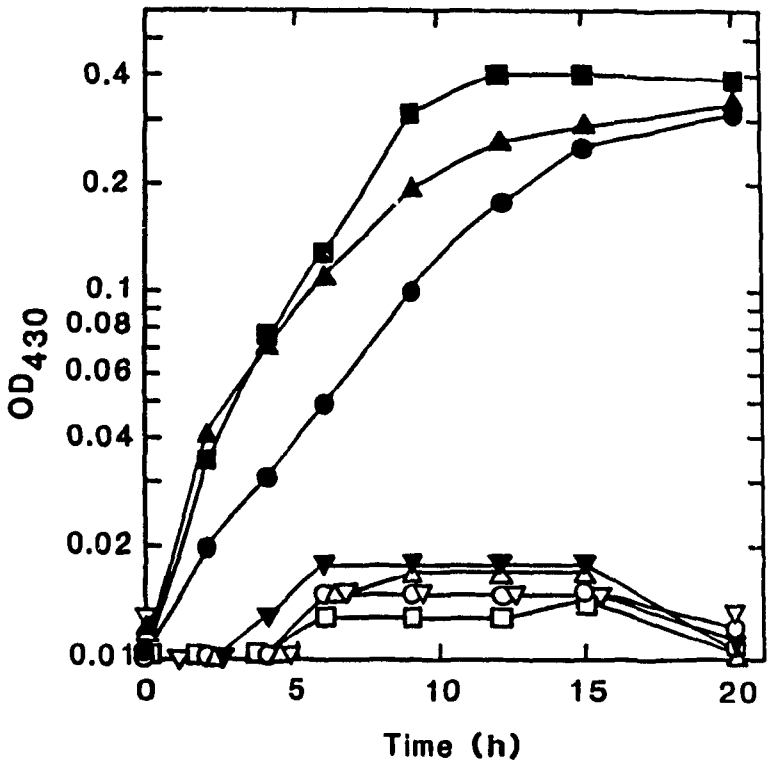


Fig. 6. Effect of addition of yeast extract, casamino acids, vitamins, and NH_4Cl on growth of *Pseudomonas saccharophila* under microaerobic condition (0.8 kPa O_2 in N_2). Cultivation was carried out in 125-mL side-arm flasks with 15 mL of medium. Difco yeast extract and NH_4Cl were supplied at 11 mg N L^{-1} and Difco casamino acids at 9 mg N L^{-1}. The vitamins were added at 100 ug L^{-1}. Except for NH_4Cl, all additions were filter-sterilized (0.45 um pore size, Millipore HA filter). The treatments were as follows: none (▽), NH_4Cl (■), yeast extract (▲), casamino acids (●), biotin (▼), vitamin B_{12} (△), pantothenic acid (○), and riboflavin (□). Data are averages of 2 replicates.

those non-supplemented or supplemented with vitamins and NH_4Cl (Table 9). The low nitrogenase activity with NH_4Cl may be due to ammonium-induced inhibition of nitrogenase.

3.3.4. *Effect of O_2 on nitrogenase activity*

Maximum nitrogenase activities were shown at O_2 headspace partial pressures of 0.25 to 0.5 kPa depending on the growth phase (Fig. 7). Nitrogenase activity was observed at all growth phases tested with the late exponential phase cells showing the highest nitrogenase activity.

To detect nitrogenase activity under strictly anaerobic conditions, N_2-fixing cells (5 mL) were transferred into pre-evacuated He-filled Pankhurst tubes. C_2H_2 (5 kPa) and alkaline pyrogallol (1 mL) were then injected into each respective port. No nitrogenase activity was detected.

3.3.5. *Chemolithotrophic N_2 fixation*

Pseudomonas saccharophila was tested first for the ability to fix N_2 chemolithotrophically in a closed batch culture (see footnote of Table 10 for experimental details). A reference culture, *Pseudomonas diazotrophicus* strain H8 (Gowda and Watanabe 1985; Watanabe *et al.* 1987) served as positive control. *P. saccharophila* grown autotrophically showed nitrogenase activity and an increase in protein content. Likewise, *P. diazotrophicus* strain H8, which was previously shown to grow chemolithotrophically and to reduce C_2H_2 (Gowda and Watanabe 1985) also showed growth and nitrogenase activity at the expense of H_2.

TABLE 9. Effect of yeast extract, casamino acids, NH_4Cl, and vitamins on C_2H_2 reduction by *Pseudomonas saccharophila*[a]

Addition	nmol C_2H_4 (mg protein)$^{-1}h^{-1}$
None	6.4 ± 1.2
Biotin	2.7 ± 0.6
Riboflavin	3.7 ± 0.6
Vitamin B_{12}	5.0 ± 1.4
Pantothenic acid	12.4 ± 5.0
NH_4Cl	1.8 ± 0.05
Casamino acids	696 ± 126
Yeast extract	1842 ± 138

[a]Twenty-hour liquid cultures were used for the assay. See Fig. 6 for experimental details. Data are averages of 2 replicates ± SEM.

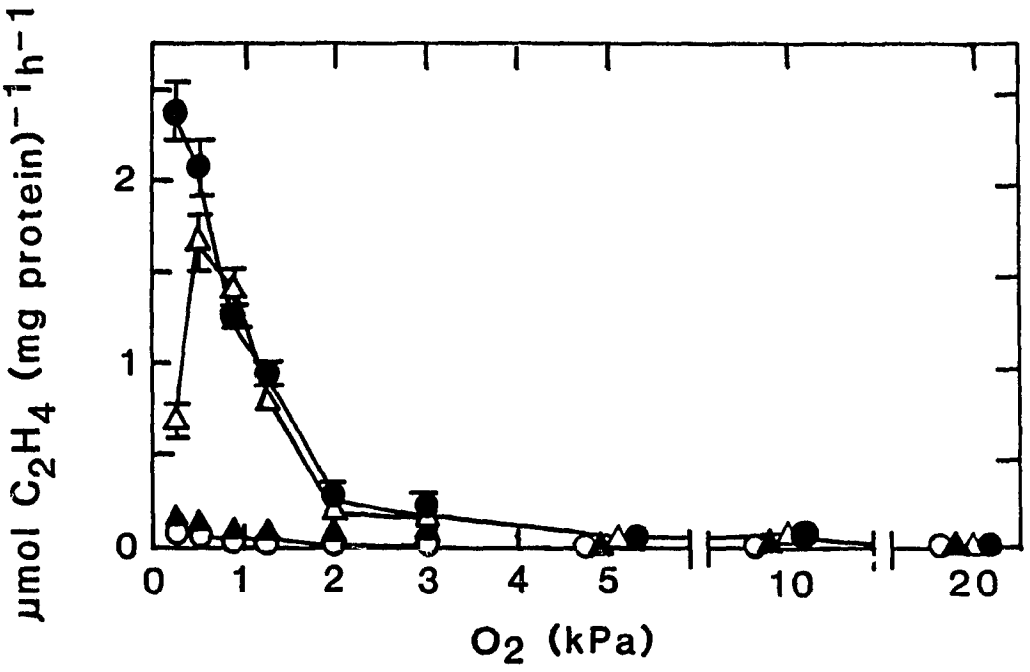


Fig. 7. Effect of O_2 on nitrogenase activity of heterotrophically-grown *Pseudomonas saccharophila*. Samples used for the assay were taken from a continuously-sparged batch culture. The different growth stages used were early exponential (○), mid-exponential (●), late-exponential (△), and early stationary (▲). Data are averages of 3 replicates. Bars indicate ± SEM.

TABLE 10. C_2H_2 reduction by two pseudomonads grown under N-limited chemolithotrophic conditions in a closed system[a]

Culture	nmol C_2H_4 (mg protein)$^{-1}$ h^{-1}	Total protein after assay (ug per flask)[b]
Pseudomonas saccharophila		
grown under		
$H_2/CO_2/O_2/N_2$	4 ± 0.5	133 ± 3
$Ar/CO_2/O_2$	0	33 ± 3
Pseudomonas diazotrophicus		
grown under		
$H_2/CO_2/O_2/N_2$	25 ± 1	147 ± 3
$Ar/CO_2/O_2$	3 ± 0.2	70 ± 5

[a]The organisms were grown in 125-mL flasks each containing 10 mL of N-limited autotrophic medium (with 50 mg L^{-1} yeast extract). The flasks were plugged as described in the Materials and methods (Section 3.2.1). One set of flasks received 4.6 kPa CO_2, 0.73 kPa O_2, 10 kPa H_2, and balance N_2; the other set had 5.1 kPa CO_2, 0.81 kPa O_2, and balance Ar. The flasks were shaken for 7 days after which they were assayed for C_2H_2 reduction activity using the gas mixture C_2H_2 (0.20 kPa); O_2 (0.35 kPa); H_2 (5.0 kPa); Ar (94.45 kPa). Incubation period was 12 h. Uninoculated medium served as negative control. Data are averages of 3 replicates ± SEM.

[b]The initial protein content for *P. saccharophila* was 5 ug and for *P. diazotrophicus*, 10 ug per flask.

The ability of *Pseudomonas saccharophila* to grow and fix N_2 chemolithotrophically was further examined in a continuously-sparged batch culture. Samples taken from this culture were analyzed for OD_{430}, nitrogenase, hydrogenase, and ribulose bisphosphate carboxylase (Fig. 8). A faster rate of growth was observed in the first few hours (6 h) of incubation, possibly due to yeast extract utilization, followed by a slower rate until about 80 h (Fig. 8a). Hydrogenase and ribulose bisphosphate carboxylase seemed to be derepressed at the same time at the early stages of growth (Fig. 8b). Detectable nitrogenase activity was observed only after about 40 h of incubation; activity peaked at about 140 h and then declined almost abruptly thereafter.

3.3.6. *Effect of hydrogen on acetylene reduction activity*

This experiment was conducted to determine the optimum partial pressure of H_2 for C_2H_2 reduction activity under N-limited chemolithotrophic conditions. Maximum activity was obtained at 2 kPa H_2 and above (Table 11).

3.4. **Discussion**

The incorporation of ^{15}N into cellular nitrogen undoubtedly confirms the N_2-fixing ability of *Pseudomonas saccharophila*. Nitrogenase activity by this organism has also been observed by Y. -K. Chan, Agriculture Canada (unpublished). Various sugars and organic acids supported its growth and N_2 fixation. Nitrogenase activity was expressed only under low oxygen partial pressure indicating

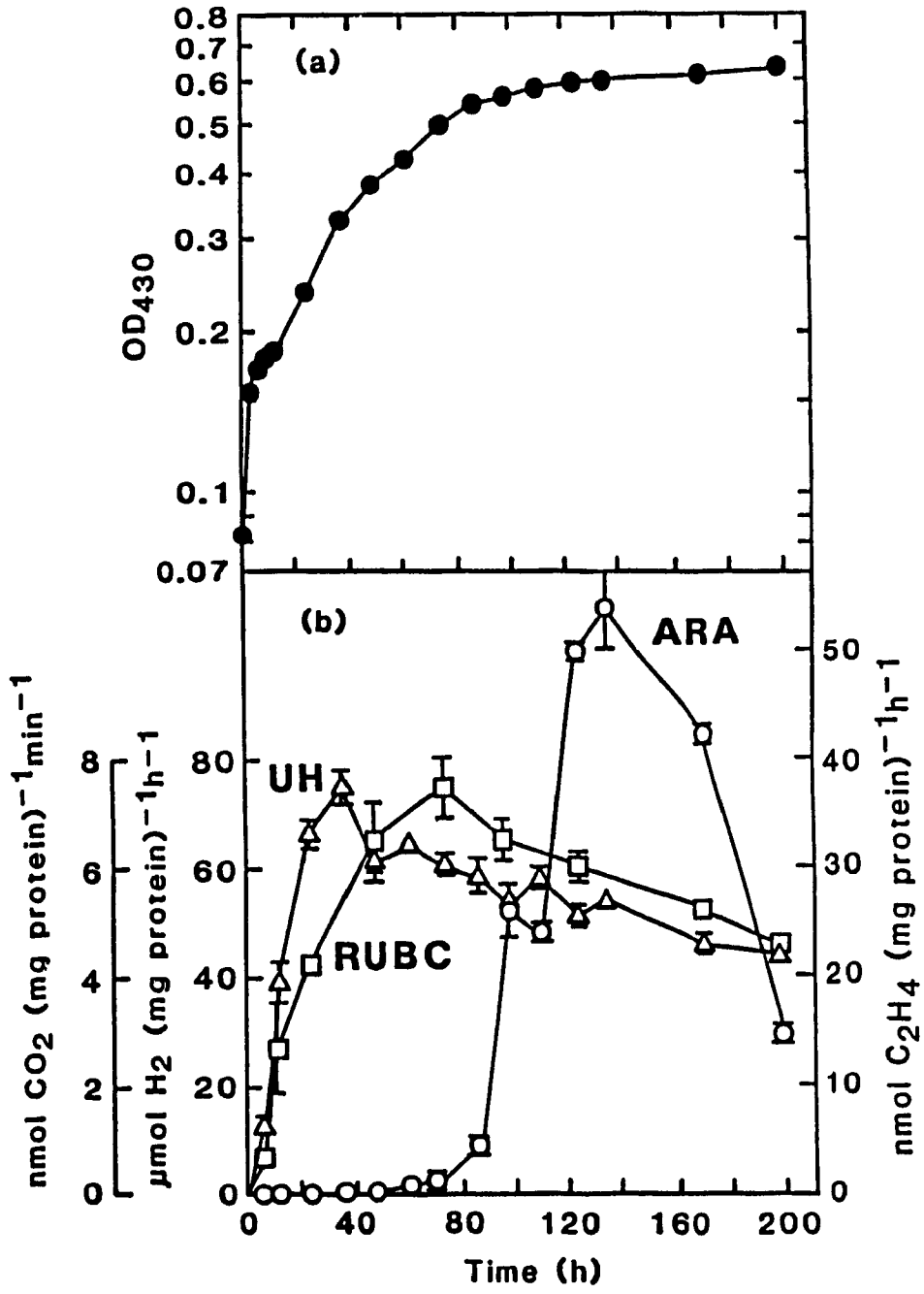


Fig. 8. (a) Typical growth curve of *Pseudomonas saccharophila* under N-limited chemolithotrophic conditions; (b) Uptake hydrogenase (UH), ribulose bisphosphate carboxylase (RUBC), and acetylene reduction (ARA) activities at various stages of growth. Methylene blue was used as the electron acceptor in the hydrogenase assay. Data for the 3 enzymes are averages of 3 replicates. Bars indicate ± SEM.

TABLE 11. Effect of H_2 headspace partial pressure on C_2H_2 reduction activity of chemolithotrophically-grown *Pseudomonas saccharophila*[a]

H_2 (kPa)	nmol C_2H_4 (mg protein)$^{-1}h^{-1}$[b]
0	7 ± 1
1	15 ± 0.2
2	17 ± 0.5
5	17 ± 0.4
10	19 ± 1.6
20	18 ± 0.4
40	18 ± 0.1

[a]Samples used for the assay were taken from a continuously-sparged batch culture (88-h) as shown in Fig. 8.

[b]Data are averages of 3 replicates ± SEM.

that the organism is a microaerophilic N_2-fixer.

Although the amino acids alone sustained better growth of *Pseudomonas saccharophila*, their failure to support nitrogenase activity is not surprising. Some amino acids have been known to regulate activity and synthesis of nitrogenase in *Klebsiella pneumoniae*, *Azotobacter vinelandii* and *Rhizobium* species (Wilson *et al.* 1943; Shanmugam & Morandi 1976; Ranga Rao 1982). The catabolism of amino acid yields ammonia which rapidly and reversibly "switches-off" nitrogenase activity (Postgate 1982) or may increase the internal NH_4^+ pool of the cell thus repressing the synthesis of nitrogenase (Brill, 1979).

Yeast extract and casamino acids added in small amounts stimulated growth of *P. saccharophila* under heterotrophic N_2-fixing conditions. The vitamins did not stimulate growth. These results suggest that it is the amino acid component of yeast extract that is responsible for growth and N_2 fixation.

The ability of *P. saccharophila* to grow and fix N_2 under N-limited chemolithotrophic conditions was demonstrated unequivocally by growth and the presence of 3 key enzymes: hydrogenase, ribulose bisphosphate carboxylase, and nitrogenase. H_2, therefore, through hydrogenase provides reducing power and ATP for both CO_2 fixation and N_2 fixation (see also Chapters 4 and 5). Organisms that are so far known to fix N_2 chemolithotrophically are *Xanthobacter autotrophicus*,

Azospirillum lipoferum, *Dervia gummosa*, *Alcaligenes latus*, and *Pseudomonas diazotrophicus* (Wiegel and Schlegel 1976; Berndt and Wölfle 1978; Pedrosa *et al.* 1980; Malik and Schlegel 1981; Bowien and Schlegel 1981; Pinkwart *et al.* 1983; Gowda and Watanabe 1983; Ravi Shankar *et al.* 1986; Barraquio *et al.* 1986; Watanabe *et al.* 1987). Since the taxonomic status of *P. diazotrophicus* is uncertain (see Table 1, Chapter 2), this makes *Pseudomonas saccharophila* the first approved species of *Pseudomonas* to fix N_2 both heterotrophically and chemolithotrophically.

4. HETEROTROPHIC AND CHEMOLITHOTROPHIC HYDROGEN OXIDATION BY *PSEUDOMONAS SACCHAROPHILA*

4.1. Introduction

Nitrogenase catalyzes not only N_2 reduction but also ATP-dependent H_2 evolution. The oxidation of evolved H_2 by a N_2-fixing culture may recover some of the energy lost through H_2 production, may prevent inhibition of nitrogenase by hydrogen, and may provide protection to nitrogenase by removal of excess oxygen (Dixon 1972). Such H_2 recycling occurs in *Azotobacter chroococcum* (Walker and Yates 1978), *Rhizobium japonicum* (Emerich *et al.* 1979), and *Rhizobium leguminosarum* bacteroids (Salminen and Nelson 1984). Uptake hydrogenase, the enzyme responsible for the unidirectional conversion of molecular H_2 to protons, is found in several other diazotrophic genera (e.g. *Azospirillum*, *Alcaligenes*, *Derxia*, *Xanthobacter*) (Bowien & Schlegel, 1981). The hydrogenase system of these organisms, however, is regulated differently by O_2, H_2, and carbon and nitrogen sources.

Since *Pseudomonas saccharophila* can utilize H_2 as electron donor during chemolithotrophic N_2-fixation (see Chapter 3), it may have the ability to utilize the evolved H_2 during heterotrophic N_2-fixation. This study was conducted to demonstrate the presence of an active uptake hydrogenase in *P. saccharophila* during heterotrophic N_2-fixation, and to examine the regulation of its activity and synthesis by H_2, sucrose and O_2.

4.2. Materials and methods

4.2.1. *Organisms, media and cultivation conditions*

The bacterial strain used in this study, *Pseudomonas saccharophila* ATCC 15946, was the same culture as used in Chapter 3. *Alcaligenes eutrophus* H16 was a gift from Dr. B. Friedrich. The media (NH_4^+-containing or N-limited ± sucrose), inoculum preparation, method of cultivation (continuously-sparged batch culture or closed batch culture), and incubation conditions were all the same as those in Chapter 3. Further details of the experiments are given in the Table or Figure legends.

4.2.2. *Intracellular distribution of hydrogenase activity*

Cells were grown for 12 h under N-limited chemolithotrophic conditions with 10 uM $NiCl_2$. Cells were harvested and washed twice with 67 mM potassium phosphate (K_2HPO_4 + KH_2PO_4) buffer (pH 7.0) at 10000 x *g* for 10 min at 4°C. Cells were resuspended in 10 mL of the same buffer and then 100 uL of 100 mM phenylmethylsulfonyl-fluoride (PMSF) was added. Cells at the start of the sonication and fractions were handled aerobically or anaerobically (under O_2-free H_2 during sonication and then subsequently under O_2-free N_2). O_2-free gases were obtained by passing them through a BASF BTS catalyst-filled column. Cells were sonicated (Cell Disruptor model W-220F, Heat Systems-Ultrasonics, Inc., Plainview, New York) 20 times with 1 min burst and 1 min cooling in a circulating ice bath (approximately 4°C). The sonicator was equipped with a cup horn and was operated at

a power output of about 95 watts. Cells were centrifuged at 10000 x *g* at 4°C for 10 min. The supernatant was the cell-free extract. The cell-free extract was centrifuged at 180000 x *g* for 1 h at 5°C. The supernatant was the soluble fraction and the pellet was the membrane fraction. Aliquots (0.9 mL) of the fractions were assayed for H^3H-uptake activity.

NAD-dependent hydrogenase (soluble hydrogenase) activity was also determined in cells grown under N-limited chemolithotrophic conditions using the method of Friedrich *et al.* (1981). *Alcaligenes eutrophus* H16 grown under chemolithotrophic conditions (with NH_4Cl) was used as the positive reference organism.

4.2.3. *Assays*

Hydrogenase activity was measured by the H^3H-uptake method (Tibelius and Knowles 1983) using O_2 or MB as electron acceptors (see Chapter 3). Nitrogenase activity was measured by the C_2H_2 reduction method (see Chapter 3).

4.2.4. *Analyses*

Analyses of H_2, O_2, C_2H_4, growth (OD_{430}), and protein were all done as in Chapter 3. Reducing sugar was determined by the phenol method (Herbert *et al.* 1971) using sucrose as the standard. All spectrophotometric determinations were done using the Beckman DU-7 spectrophotometer. Dissolved H_2 and C_2H_4 were calculated using the appropriate Ostwald coefficients (Wilhelm *et al.* 1977).

4.3. Results

4.3.1. *Kinetics of hydrogen uptake*

The apparent K_m for H_2 of hydrogenase was determined in whole cells of a stationary phase N_2-fixing heterotrophic culture. From an Eadie-Hofstee plot, the apparent K_m for H_2 in the O_2- and MB-dependent assays was 4.3 uM and 1.82 uM in solution, respectively. In the gas phase, these values correspond to 0.56 kPa and 0.24 kPa for O_2- and MB-dependent activities, respectively. These values are lower than that for *Azospirillum brasilense* which was 1.2 kPa to 3 kPa (Pedrosa *et al.* 1982; Tibelius and Knowles 1983). The value for MB-dependent activity is similar to that for *Rhizobium japonicum* which was 0.23 kPa (Lim and Shanmugan 1979). The 3 kPa H^3H used in this study was sufficient to saturate the O_2- and MB-dependent components of the hydrogenase system and in both cases the uptake of H^3H was linear with time up to about 90 min (Fig. 9).

4.3.2. *Growth and H_2 oxidation under N_2-fixing and non-N_2-fixing conditions*

The growth rates in both N_2-fixing and NH_4^+-grown microaerobic cultures were practically the same in the first 6 h (Fig. 10a) no doubt due to the initial utilization of the yeast extract. Thereafter, the N_2-fixing culture grew relatively slowly, but during this period a marked increase in hydrogenase activity was observed (Fig. 10b). Hydrogenase activity was very much higher in the N_2-fixing culture than in the NH_4^+-grown culture.

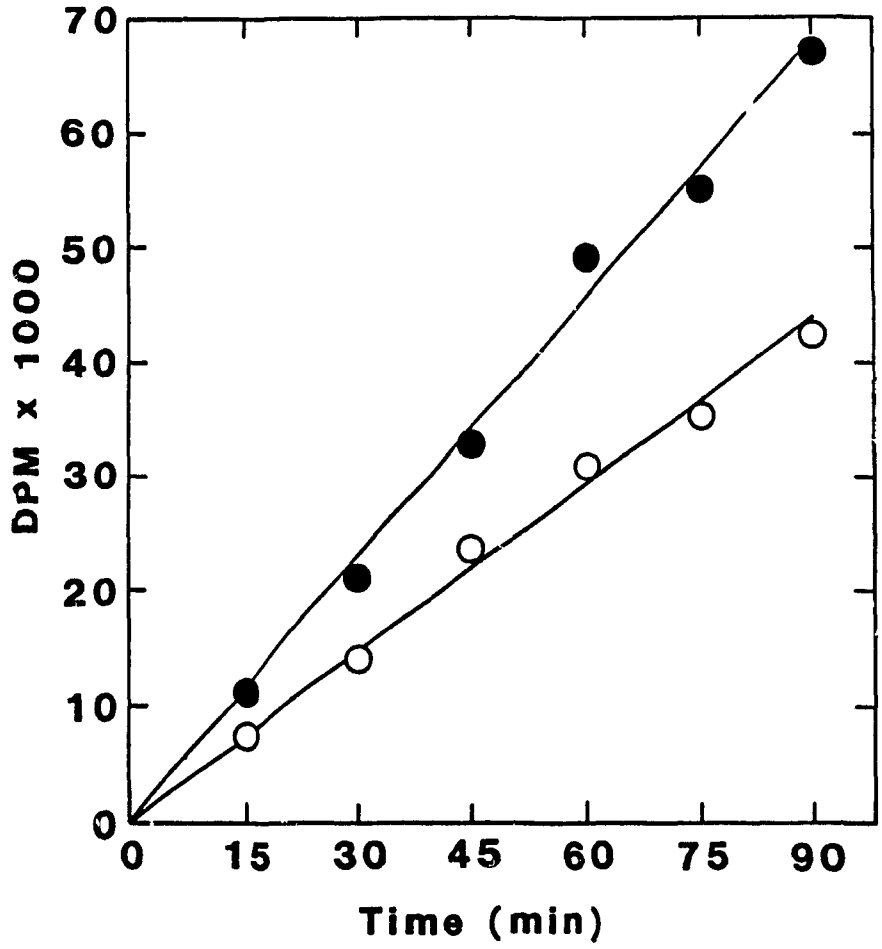


Fig. 9. Time course of H^3H uptake by *Pseudomonas saccharophila*. Late stationary N_2-fixing cells (2-mL samples) from a heterotrophic batch culture (continuously-sparged) were transferred into 14-mL serum bottles and then assayed for H^3H uptake activity with 2 kPa O_2 (○) and 5mM methylene blue (●) as electron acceptors. Data are averages of 3 replicates.

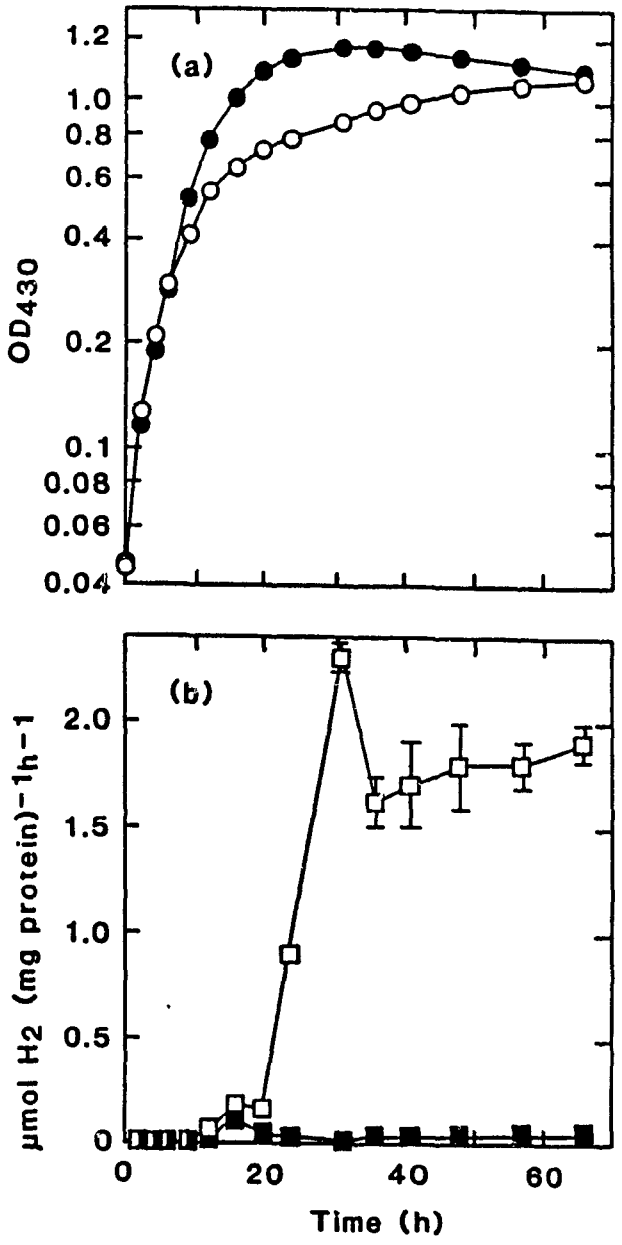


Fig. 10. Growth and hydrogenase activity of *Pseudomonas saccharophila* under N_2-fixing (open symbols) and NH_4^+-grown (closed symbols) heterotrophic conditions. Both cultures were grown under 0.71 kPa O_2 in N_2. (a) Growth curve. (b) Hydrogenase activity. Methylene blue was used as the electron acceptor in the hydrogenase assay. Data are averages of 3 replicates for hydrogenase activity. Bars indicate ± SEM.

4.3.3. *Hydrogenase induction*

The results of the above experiment could indicate that the H_2 evolved during N_2 fixation stimulated synthesis of hydrogenase. Therefore, an experiment was done to determine if such synthesis could be induced by exogenous H_2. Indeed, the level of hydrogenase under non-N_2-fixing autotrophic conditions increased greatly in the presence of H_2 (Fig. 11). Addition of chloramphenicol prevented the increase. The results suggest that H_2 did not activate preformed enzyme but rather caused derepression of synthesis of hydrogenase. Cultures supplied with Ar instead of H_2 had very low or negligible activity (Fig. 11).

4.3.4. *Expression of hydrogenase activity in ammonium-grown culture under air plus hydrogen*

This experiment was conducted to determine if synthesis of hydrogenase could be induced by H_2 under aerobic heterotrophic conditions. NH_4^+-grown batch cultures were continuously sparged with air alone or with air containing 2.2 kPa H_2. The growth and sucrose utilization patterns were nearly identical for both cultures (Fig. 12a). However, the hydrogenase activity remained negligible in the absence of exogenous H_2 (Fig. 12b). In contrast, the H_2-sparged culture had much higher hydrogenase activity and the maximum activity was reached when the level of sucrose in the medium was relatively low. In this experiment, MB-dependent hydrogenase was compared with O_2-dependent activity at different growth stages of the H_2-sparged

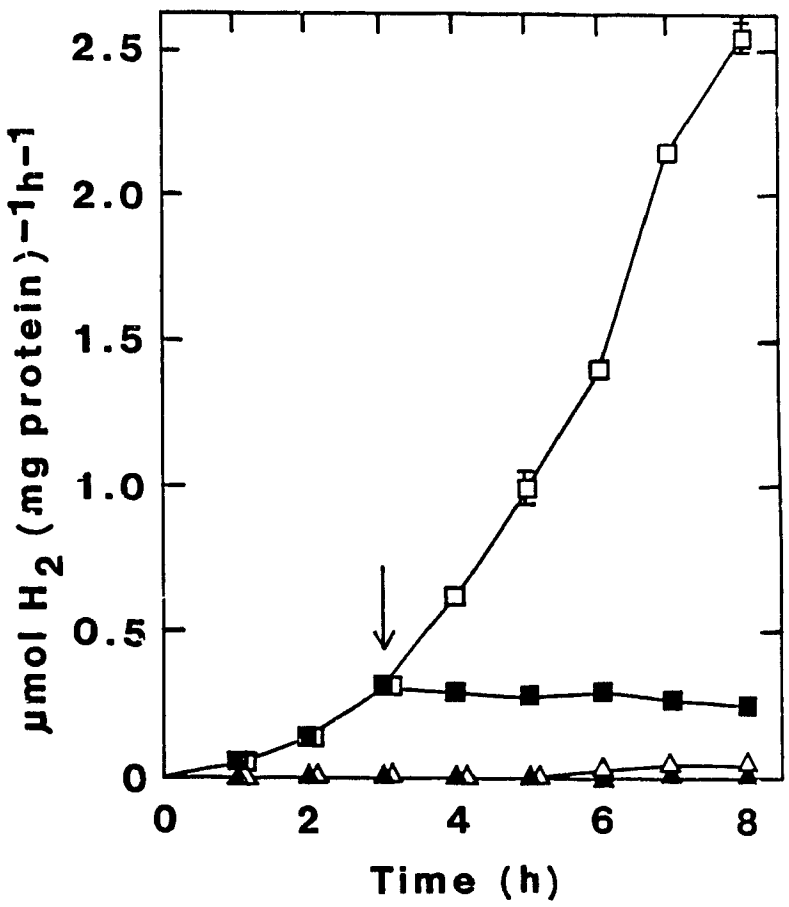


Fig. 11. Effect of H_2 on hydrogenase synthesis in *Pseudomonas saccharophila*. A mid-exponential NH_4^+-grown heterotrophic culture was washed, then resuspended in a small quantity of the same medium but without sucrose. Samples (5 mL, OD_{430} = 18 as determined by dilution, 1.5 mg protein mL^{-1}) were inoculated into 500-mL vacuum flasks each containing 150 mL of the same medium. Zero-h samples were taken, then the flasks were evacuated and backfilled with N_2. Twenty per cent of the headspace was withdrawn then replaced individually with 5 kPa CO_2, 5 kPa O_2, and 10 kPa H_2 or Ar. Chloramphenicol (Cm) at 100 ug mL^{-1} was added as indicated (arrow). The flasks were shaken at 30°C, and samples were taken each hour. Hydrogenase assays were carried out under air. The treatments were as follows: plus H_2 (□), plus H_2 and Cm (■), plus Ar (△), plus Ar and Cm (▲). Data are averages of triplicate samples. Bars indicate ± SEM.

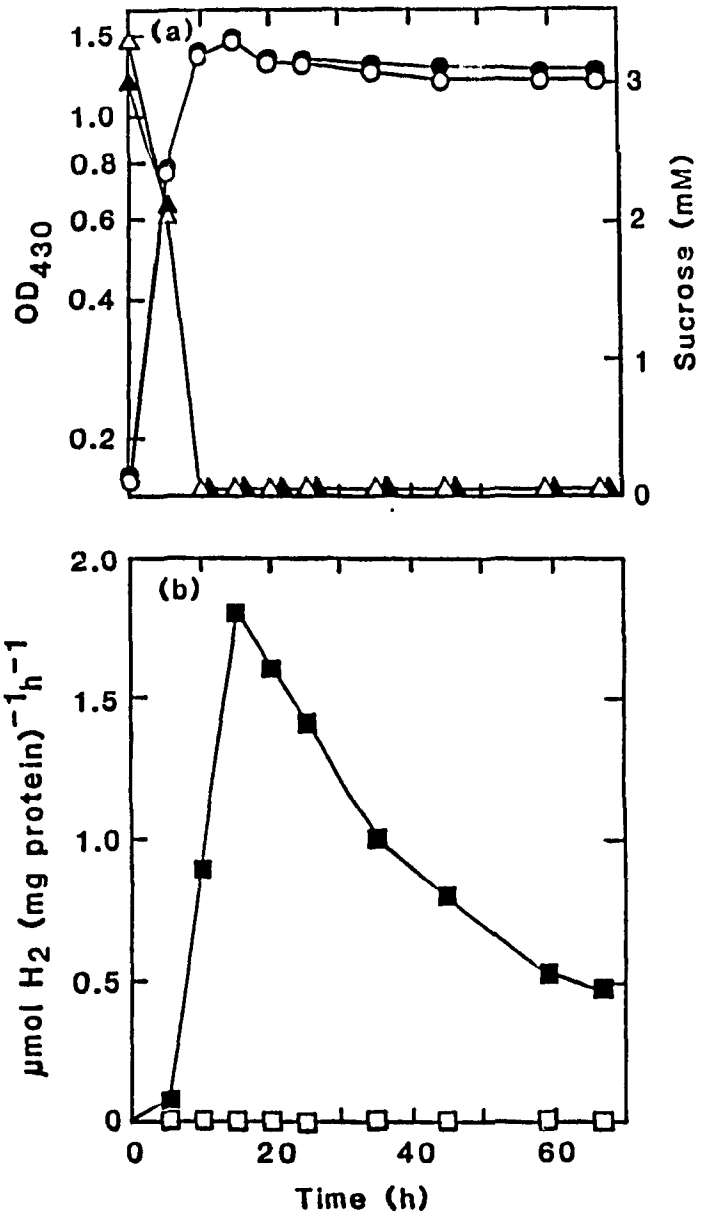


Fig. 12. Growth and hydrogenase activity of *Pseudomonas saccharophila* with (closed symbols) and without (open symbols) H_2 (2.2 kPa) under non-N_2-fixing heterotrophic conditions (aerobically with NH_4Cl). (a) Growth (○, ●) and residual reducing sugar as sucrose (△, ▲). (b) Hydrogenase activity. Activity was measured using 2 kPa O_2 as the electron acceptor. Data are averages of 3 replicates for residual sucrose and hydrogenase activity. SEM ranged from 1 to 6% for hydrogenase activity and 4 to 7% for residual sucrose.

culture. MB-dependent hydrogenase activity was higher than the O_2-dependent activity by 1.6 ± 0.18 times (average ± SEM of determinations from 9 time intervals). A similar ratio was obtained by Berndt and Wolfle (1978) in *Xanthobacter autotrophicus*. The results might suggest that in the O_2-dependent assay, some components of the electron transport chain were limiting. The O_2- and MB-dependent hydrogenase activities at different growth stages were proportional with a correlation coefficient of 0.94. Of the 5 MB concentrations tested, 5 mM showed the highest hydrogenase activity (Fig. 13).

4.3.5. *Effect of O_2 on hydrogenase activity and synthesis*

Nitrogen-fixing continuously-sparged batch cultures showed maximum hydrogenase activity in the stationary phase (Fig. 14). The hydrogenase showed wide tolerance to O_2, maximum activity being observed from pO_2 0.9 to 20 kPa. Early-exponential cultures did not show hydrogenase activity. Low O_2 levels became limiting in late exponential and early stationary phases but not in mid exponential phase, probably because of the low hydrogenase activity and low population density resulting in lower O_2 uptake rate in the latter.

It is possible that O_2 may become limiting when sucrose is available in the medium, thus causing a low level of hydrogenase synthesis (Van Berkum and Maier 1988). To test this hypothesis, hydrogenase formation was induced under microaerobic and aerobic conditions in the presence

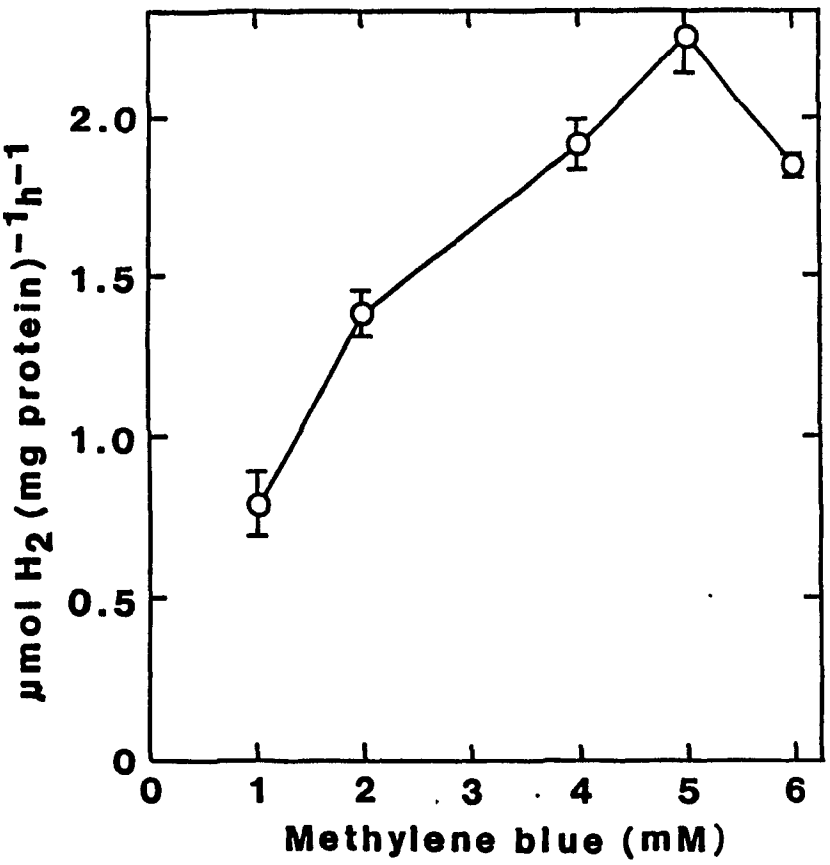


Fig. 13. Effect of methylene blue concentration on hydrogenase activity of *Pseudomonas saccharophila*. Stationary phase N_2-fixing cells (2-mL samples) from a heterotrophic batch culture (continuously-sparged) were transferred into 14-mL serum bottles and then assayed for hydrogenase activity. Data are averages of 3 replicates. Bars indicate ± SEM.

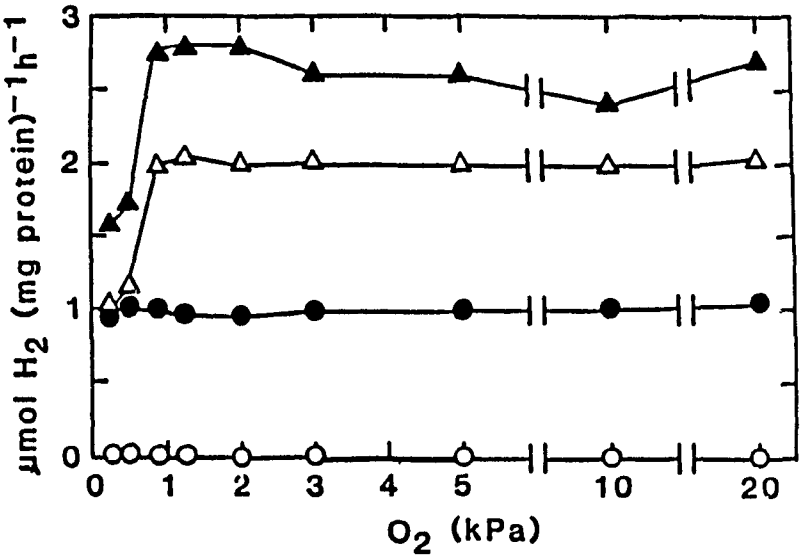


Fig. 14. O_2 optima for hydrogenase activity of *Pseudomonas saccharophila*. Activity was measured using early exponential (○), mid-exponential (●), late exponential (△) and early stationary (▲) N_2-fixing cells from a heterotrophic batch culture (continuously-sparged). Data are averages of 3 replicates. SEM ranged from 0.2 to 7%.

and absence of sucrose. In the absence of sucrose, hydrogenase formation was partially repressed by 18 kPa O_2, the activity being 80% lower than that under 2.8 kPa O_2 (Table 12). In the presence of sucrose, much lower activity developed. O_2 may have been limiting in the 2.8 kPa O_2 plus sucrose treatment, but since 5.8 kPa O_2 remained in the 18 kPa O_2 treatment with little appearance of activity, it is probable that sucrose and not O_2 availability was the important factor.

4.3.6. *Effect of carbon sources on hydrogenase activity and synthesis*

The results of the previous experiments suggested that the concentration of sucrose may affect hydrogenase formation. Carbon substrates which are used to different extents for growth of *Pseudomonas saccharophila* (Section 3.3.2) were therefore examined for their effects on hydrogenase formation. Carbon sources which are utilized only slightly or not at all, such as mannose, fructose, xylose, and glucose, repressed hydrogenase formation either not at all or at the most by 40% (Table 13). Pyruvate, a better substrate, showed 80% repression. Those carbon sources which support vigorous growth of the organism exhibited 97-100% repression. In the absence of carbon source, and with CO_2 replaced by Ar, there was about a 20% reduction in hydrogenase expression.

The kinetics of repression of hydrogenase synthesis and of inhibition of preformed enzyme by sucrose was then

TABLE 12. Effect of O_2 on formation of hydrogenase in *Pseudomonas saccharophila*[a]

Oxygen (kPa)	nmol H_2 (mg protein)$^{-1}$ h^{-1}	
	- Sucrose	+ Sucrose
2.8	2107 ± 288 (2.4 ± 0.2)	12 ± 2 (0.21 ± 0.05)
18.26	443 ± 32 (15.8 ± 0.05)	22 ± 1 (5.8 ± 0.2)

[a]A mid-exponential phase NH_4^+-grown aerobic heterotrophic culture was washed with NH_4Cl-containing (minus sucrose) medium and then resuspended in a small quantity of the same medium. Samples (0.2 mL, OD_{430} = 6 as determined by dilution, 0.50 mg protein mL^{-1}) were dispensed into vials containing 1.8 mL of the same medium without or with sucrose (15 mM). One set of vials received 10 kPa H_2, 5 kPa CO_2, 2.8 kPa O_2, and balance N_2; the other set received 10 kPa H_2 and 5 kPa CO_2 in air. The cultures were shaken for 8 h, after which they were analyzed for O_2, then evacuated and backfilled with N_2. The H_2-uptake assay was carried out with MB as electron acceptor. Values in parentheses are kPa O_2 at the end of the derepression period. Data are averages of 4 replicates ± SEM.

TABLE 13. Effect of different carbon sources on formation of hydrogenase in *Pseudomonas saccharophila* previously grown under N_2-fixing conditions[a]

Carbon source	Hydrogenase activity[b] (nmol H_2 (mg protein)$^{-1}h^{-1}$)	Relative activity
None (control)	7414 ± 1891	100
D(+)-Fructose	9589 ± 828	129
D(+)-Mannose	8477 ± 790	114
D(+)-Xylose	4939 ± 569	67
D(+)-Glucose	4630 ± 568	62
Na-Pyruvate	1489 ± 106	20
Na-Citrate	223 ± 44	3
L(+)-Arabinose	133 ± 10	1.8
Sucrose	89 ± 26	1.2
Na-Acetate	53 ± 5	0.7
Na-Succinate	20 ± 20	0.2
D(+)-Galactose	4 ± 1.4	0.05
Na-Malate	0	0
None (no CO_2, plus Ar)	6063 ± 1450	82

[a]A mid-exponential phase N_2-fixing heterotrophic culture was washed thrice with N-free medium lacking carbon source. The pellet was resuspended in the same medium, then 0.2-mL samples of the concentrated cell suspension (OD_{430} = 8 as determined by dilution, 0.56 mg protein ml^{-1}) were dispensed into 14-mL vials containing 1.8 mL of the same medium plus or minus carbon source (15 mM). The vials were stoppered then evacuated and backfilled thrice with N_2. Zero-h samples were then assayed for hydrogenase activity using 2 kPa O_2 as the electron acceptor. The gas phase of the remaining vials contained: 5 kPa O_2, 5 kPa CO_2, 10 kPa H_2, and balance N_2. The vials were shaken for 13 h at 30°C after which the cultures were assayed for hydrogenase activity. Data are averages of 2 replicates (except control and no CO_2 treatments which had 4 replicates each) ± SEM.

[b]Difference between 13-h and 0-h activities. Zero-time activity in all treatments ranged from 22 to 81 nmol H_2 (mg protein)$^{-1}h^{-1}$ with an average of 44 ± 6.

studied. In the presence of sucrose, preformed hydrogenase activity was not affected but synthesis was almost completely repressed throughout the 8-h experiment (Fig. 15). Hydrogenase formation was greatly stimulated in the absence of sucrose. In the absence of sucrose but with chloramphenicol there was a rapid decline in activity. Sodium azide prevented this decline whereas PMSF did so only partially.

4.3.7. *Effect of acetylene on hydrogenase activity*

This experiment was done since C_2H_2 was reported to inhibit hydrogenase activity in N_2-fixing bacteria (Smith *et al.* 1976). Thirty-min exposure to C_2H_2 (0.1 to 50 kPa) did not inhibit hydrogenase activity of *Pseudomonas saccharophila* (Table 14).

4.3.8. *Effect of hydrogenase activity on O_2 sensitivity of nitrogenase*

This experiment was done to determine if hydrogenase would provide some protection to nitrogenase activity against O_2 inactivation. The presence or absence of H_2 and the O_2-dependent H_2 uptake had no effect on the O_2 sensitivity of nitrogenase at different growth stages (Fig. 16). Perhaps hydrogenase activity is still too low (from about 0.5 to 2.0 umol H_2 (mg protein)$^{-1}h^{-1}$) to be able to perform a vigorous oxyhydrogen reaction.

4.3.8. *Effect of medium pH on expression of hydrogenase activity*

Of the 5 pH levels tested for hydrogenase expression

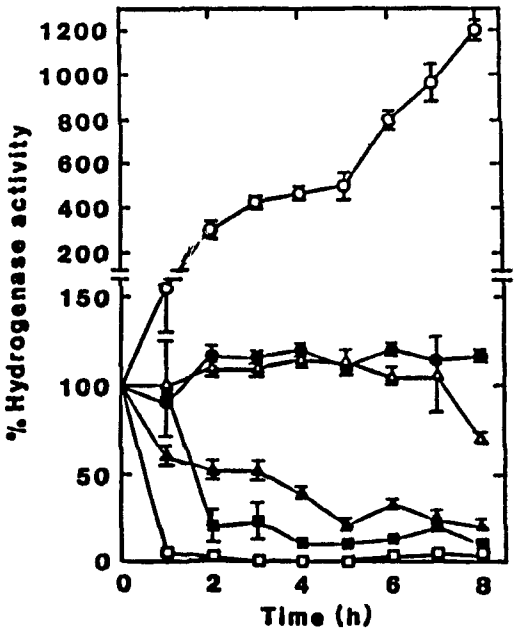


Fig. 15. Effect of sucrose on preformed activity and synthesis of hydrogenase in *Pseudomonas saccharophila*. A late-exponential phase N_2-fixing heterotrophic culture was washed, then resuspended in a small quantity of NH_4Cl-containing (minus sucrose) medium. Samples (5 mL, OD_{430} = 12.4 as determined by dilution, 0.86 mg protein mL^{-1}) were inoculated into 500-mL vacuum flasks each containing 150 mL of the same medium. Zero-time samples were taken, then sucrose (15 mM), sodium azide (1 mM), phenylmethylsulfonylfluoride (PMSF, 1 mM), and chloramphenicol (Cm, 100 ug mL^{-1}) were injected immediately as desired. The flasks were evacuated and backfilled with N_2, then 20% of the headspace was withdrawn and replaced individually with 10 kPa H_2, 5 kPa CO_2, and 5 kPa O_2. The flasks were shaken at 30°C, and samples were taken each hour. Methylene blue was used as the electron acceptor in the hydrogenase assay. The treatments were as follows: minus sucrose (○), plus sucrose (●), minus sucrose plus Cm (□), plus sucrose and Cm (■), minus sucrose plus Cm and sodium azide (△), minus sucrose plus Cm and PMSF (▲). Data are averages of triplicate samples. Bars indicate ± SEM.

TABLE 14. Effect of C_2H_2 on hydrogenase activity of *Pseudomonas saccharophila*[a]

C_2H_2 (kPa)	umol H_2 (mg protein)$^{-1}h^{-1}$	% Activity
0	2.9 ± 0.04	100
0.1	2.7 ± 0.01	93
0.5	2.9 ± 0.08	100
1	2.9 ± 0.07	100
5	3.1 ± 0.03	107
10	3.2 ± 0.03	110
25	2.9 ± 0.08	100
50	2.9 ± 0.01	100

[a]A late stationary phase heterotrophic N_2-fixing culture was used. Cells (2-mL samples) were transferred into 14-mL serum bottles and then assayed for hydrogenase activity under an atmosphere of 3 kPa H^3H and C_2H_2 in air for 30 min. Data are averages of 3 replicates ± SEM.

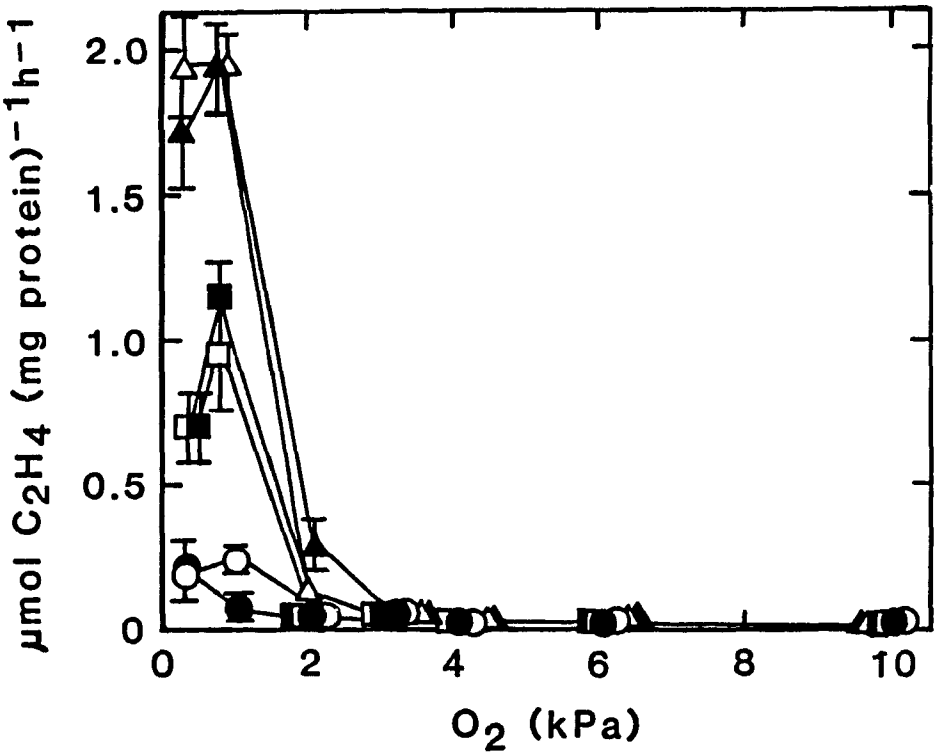


Fig. 16. Effect of exogenous H_2 (10 kPa) on O_2 sensitivity of nitrogenase of *Pseudomonas saccharophila*. Cells (2-mL samples) from an N_2-fixing heterotrophic batch culture (continuously-sparged) at different growth stages were taken and then assayed for nitrogenase activity (see Chapter 3 for C_2H_2 reduction assay) at different O_2 levels. Open and closed symbols represent absence and presence of H_2, respectively. The different growth stages were as follows: mid-exponential (○, ●), late exponential (△, ▲), and early stationary (□, ■). Data are averages of 3 replicates. Bars indicate ± SEM.

under N-limited chemolithotrophic conditions, pH 6.4 showed the highest activity (Table 15). Unless otherwise stated, this pH was used in subsequent experiments that involved hydrogenase derepression under N-limited chemolithotrophic conditions.

4.3.9. *Distribution of hydrogenase activity in cell-free extract*

Nearly all of the methylene blue-dependent hydrogenase activity was found in the membrane fraction whether prepared aerobically or anaerobically (Table 16). NAD reduction by cetyltrimethylammonium bromide-permeabilized cells was negative indicating the absence of soluble hydrogenase. *Alcaligenes eutrophus* H16 had specific activity of 0.54 units (mg protein)$^{-1}$ (1 unit = 1 umol NAD reduced per min).

4.4. Discussion

The results clearly show the presence of an active uptake hydrogenase in N_2-fixing cultures of *Pseudomonas saccharophila*. Hydrogenase was expressed significantly more in N_2-fixing than in non-N_2-fixing culture, as in *Azotobacter chroococcum* (Partridge *et al.* 1980), *Xanthobacter autotrophicus* (Berndt and Wolfle 1978) and *Azotobacter vinelandii* (Lee and Wilson 1943) but in contrast to *Azospirillum brasilense* where an NH_4^+-grown culture had hydrogenase activity similar to or slightly higher than that in a N_2-fixing culture (Tibelius and Knowles 1983). Our results show that NH_4^+ *per se* does not

TABLE 15. Effect of pH on expression of hydrogenase activity by *Pseudomonas saccharophila* under N-limited chemolithotrophic conditions[a]

pH of medium	umol H_2 (mg protein)$^{-1}h^{-1}$
5.6	28.3 ± 4.3
6.0	28.4 ± 1.9
6.4	37.0 ± 0.1
6.8	20.9 ± 1.4
7.2	21.0 ± 6.1

[a]Hydrogenase derepression was carried out in 14-mL serum bottles containing 2 mL medium with 10 uM $NiCl_2$ under 2 kPa O_2, 5 kPa CO_2, 20 kPa H_2, and balance N_2 for 20 h. Inoculum was 30 uL cell suspension (1.98 mg mL^{-1} protein) per bottle. O_2 (2 kPa) was used as the electron acceptor. Data are averages of 3 replicates ± SEM.

TABLE 16. Distribution of methylene blue-dependent hydrogenase activity in cell-free extracts of *Pseudomonas saccharophila* derepressed for hydrogenase under N-limited chemolithotrophic conditions[a]

Fraction[b]	Specific activity[c]	Total protein (mg)	Total activity[d]	%
Aerobic handling				
CFE	326 ± 18	0.84	274	100
SF	10 ± 2	0.53	5.3	1.9
MF	961 ± 28	0.26	250	91.2
Anaerobic handling				
CFE	453 ± 32	4.59	2079	100
SF	73 ± 5	3.06	223	10.7
MF	1298 ± 59	1.15	1493	71.8

[a]Derepression was carried out for 12 h under N-limited chemolithotrophic conditions (30 kPa H_2, 5 kPa CO_2, 5 kPa O_2, balance N_2) with 10 uM $NiCl_2$ added to the medium.

[b]CFE, cell-free extract; SF, soluble fraction; and MF, membrane fraction.

[c]nmol H_2 (mg protein)$^{-1}$min^{-1}. Data are averages of 3 replicates ± SEM.

[d]nmol H_2 min^{-1}.

inhibit hydrogenase activity, and in the presence of H_2, synthesis of the enzyme is greatly induced. Thus, the inducible hydrogenase system of *Pseudomonas saccharophila* resembles that of *Xanthobacter autotrophicus* (Berndt and Wölfle 1978), *Aquaspirillum autotrophicum* (Aragno and Schlegel 1978), *Bradyrhizobium japonicum* (Maier *et al.* 1979; Van Berkum 1987), *Rhodobacter capsulatus* (Colbeau and Vignais 1983), *Alcaligenes hydrogenophilus* (Friedrich *et al.* 1984), *Rhizobium* ORS 571 (de Vries *et al.* 1984), *Alcaligenes latus* (Doyle and Arp 1987), and *Azotobacter vinelandii* (Prosser *et al.* 1988) but differs from that of *Azospirillum brasilense* (Tibelius and Knowles 1983), and most strains of *Paracoccus denitrificans* (Nokhal and Schlegel 1980). In *R. capsulatus* (Colbeau and Vignais 1983) the H_2 evolved during N_2 fixation is probably responsible for the stimulated hydrogenase expression in N_2-fixing culture. In both *R. capsulatus* (Colbeau and Vignais 1983) and *P. saccharophila*, expression of hydrogenase in both autotrophic and heterotrophic NH_4^+-grown cultures provided with exogenous H_2 suggests that hydrogenase is independent of nitrogenase expression.

Hydrogenase activity of *P. saccharophila* was not sensitive to O_2 and in this respect resembles the H_2 uptake system of *A. autotrophicum*, *Alcaligenes eutrophus*, and *Azospirillum amazonense* (Aragno and Schlegel 1978; Fu and Knowles 1986; Wilde & Schlegel, 1982) but differs from that of other diazotrophic bacteria such as *A. brasilense* and

Azospirillum lipoferum (Fu and Knowles 1986; Pedrosa *et al.* 1980; Tibelius and Knowles 1983), and *Rhizobium japonicum* and its bacteroids (Maier *et al.* 1979; Ruiz-Argueso *et al.* 1979). However, 18 kPa O_2 partially repressed hydrogenase formation. Hydrogenase formation by *Bradyrhizobium japonicum* was repressed under aerobic conditions (Van Berkum 1987). Above 12.5 kPa O_2, hydrogenase synthesis by *Alcaligenes latus* was repressed (Doyle and Arp 1987). Complete repression was reported in *Aquaspirillum autotrophicum* at 30 kPa O_2 whereas enzyme synthesis occurred at 20 kPa O_2 but at a much lower rate than at 7.5 kPa O_2 (Aragno and Schlegel 1978).

Under carbon-limited conditions hydrogenase activity of *Azotobacter chroococcum* was consistently high (Partridge *et al.* 1980; Waller and Yates 1978). Likewise, maximum hydrogenase activity of *Pseudomonas saccharophila* was observed when residual sucrose was low or nil. In the presence of 15 mM sucrose, the synthesis of hydrogenase was repressed but the activity of preformed enzyme was not inhibited. Other utilizable carbon substrates tested also repressed hydrogenase synthesis whereas those which were little or not at all utilized showed negligible repressive effect. Similar behavior is seen in *Rhizobium japonicum* (Maier *et al.* 1979) and *A. latus* (Doyle and Arp 1987). However, in *A. autotrophicum* (Aragno and Schlegel 1978), *Rhizobium* strain ORS 571 (De Vries *et al.* 1984) and *R. japonicum* strain JH (Graham *et al.* 1984), some utilizable

carbon substrates do not repress enzyme formation. Recently, Van Berkum (1987) and Van Berkum and Maier (1988) found no repression of hydrogenase expression by carbon substrate in *Bradyrhizobium japonicum*. They suggested that acidification of the induction medium and limiting O_2 were the factors responsible for decreased hydrogenase activity. In the study of *Rhizobium japonicum* mutants, Merberg *et al.* (1983) found that a common element is involved in the regulation of hydrogenase by oxygen and by carbon substrates. In the present study, it seems that sucrose repression of hydrogenase formation is independent of repression by O_2. Catabolite repression involving cyclic AMP and possibly other effector molecules is implicated in malate-mediated repression of H_2 uptake in *R. japonicum* (Lim and Shanmugam 1979; McGetrick *et al.* 1985). The discrepancies among these reports may indicate only the presence of different mechanisms by which carbon substrates regulate H_2-oxidation. Nevertheless, O_2 and carbon substrates may be essential co-regulators especially in O_2-sensitive hydrogenase systems.

The decline in activity in the presence of sucrose plus chloramphenicol was prevented by addition of 1 mM sodium azide, a known inhibitor of ATP production, but not by PMSF, a proteolysis inhibitor. Schlesier and Friedrich (1981) reported inactivation of soluble hydrogenase in *Alcaligenes eutrophus* strain H16 when intact cells were exposed to a $H_2/O_2/CO_2$ atmosphere, and this inactivation

was increased in the presence of chloramphenicol; cells exposed to H_2/O_2 with 1 mM sodium azide retained only about 10% of the activity of cells exposed to either air or H_2 but 27% of the activity was retained in the presence of 5 mM sodium azide. However, addition of 2 mM ATP to cell extracts did not enhance inactivation of hydrogenase and it was concluded that inactivation was due to superoxide radical anions produced by hydrogenase itself during catalysis. The present results seem to suggest that inactivation is ATP-supported and, as in *Alcaligenes eutrophus*, is not due to simple proteolysis.

Hydrogenase activity of *Pseudomonas saccharophila* was not inhibited by C_2H_2. Likewise, the H_2-uptake systems of *Azospirillum amazonense*, *Azospirillum lipoferum*, *Derxia gummosa*, *Xanthobacter autotrophicus* (intact cells and partially purified hydrogenase), and *Bradyrhizobium japonicum* (bacteroids) are not very sensitive to C_2H_2 (Berndt and Wolfle 1978; Ruiz-Argüeso *et al.* 1979; Ravi Shankar *et al.* 1986; Fu and Knowles 1988). On the other hand, hydrogenase activity of *Azotobacter chroococcum* (Smith *et al.* 1976; Walker and Yates 1978) and *Azospirillum brasilense* (Chan *et al.* 1980; Tibelius and Knowles 1984) were sensitive to C_2H_2. Purified hydrogenases from *Azotobacter vinelandii*, *Rhizobium japonicum*, and *A. eutrophus* H16 were inhibited reversibly by C_2H_2 (Hyman and Arp 1987). H_2 and CO were shown to protect these hydrogenases against C_2H_2 inhibition (Hyman and Arp 1987).

Likewise, Van der Werf and Yates (1978) showed that inhibition of partially purified hydrogenase from *Azotobacter chroococcum* could be reversed by flushing with H_2. It is quite possible that the insensitivity of the hydrogenase activities to C_2H_2 observed in *Pseudomonas saccharophila*, *Azospirillum amazonense*, *Derxia gummosa*, *Xanthobacter autotrophicus*, and *Bradyrhizobium japonicum* bacteroids is due to protection provided by H_2 which was present during the assays.

The presence of exogenous H_2 and hydrogenase did not affect the O_2 sensitivity of *P. saccharophila* nitrogenase. This suggests that H_2-dependent respiration does not keep the O_2 away from nitrogenase. This is in agreement with the data for *Azospirillum brasilense* (Pedrosa *et al.* 1982), *Azospirillum lipoferum*, and *A. amazonense* (Fu and Knowles 1988), but contrasts with that for *A. chroococcum* (Walker and Yates 1978) where oxidation of exogenous H_2 afforded some protection for nitrogenase.

Bone (1960) reported the presence of H_2-activating enzymes in the membrane and soluble fractions of *P. saccharophila*. However, Podzuweit *et al.* (1983) did not find any hydrogenase activity in the soluble fraction. The present data are in agreement with the results of Podzuweit *et al.* (1983). The absence of NAD-dependent soluble hydrogenase in *P. saccharophila* implies that the organism has to generate NADH by reverse electron flow (Gottschalk 1986) when growing under chemolithotrophic conditions.

5. NICKEL UTILIZATION BY *PSEUDOMONAS SACCHAROPHILA*

5.1. Introduction

In H_2-dependent chemolithotrophic N_2-fixation, three key reactions are involved: H_2 uptake, N_2 reduction, and CO_2 fixation. For an organism to grow under such conditions, the large ATP demand (16 and 3 ATPs per N_2 and CO_2 fixed, respectively) (Gottschalk 1986) of the last two processes must be satisfied. The charge separation catalyzed by uptake hydrogenase ultimately generates ATP by oxidative phosphorylation (Bowien and Schlegel 1981). However, H_2 itself is a known competitive inhibitor of N_2 reduction (Hwang *et al.* 1973). Therefore, the possession of an efficient uptake hydrogenase system is essential for H_2/N_2-dependent chemolithotrophic growth.

Pseudomonas saccharophila, *Xanthobacter autotrophicus*, *Alcaligenes latus*, and *Derxia gummosa* fix N_2 heterotrophically and chemolithotrophically (Barraquio *et al.* 1986; Berndt and Wolfe 1978; Finkwart *et al.* 1983; Kavi Shankar *et al.* 1986; see Chapter 3). The growth of these organisms under either NH_4^+-utilizing or N_2-fixing chemolithotrophic conditions is normally scanty. Investigators have manipulated the composition of the gas phase and the mineral constituents of the medium to improve autotrophic growth (Repaske and Repaske 1976; Kavi Shankar *et al.* 1986;). Nickel was found to greatly stimulate NH_4^+/H_2-dependent autotrophic growth of *Bradyrhizobium*

japonicum and *Alcaligenes eutrophus* (Bartha and Ordal 1965; Tabillon *et al.* 1980; Klucas *et al.* 1983). Subsequently, it was shown to be a component of most hydrogenases, urease, carbon monoxide dehydrogenase, and coenzyme F_{430} in methyl reductase (Hausinger 1987; Andrews *et al.* 1988) and to be involved in the regulation of hydrogenase synthesis in *Bradyrhizobium japonicum* and *Alcaligenes latus* (Stults *et al.* 1986; Doyle and Arp 1988).

Microorganisms that require nickel for growth and enzyme synthesis take up nickel from the environment with or without the utilization of energy (chemical or electrochemical) (Hausinger 1987; Drake 1988). Most of the nickel-uptake systems so far reported are energy-dependent and can further be categorized principally according to the presence or absence of a magnesium-transport system which couples the uptake of other metals including nickel (Jasper and Silver 1977; Hausinger 1987; Bryson and Drake 1988; Drake 1988; Lundie *et al.* 1988). Energy-independent nickel-uptake systems are known only in *B. japonicum*, *Azotobacter chroococcum*, and *Methanothrix concilii* (Partridge and Yates 1982; Stults *et al.* 1987; Drake 1988; Baudet *et al.* 1988).

This study examines the effects of nickel on *Pseudomonas saccharophila* under N-limited chemolithotrophic conditions, and the organism's nickel-uptake system.

5.2. Materials and methods

5.2.1. *Organism and growth*

Pseudomonas saccharophila ATCC 15946 was used (see

Chapter 3). The organism was grown aerobically in NH_4Cl + sucrose + mineral salts + yeast extract (100 mg L^{-1}) medium (pH 6.8) (see Chapter 3). Washed cells were inoculated into culture vessels containing the autotrophic medium (basal medium) + yeast extract (100 mg L^{-1}) (see Chapter 3 for the composition of the medium). The pH of the medium was 6.4 (see Section 4.3.8) except otherwise stated. The medium is referred to as nitrogen-limited because of the presence of yeast extract. These media are all nickel-deficient and when $NiCl_2$ is added, the medium is nickel-enriched. The nickel contaminant in the nickel-deficient medium was not detected by atomic absorption spectroscopy (Dr. F. Archibald, personal communication). Nickel contamination from the glassware was minimized by overnight soaking in 2 N HCl and then finally rinsing with deionized distilled water. This washing procedure was later modified by using 2 N HNO_3 followed by 10 mM EDTA (pH 7.0) then finally rinsing with deionized distilled water. For monitoring growth, 125-mL side-arm flasks containing 15 mL of medium were used. Flasks were plugged with rubber stoppers fitted with cut plastic syringes containing cotton filters. The cut syringes were capped with Suba Seals. The headspace consisted of H_2, CO_2, O_2, and balance N_2, the partial pressures of which are indicated in the text. The culture vessels were incubated at 30°C on a gyratory shaker at 250 rpm.

For nickel uptake experiments, mid-log phase cells

were harvested by centrifugation (10000 x *g*) at 4°C for 10 min, washed twice with 16.7 mM phosphate buffer ($Na_2HPO_4 \cdot 7H_2O$ + KH_2PO_4, pH 6.4) and then resuspended in the same buffer. The optical density (OD) at 430 nm was adjusted such that the final OD of the assay mixture would be approximately 1.0. Depending on the final volume of the assay mixture, 0.5 or 1.0 mL of the cell suspension was used. The cells were kept on ice until analysis.

5.2.2. *Hydrogenase derepression*

Either 14-mL serum bottles each containing 2 mL of medium and capped with butyl stoppers, or 500-mL vacuum flasks each containing 150 mL of medium and plugged as for the 125-mL flasks were used in the derepression of hydrogenase. Boiled cells served as control. The headspace was the same as above with partial pressures given in the text. The incubation conditions were the same as above except for the 14-mL bottles which were incubated on a water bath shaker. After incubation the bottles were evacuated and backfilled thrice with N_2. The final backfilling was done to the appropriate partial pressure and then O_2 and H^3H were injected for the O_2-dependent H^3H uptake assay (see Chapter 3). Culture samples (1.8 mL) from the 500-mL flasks were transferred by syringe into 14-mL bottles prefilled with N_2 and 0.2 mL of 50 mM methylene blue (MB) for the MB-dependent H^3H uptake assay.

Hydrogenase-derepressed cells for the nickel uptake experiments were prepared in vacuum-flasks (500-mL) each

containing 100 mL of N-limited autotrophic medium and inoculated with heterotrophically-grown washed cells. The flasks were incubated at 30°C under shaking (250 rpm) with a headspace of 20 kPa H_2, 5 kPa O_2, 5 kPa CO_2, and balance N_2. Cells which were not derepressed for hydrogenase were prepared similarly except that no H_2 was added to the headspace. After 10 h, the cells were harvested as above and then resuspended in 16.7 mM Na-K phosphate buffer (pH 6.4) with an optical density of about 1.0. Prior to nickel uptake assay, samples were taken for MB-dependent H^3H uptake assay.

5.2.3. *$^{63}Ni^{2+}$ incorporation*

Vacuum flasks (500-mL) each containing 100 mL of autotrophic medium + yeast extract were inoculated with heterotrophically-grown washed cells. Filter-sterilized $^{63}NiCl_2$ (specific activity, 0.2 uCi $nmol^{-1}$) and non-radioactive $NiCl_2$ were added by syringe at a final concentration of 1 uM, to two separate series of flasks for measurement of nickel incorporation and hydrogenase activity, respectively. The flasks were incubated as above with a headspace of ± 20 kPa H_2, 5 kPa CO_2, 5 kPa O_2, and balance N_2. Nickel incorporation was determined by filtering (Millipore HA filter, 0.45 um pore size, 25 mm diam.) 1-mL samples and then washing thrice with 16.7 mM Na-K phosphate buffer (pH 6.4). Filters were transferred into vials containing 4 mL of Universol scintillation fluid. $^{63}Ni^{2+}$ binding on the filter paper was subtracted

from the sample counts.

5.2.4. *Intracellular distribution of* $^{63}Ni^{2+}$

Cells were grown for 13 h under N-limited chemolithotrophic conditions with $^{63}NiCl_2$ (3.16 uM with specific activity of 0.05 uCi $nmol^{-1}$) (see Section 5.2.3). Cells were harvested and washed twice with 67 mM phosphate (K_2HPO_4 + KH_2PO_4) buffer (pH 7.0) at 10000 x *g* for 10 min at 4°C. Cells were resuspended in 10 mL of the same buffer and then 100 uL of 100 mM phenylmethylsulfonyl-fluoride was added. Cells were sonicated aerobically (Cell Disruptor Model W-220F, Heat Systems-Ultrasonics, Inc., Plainview, New York) 20 times with 1 min burst and 1 min cooling in circulating ice water (approximately 4°C). The sonicator was equipped with a cup horn and was operated at a power output of about 95 watts. Cells were centrifuged at 10000 x *g* at 4°C for 10 min. The supernatant was the cell-free extract. The cell-free extract was centrifuged (Beckman L8-70M ultracentrifuge) at 180000 x *g* for 1 h at 5°C. The supernatant was the soluble fraction and the pellet was the membrane fraction. Aliquots (0.1 mL) from each fraction were taken and transferred into scintillation vials containing 4 mL of Universol scintillation fluid. The cell-free preparations were also used for detection of urease activity.

5.2.5. *Nickel uptake*

The assay was carried out in 60-mL serum bottles using 16.7 mM Na-k phosphate buffer (pH 6.4) as test buffer with a total volume of 5 or 10 mL. The cells (143 to 165 ug protein mL^{-1}) in buffer were preincubated aerobically for 10 min at 30°C in a water bath shaker (300 rpm). $^{63}NiCl_2$ diluted with non-radioactive $NiCl_2$ to specific activity of 0.05 uCi $nmol^{-1}$ was added to a final concentration of 5 uM unless otherwise indicated. The 5 uM concentration was enough to obtain significant radioactive counts and at the same time minimize the use of the expensive radiolabel. The cells were incubated as above, and then aliquots (0.3 mL) were withdrawn from the assay vessel after 1 min and filtered rapidly through a Millipore HA filter (0.45 um pore size, 25 mm diam.). The filters were immediately rinsed thrice (3 mL per rinse) with the test buffer. Further rinsing and adding non-radioactive $NiCl_2$ (800-fold excess) to the washing buffer did not significantly reduced the incorporated radioactivity. The filters were transferred to scintillation vials each containing 4 mL of Universol scintillation fluid.

5.2.6. *Analyses*

Hydrogenase activity was measured using the H^3H uptake method with O_2 (2 kPa) or MB (5 mM) as electron acceptors for 20 min (see Chapters 3 and 4). Nitrogenase assay was done by C_2H_2 reduction with 1 kPa C_2H_2, 0.75 kPa O_2, 20 kPa H_2, and balance He. C_2H_4, H_2, CO_2, and O_2 were

analyzed by gas chromatography as described by Chan *et al.* (1980) and Tibelius and Knowles (1983). ^{63}Ni was analyzed using the ^{14}C channel of an LS 7500 Beckman liquid scintillation counter. Urease activity was determined by measuring colorimetrically the ammonia release (Weatherburn 1967). Cell fractions (0.2 mL) were incubated with 40 uL of urea solution containing 6.0 ug N for 15 min at 37°C prior to addition of reagents. Cell fractions without urea addition served as control. Protein was analyzed by the modified Lowry method (Markwell *et al.* 1978) using bovine serum albumin as standard.

5.2.7. *Chemicals*

Reagent-grade chemicals and deionized distilled water were used throughout the study. $^{63}NiCl_2$ (with specific activity of 0.85 uCi $nmol^{-1}$) was purchased from Amersham Canada Ltd. Sodium azide, N,N'-dicyclohexylcarbodiimide (DCCD), carbonyl cyanide chlorophenyl hydrazone (CCCP) were from Sigma; 2, 4-dinitrophenol from Matheson Coleman and Bell; and sodium cyanide from J. T. Baker Chemical Co. The scintillation fluid was Universol from ICN Biomedicals Inc.

5.3. **Results**

5.3.1. *Effect of nickel on growth, nitrogenase activity and expression of hydrogenase activity*

Growth and expression of hydrogenase activity of *Pseudomonas saccharophila* was significantly stimulated by addition of nickel to the nickel-deficient medium (Table 17). Nitrogenase activity was stimulated too.

TABLE 17. Effect of nickel on growth, nitrogenase activity, and expression of hydrogenase activity by *Pseudomonas saccharophila* under N-limited chemolithotrophic conditions[a]

Culture	Growth[b] (OD_{430})	Hydrogenase activity[c]	Nitrogenase activity[d]
Nickel-deficient	0.149	17.8 ± 1.6	0.49 ± 0.09
Nickel-enriched (10 uM $NiCl_2$)	0.262	49.5 ± 1.3	3.77 ± 0.58

[a]A 48-h culture (N_2-fixing mid-log phase) grown in a closed batch cultivation system was used. The headspace was 2 kPa O_2, 5 kPa CO_2, 20 kPa H_2, balance N_2. Data are averages of 4 replicates ± SEM.

[b]Initial OD was 0.035. Fifty microliters of cell suspension (nickel-deficient) containing 1.67 mg protein mL^{-1} was inoculated into 15 mL of medium contained in 125-mL sidearm flasks.

[c]umol H_2 (mg protein)$^{-1}h^{-1}$; methylene blue was the electron acceptor.

[d]nmol C_2H_4 (mg protein)$^{-1}h^{-1}$; assay was done for 6 h.

5.3.2. *Effect of chelators on hydrogenase expression*

Two chelators, ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA), were tested for their effect on derepression of hydrogenase in the nickel-deficient medium. Expression of hydrogenase activity declined rapidly in response to chelator concentration (Fig. 17). With 50 uM chelator, the inhibition was 99% and 93% for EDTA and NTA, respectively.

5.3.3. *Effect of nickel concentration on expression of hydrogenase activity*

Inhibition of hydrogenase expression by EDTA (50 uM) in the nickel-deficient medium was relieved by the addition of nickel in a wide range of concentrations (1 to 50 uM) (Fig. 18). Inhibition was also relieved at 100 and 200 uM $NiCl_2$ but hydrogenase activity was only 50 and 13.5%, respectively, of the maximum activity seen at 50 uM.

5.3.4. *Effect of other metals on expression of hydrogenase activity*

$CoCl_2$, $ZnSO_4$, $MnCl_2$, and $CuSO_4$ stimulated the expression of hydrogenase activity but to a much lesser degree than nickel (Table 18). At 25 uM, $CoCl_2$ and $ZnSO_4$ showed 40 and 28%, respectively, of the activity exhibited by nickel at the same concentration, but it should be noted that the $CoCl_2$ used contained 0.1% nickel as contaminant. Addition of $FeSO_4$, $MgSO_4$, $NaSeO_3$, or $NaSeO_4$ did not affect hydrogenase expression. Activities in media with nickel in combination individually with $MgSO_4$, $FeSO_4$, $NaSeO_3$, and

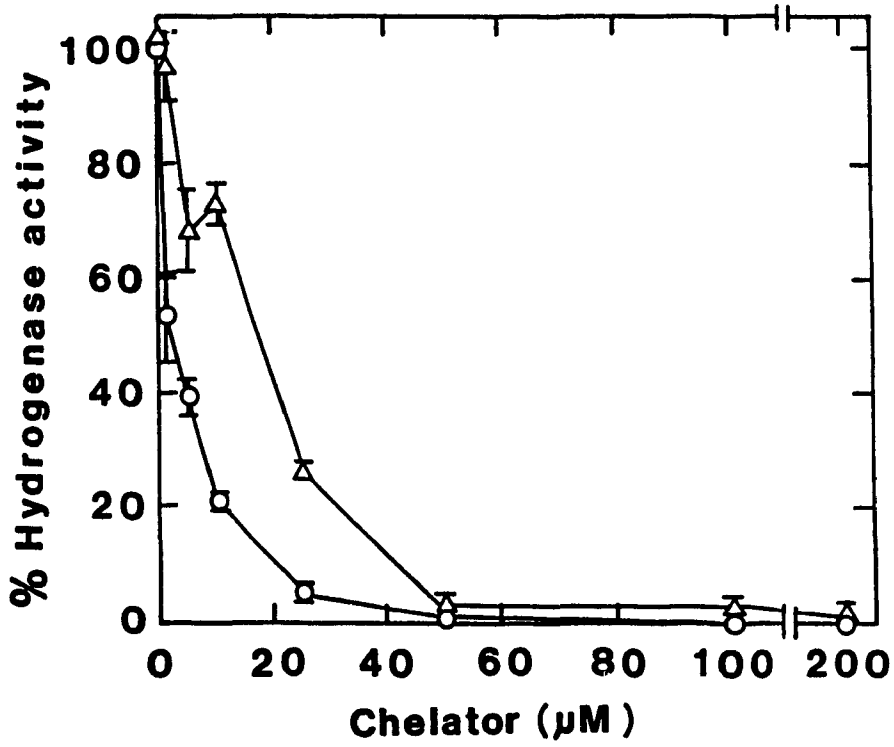


Fig. 17. Effect of EDTA (○) and NTA (△) on expression of hydrogenase activity by *Pseudomonas saccharophila* under N-limited chemolithotrophic nickel-deficient conditions. Fourteen-mL bottles each containing 2 mL of medium were inoculated with 30 uL of cell suspension (1.30 mg protein mL^{-1}). Derepression was carried out for 20 h under an atmosphere of 0.7 kPa O_2, 3 kPa CO_2, 10 kPa H_2, and balance N_2. The medium had a pH of 6.8. O_2 was the electron acceptor in the assay. The 100% activity at zero chelator was 14.6 ± 0.8 umol H_2 (mg protein)$^{-1}h^{-1}$ for EDTA and 12.0 ± 0.5 for NTA. Data are averages of 3 replicates. Bars indicate ± SEM.

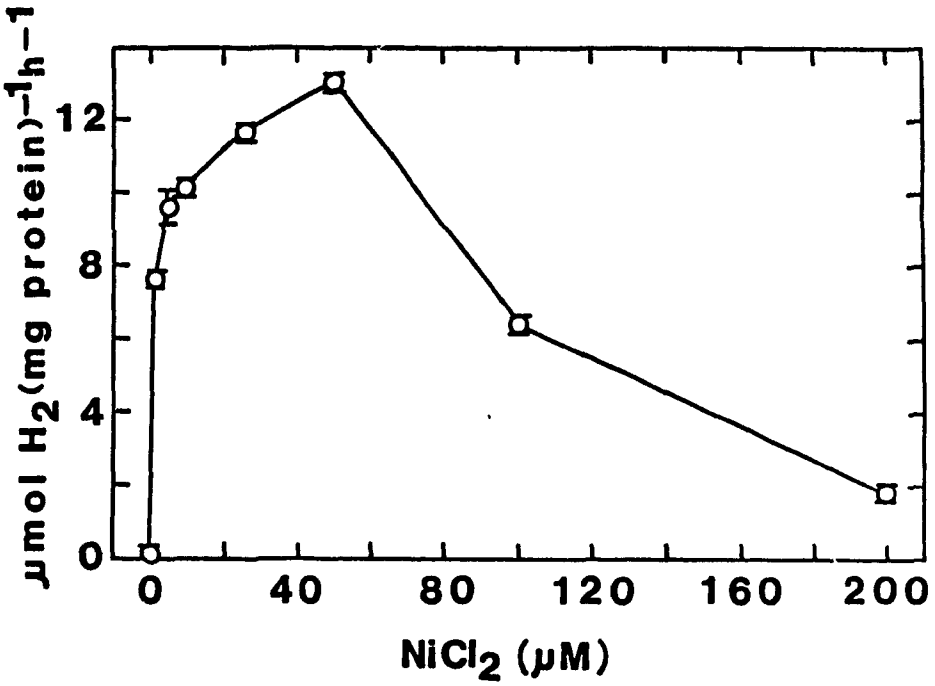


Fig. 18. Effect of nickel concentration on expression of hydrogenase activity by *Pseudomonas saccharophila* under N-limited chemolithotrophic conditions. Fourteen-mL bottles each containing 2 mL of medium were inoculated with 30 uL of cell suspension (2.08 mg protein mL^{-1}). Derepression was carried out for 20 h in a medium (pH 6.8) containing 50 uM EDTA under the same atmosphere as that in Fig. 17. O_2 was the electron acceptor in the assay. Data are averages of 3 replicates. Bars indicate ± SEM.

TABLE 18. Effect of addition of several metals on expression of hydrogenase activity by *Pseudomonas saccharophila* under N-limited chemolithotrophic conditions with 50 uM EDTA in the medium[a]

Metal added	Relative activities with metal additions at:		
	0.1 uM	2.5 uM	25 uM
$NiCl_2$		87.3	100.0
$CoCl_2$		2.9	40.4
$ZnSO_4$		7.2	27.6
$MnCl_2$		1.3	6.2
$CuSO_4$		1.8	4.5
$FeSO_4$		0.0	0.5
$MgSO_4$		0	0.01
$NaSeO_3$	0.07	0	0
$NaSeO_4$	0.06	0	0.1
$NiCl_2$ (25 uM) + $MgSO_4$[b]		113.0	104.0
" + $FeSO_4$		70.5	119.0
" + $NaSeO_3$	105.0	99.0	1.1
" + $NaSeO_4$	92.0	104.0	62.0

[a]Trace elements were not added to the medium (pH 6.8). The inoculum (30 uL) had an average protein concentration of 1.90 mg mL^{-1}. Derepression was carried out for 20 h under 0.70 to 0.80 kPa O_2, 3 kPa CO_2, 10 kPa H_2, and balance N_2 in 14-mL serum bottles each containing a final volume of 2 mL. O_2 was the electron acceptor in the assay. Activities obtained without metal addition in 5 separate determinations ranged from 0.08 to 1.8% of that of $NiCl_2$ at 25 uM. The 100% activity at 25 uM $NiCl_2$ corresponds to 14.0 $\pm$ 2.3 (SEM) umol H_2 (mg protein)$^{-1}h^{-1}$ (from 5 separate experiments). Data are averages of 3 replicates.

[b]$MgSO_4$ at 500 and 1000 uM showed 93.2 and 125% activity, respectively.

$NaSeO_4$ were essentially the same as that with nickel alone except in the case of 25 uM $NaSeO_3$ which appeared to be toxic as shown by decreased growth (data not shown).

5.3.5. *Role of nickel in hydrogenase synthesis*

Cells derepressed with 25 uM nickel added at zero time expressed high levels of hydrogenase activity (Fig. 19). Without nickel addition, there was no expression of hydrogenase activity until 24 h when there was detectable but very low activity. Addition of 25 uM nickel at 10 h resulted in high levels of hydrogenase activity. However, addition of 25 uM nickel plus 100 ug mL^{-1} chloramphenicol at 10 h resulted only in barely detectable hydrogenase activity compared to that with nickel alone.

5.3.6. *Effect of nickel on the sensitivity of hydrogenase synthesis to* O_2

Increasing the concentration of nickel in the medium alleviated the sensitivity of hydrogenase synthesis to O_2. With 25 uM Ni^{2+}, there was about a 30% decrease in expression of hydrogenase activity in contrast to that with 2.5 uM Ni^{2+} which showed about a 100% decrease under 10 and 20 kPa O_2.

5.3.7. ^{63}Ni *incorporation during hydrogenase derepression*

Cells incubated under hydrogenase-derepression conditions incorporated $^{63}Ni^{2+}$ in a progressive manner (Fig. 20). For cells placed under non-derepression conditions, there was an initial uptake at 2 h of incubation and thereafter a gradual decline of radioactivity was observed.

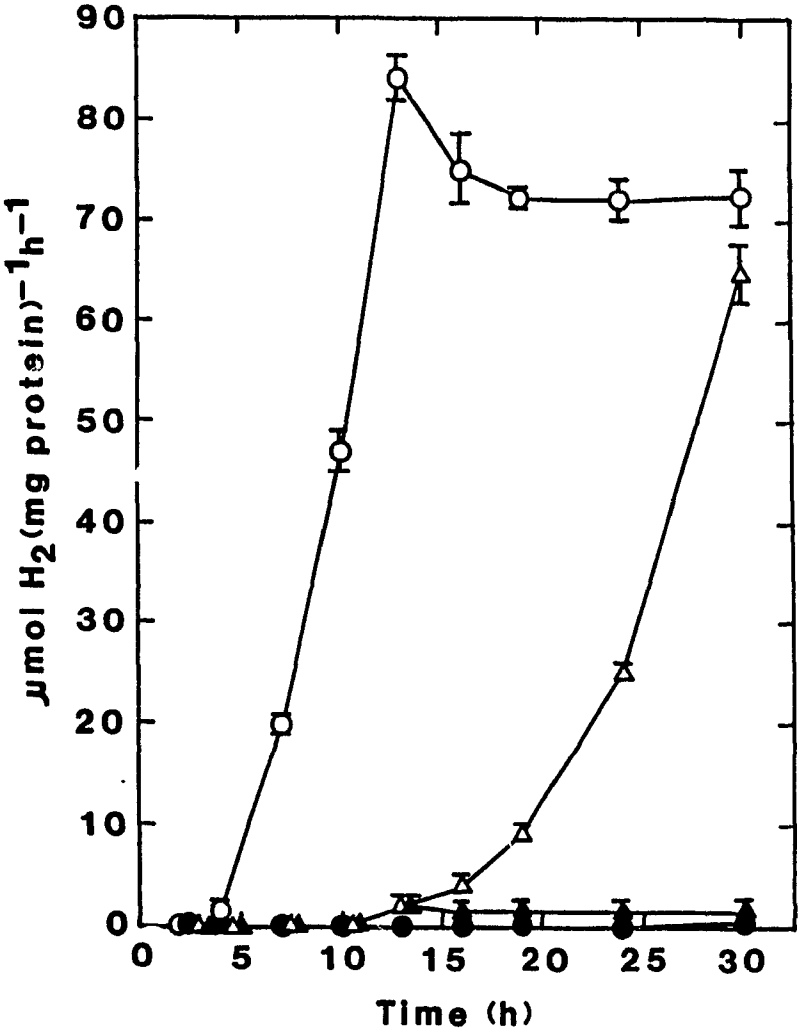


Fig. 19. Effect of nickel (25 uM) and chloramphenicol (100 ug mL^{-1}) on expression of hydrogenase activity by *Pseudomonas saccharophila*. The treatments were as follows: plus Ni^{2+} at 0 h (○), plus Ni^{2+} at 10 h (△), minus Ni^{2+} (●), and plus Ni^{2+} plus Cm at 10 h (▲). Five-hundred mL flasks each containing 150 mL medium plus EDTA (50 uM) were inoculated with 0.3 mL of cell suspension (2.04 mg protein mL^{-1}). The atmosphere during derepression was 20 kPa H_2, 5 kPa CO_2, 2 kPa O_2, balance N_2. Methylene blue was the electron acceptor in the assay. Data are averages of 3 replicates. Bars indicate ± SEM.

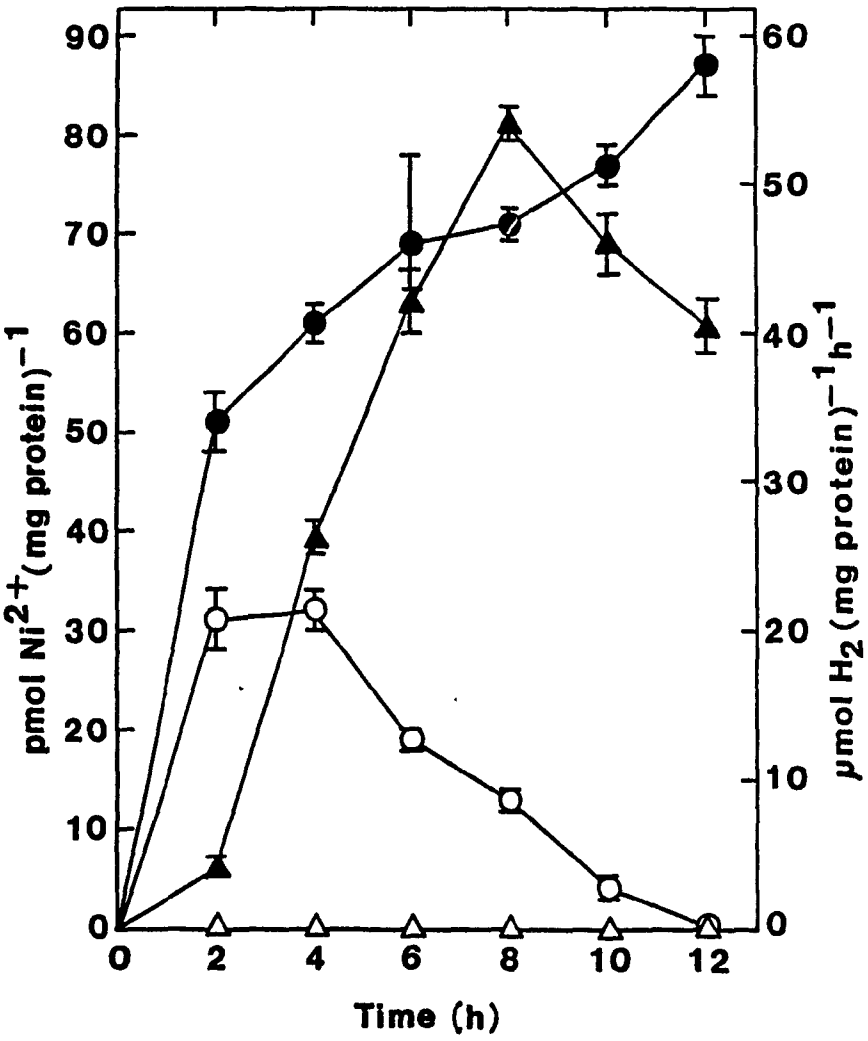


Fig. 20. Nickel accumulation (○,●) and hydrogenase expression (△,▲) in *Pseudomonas saccharophila*. Closed and open symbols represent incubation with and without exogenous H_2 in the headspace, respectively. Data are averages of 3 replicates. Bars indicate ± SEM.

5.3.8. *Kinetics of nickel uptake*

Nickel uptake by resting cells from NH_4^+-grown heterotrophic culture of *Pseudomonas saccharophila* using 5 uM $^{63}NiCl_2$ was relatively linear to at least 4 min (Fig. 21). It followed Michaelis-Menten saturation kinetics over the concentration range from 5 to 220 uM $^{63}NiCl_2$ (Fig. 22). The apparent K_m was determined by linear regression analysis to be 31.7 uM; the maximum specific rate of uptake, V_{max}, was 3.8 nmol Ni^{2+} (mg protein)$^{-1}$ min^{-1}.

5.3.9. *Nickel uptake by hydrogenase-derepressed cells*

Cells with preformed hydrogenase had essentially the same nickel uptake rates as cells without hydrogenase (Table 19). The presence of exogenous H_2 did not affect the nickel uptake rate of cells with preformed hydrogenase.

5.3.10. *Effect of energy source addition on nickel uptake*

Addition of sucrose and placing the cells under hydrogenase-derepression conditions had no significant effect on nickel uptake rates (Table 20).

5.3.11. *Effect of metabolic inhibitor and EDTA on nickel uptake*

Of the inhibitors tested, only NaCN had a significant effect on nickel uptake (Table 21). The inhibitory effect of NaCN is probably due to increased pH upon its addition or probably due to formation of a cyano-nickel complex as was suggested by other investigators (Partridge and Yates 1982; Lohmeyer and Friedrich 1987). EDTA completely inhibited uptake, most likely by chelating nickel.

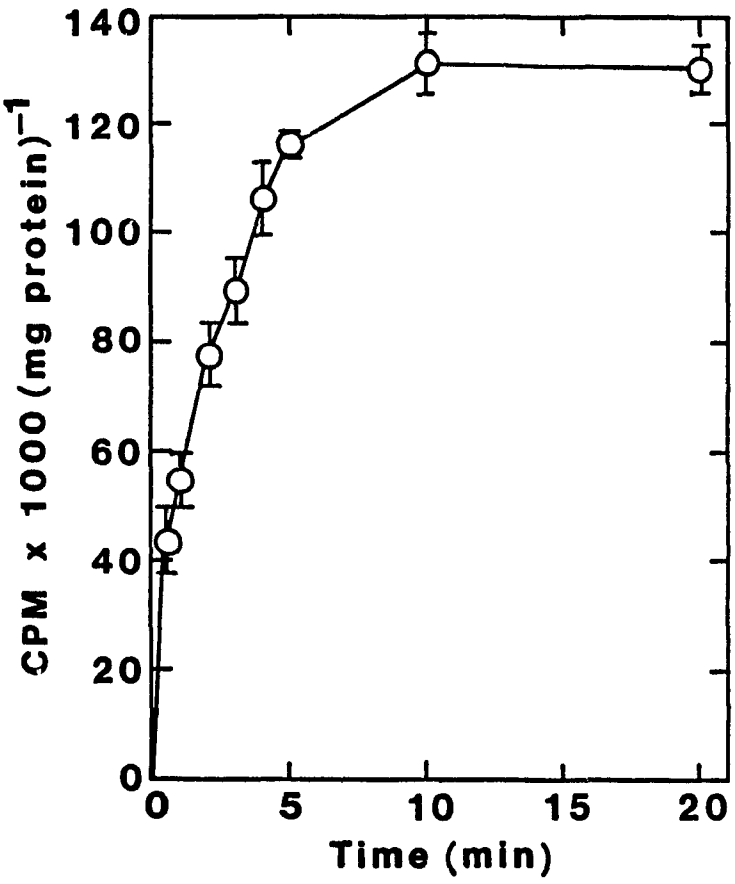


Fig. 21. Time course of nickel uptake by *Pseudomonas saccharophila*. The concentration of $^{63}NiCl_2$ used was 5 uM. Data are averages of 3 replicates. Bars indicate ± SEM.

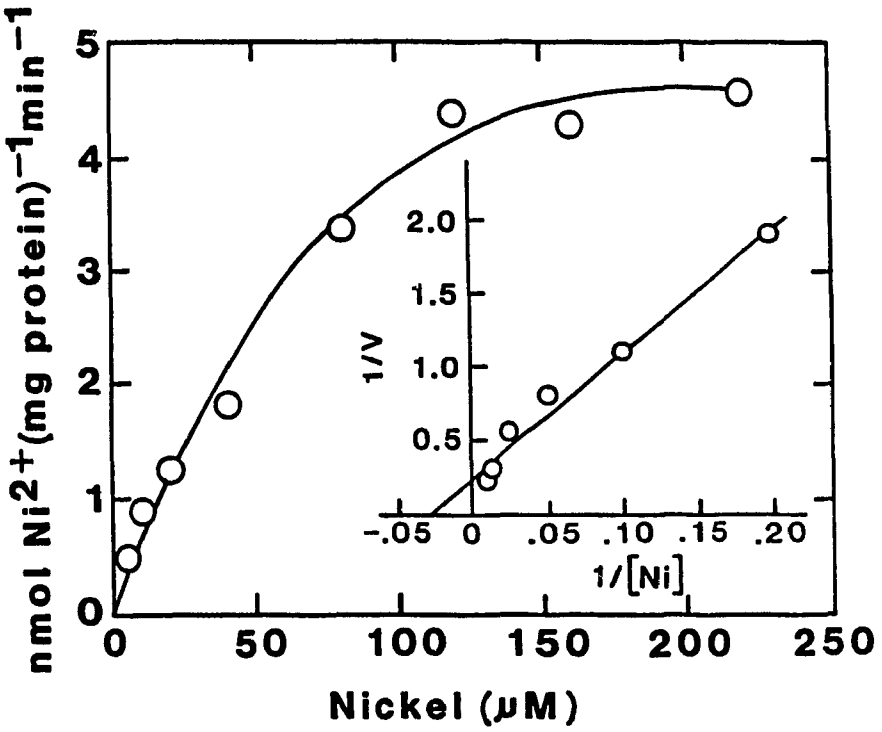


Fig. 22. Kinetics of nickel uptake by *Pseudomonas saccharophila*. A double reciprocal plot of the data calculated by regression analysis is shown in the inset. Data are averages of 3 replicates.

TABLE 19. Nickel uptake by *Pseudomonas saccharophila* cells derepressed and not derepressed for hydrogenase chemolithotrophically[a]

Type of cells	nmol Ni^{2+} (mg protein)$^{-1}$ min^{-1}	
	$-H_2$	$+H_2$
Without hydrogenase	1.4 ± 0.12	1.3 ± 0.06
With hydrogenase[b]	1.1 ± 0.09	1.3 ± 0.08

[a]Cells were incubated under N-limited chemolithotrophic conditions with H_2 (for hydrogenase-derepressed cells) and without H_2 (for cells not derepressed) in the headspace. Nickel uptake assay was carried out with H_2 (10 kPa in air) and without H_2 in the headspace. Data are averages of 4 replicates ± SEM.

[b]Hydrogenase activity of the cells was 10.9 ± 0.12 umol H_2 (mg protein)$^{-1}h^{-1}$.

TABLE 20. Effect of addition of energy source on nickel uptake by *Pseudomonas saccharophila*[a]

Energy source	nmol Ni^{2+} (mg protein)$^{-1}$ min^{-1}	% Uptake rate
None	0.66 ± 0.04	100
Sucrose (3 mM)	0.66 ± 0.02	100
H_2[b]	0.70 ± 0.05	106

[a]Conditions were established 10 min prior to addition of $^{63}NiCl_2$. Data are averages of 4 replicates ± SEM.

[b]20 kPa H_2, 5 kPa CO_2, 2 kPa O_2, balance N_2.

TABLE 21. Effect of metabolic inhibitors and EDTA on nickel uptake by *Pseudomonas saccharophila*[a]

Inhibitor[b]	Concentration	nmol Ni^{2+} (mg protein)$^{-1}$ min^{-1}	% Uptake rate
None	-	0.59 ± 0.04	100
2,4-DNP	1 mM	0.54 ± 0.03	92
DCCD	50 uM	0.68 ± 0.02	115
CCCP	50 uM	0.63 ± 0.02	107
NaCN	10 mM	0	0
NaN_3	10 mM	0.72 ± 0.02	122
EDTA	100 uM	0	0

[a]Cells plus inhibitor were incubated for 10 min prior to addition of $^{63}NiCl_2$. Data are averages of 4 replicates ± SEM.

[b]2,4-DNP, dinitrophenol; DCCD, N, N'-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NaCN, sodium cyanide; NaN_3, sodium azide; EDTA, ethylenediamine-tetraacetic acid.

5.3.12. *Effect of other ions on nickel uptake*

Among the monovalent and divalent ions tested, Co^{2+}, Zn^{2+}, Mg^{2+}, and Mn^{2+} inhibited uptake of nickel by 51 to 30% when present at a concentration 100-fold higher than the ^{63}Ni (Table 22). Nonradioactive Ni^{2+} exhibited about 90% inhibition of uptake of radioactive nickel. In the presence of Fe^{2+} the uptake rate of nickel was substantially increased most likely because of nickel adsorption to the precipitate of $Fe(OH)_3$ formed.

5.3.13. *Exchangeability of $^{63}Ni^{2+}$*

Cells exposed to non-radioactive nickel after exposure to $^{63}Ni^{2+}$ showed removal of radioactive Ni^{2+} that was 58% of that shown by cells not exposed to non-radioactive nickel (Table 23). The exchange reaction seemed to be energy-dependent to a certain degree since 2,4-dinitrophenol decreased the exchange to 39%.

5.3.14. *Intracellular distribution of $^{63}Ni^{2+}$*

About 67% and 27% of the $^{63}Ni^{2+}$ incorporated by cells grown under nitrogen-limited chemolithotrophic conditions with $^{63}NiCl_2$ was found in the soluble and membrane fractions, respectively (Table 24).

5.3.15. *Intracellular distribution of urease activity*

Urease activity was detected mostly in the soluble fraction but some was found in the membranes probably due to some contamination of the soluble fraction (Table 25). There was no urease activity detected in cells grown heterotrophically under aerobic conditions with NH_4^+.

TABLE 22. Effect of other cations on nickel uptake by *Pseudomonas saccharophila*[a]

Cation	% Uptake rate[b]
None (control)	100
$^{58}NiCl_2$	11
$MgCl_2$	61
$ZnCl_2$	58
$CoCl_2$	49
$CuCl_2$	82
$MnCl_2$	70
NH_4Cl	108
$FeCl_2$	257
$CaCl_2$	115
LiCl	127

[a]Cells and individual cations (final conc., 500 uM) were incubated for 10 min at 30°C prior to addition of $^{63}NiCl_2$. Data are averages of 3 replicates.

[b]Nickel uptake rate of control was 0.74 ± 0.09 nmol Ni^{2+} (mg protein)$^{-1}$min^{-1}.

TABLE 23. Exchangeability of $^{63}Ni^{2+}$ taken up by *Pseudomonas saccharophila*[a]

Treatment	nmol Ni^{2+} (mg protein)$^{-1}$	% Exchange
1 min with $^{63}NiCl_2$	0.84 ± 0.07	-
1 min with $^{63}NiCl_2$ then 15 min with $^{58}NiCl_2$	0.35 ± 0.02	58.3
1 min with $^{63}NiCl_2$ then 15 min with 2,4-DNP then 15 min with $^{58}NiCl_2$	0.51 ± 0.03	39.3

[a]The concentration of non-radioactive nickel ($^{58}NiCl_2$) was 500 uM and that of 2,4-DNP (dinitrophenol) was 1 mM. Data are averages of 4 replicates ± SEM.

TABLE 24. Distribution of incorporated $^{63}Ni^{2+}$ in cell-free extract of *Pseudomonas saccharophila* previously grown under N-limited chemolithotrophic conditions[a]

Fraction	nmol Ni^{2+} $(mg\ protein)^{-1}$	Total protein (mg)	Total Ni^{2+} (nmol)	% Ni^{2+}
Cell-free extract	0.17 ± 0.003	27.0	4.5 ± 0.09	100
Soluble	0.18 ± 0.003	16.6	3.0 ± 0.05	67
Membrane	0.12 ± 0	10.2	1.2 ± 0	27

[a]Cells were incubated under N-limited chemolithotrophic conditions for 13 h with 3.16 μM $^{63}NiCl_2$. Data are averages of 3 replicates ± SEM.

TABLE 25. Distribution of urease activity in cell-free extract of *Pseudomonas saccharophila* previously grown under N-limited chemolithotrophic conditions[a]

Fraction	ug NH_4^+-N released (mg protein)$^{-1}$	Total protein (mg)	Total NH_4^+-N (ug)	% Activity
Cell-free extract	14.6 ± 0.2	27.0	394	100
Soluble	20.0 ± 1.0	16.6	332	84
Membrane	5.5 ± 0.6	10.2	56	14

[a]The fractions used were those prepared in the $^{63}Ni^{2+}$ distribution experiment shown in Table 24. Activities were based on 15 min incubation period. Data are averages of 5 replicates ± SEM.

5.4. Discussion

The results clearly show that in the presence of added nickel under N-limited chemolithotrophic conditions, growth of *Pseudomonas saccharophila* and expression of hydrogenase activities were significantly stimulated. Nitrogenase activity was also enhanced in the nickel-enriched culture. Stimulation of chemolithotrophic growth and hydrogenase expression by nickel have been reported in other N_2-fixing organisms (Takakuwa and Wall 1981; Colbeau and Vignais 1983; Klucas *et al.* 1983; Pedrosa and Yates 1983) but there was no mention of the effect of nickel on nitrogenase activity. Bertrand (1974) and Bertrand and DeWolff (1973) reported the stimulatory effect of nickel on soil nitrogenase activity and on yield of soybean plants, respectively. Slater and Capone (Duxbury 1985) reported the stimulation of nitrogenase activity of lake sediments by addition of nickel. In pure cultures, addition of nickel to the heterotrophic semisolid cultures of *Azospirillum brasilense* Sp7 did not have any effect on diazotrophic growth (Pedrosa and Yates 1983). In *Anabaena inaequalis*, photosynthesis and acetylene reduction were completely inhibited by 170 and 340 uM Ni^{2+}, respectively (Stratton and Corke 1979), both relatively high concentrations. The exponential growth rate of nitrogen-fixing *Anabaena cylindrica* was found to be unaffected by omission of nickel from the growth medium (Daday *et al.* 1985). However, nickel was recently shown to facilitate the transition of *Anabaena*

cylindrica from a metabolic state in which nitrogenase activity is repressed to one of active nitrogen fixation (Daday *et al.* 1988). The occurrence of growth and hydrogenase activity of *Pseudomonas saccharophila* in the nickel-deficient medium suggests that there is nickel contaminant in the medium. Difco yeast extract at 0.1% was reported to contain 0.3 uM $NiCl_2$ (Hammel *et al.* 1984). The above observations suggest that hydrogenase formation as well as chemolithotrophic growth in the nickel-deficient medium is limited by the supply of nickel.

Addition of EDTA or NTA inhibited hydrogenase expression most likely due to the unavailability of metals such as nickel. EDTA was reported to effectively inhibit hydrogenase expression in *Bradyrhizobium japonicum* (Stults *et al.* 1984), *Alcaligenes eutrophus* H16 (Friedrich *et al.* 1981), *Xanthobacter autotrophicus* Y38 (Nakamura *et al.* 1985), *Azotobacter chroococcum* (Partridge and Yates 1982), *Azospirillum brasilense* Sp7, *Azospirillum lipoferum* 208b, and *Derxia gummosa* DA (Pedrosa and Yates 1983). The inhibition of hydrogenase expression by EDTA in *P. saccharophila* was overcome by the addition of increasing concentrations of nickel, and other metals were not as effective. This specificity of nickel to stimulate hydrogenase synthesis was also reported in the above mentioned organisms and in *Rhodobacter capsulatus* (Takahwa and Wall 1981). The stimulation of hydrogenase expression by nickel was not affected by Mg^{2+}, SeO_3^{2-}, or SeO_4^{2-}. This

may indicate that (a) nickel uptake by *Pseudomonas saccharophila* occurs by some mechanism (Hausinger 1987) other than that coupled to the Mg^{2+}-transport system (Jasper and Silver 1977; Takakuwa 1987; Bryson and Drake 1988) and (b) the hydrogenase of *P. saccharophila* is not a selenium-containing enzyme in contrast to that of *Bradyrhizobium japonicum* (Boursier *et al.* 1988).

The very low and very high expression of hydrogenase activity in cultures with chloramphenicol plus nickel and with nickel alone, respectively, suggests that nickel is required for the synthesis of hydrogenase in *P. saccharophila*. A similar role of nickel was reported in *Alcaligenes latus* (Doyle and Arp 1988), *Alcaligenes eutrophus* (Friedrich *et al.* 1981), and *B. japonicum* (Stults *et al.* 1986). In *B. japonicum*, it was suggested that nickel regulates hydrogenase synthesis at the transcriptional level (Stults *et al.* 1986) but Doyle and Arp (1988) who worked on *A. latus* proposed that hydrogenase apoprotein could be made in the absence of nickel, but with no nickel present to stabilize the tertiary structure of the enzyme, the apoprotein would be rapidly degraded. The above findings are in contrast to that of *Methylosinus trichosporium* OB3b (Chen and Yoch 1987) and *Nocardia opaca* 1b (Schneider *et al.* 1984) in which nickel is required only for activity.

Hydrogenase formation in *P. saccharophila* is partially repressed by high levels of O_2 (Barraquio and

Knowles 1988) as in other H_2-oxidizing bacteria (Bowien and Schlegel 1981). Nakamura *et al.* (1985) observed oxygen-resistant autotrophic growth of *Xanthobacter autotrophicus* Y38 in the presence of nickel and concluded that the vigorous hydrogenase synthesis under high oxygen tension was the main factor in the oxygen resistance of strain Y38. In the present study, the decrease in sensitivity of hydrogenase synthesis to O_2 was brought about by addition of a higher nickel concentration which resulted in a significant increase in the level of hydrogenase.

The increased tolerance of hydrogenase formation to O_2 upon nickel addition may be due partially to increased stability of the enzyme being formed against O_2 inactivation. Nickel with magnesium were found to be essential not only for the NAD-reducing activity of soluble hydrogenase but also for its stability (at 4°C under air over a range of pH levels between 6 and 8) in *Nocardia opaca* 1b (Schneider *et al.* 1984). Hydrogenase activity of *Pseudomonas saccharophila* is not sensitive to O_2 in a 20-min ^3H-uptake assay (Barraquio and Knowles 1988).

The incorporation of nickel during hydrogenase derepression suggests that hydrogenase may act as a sink for nickel. It is apparent that uptake not associated with hydrogenase synthesis occurs in cells which were not derepressed for hydrogenase since nickel accumulation peaked initially at 2 h incubation. However, the progressive decline in nickel taken up by these cells might

suggest that the ion is not tightly bound to cell components and that there is no readily available sink for nickel. Nickel incorporation under hydrogenase derepression conditions was also demonstrated in *Azotobacter chroococcum*, *Bradyrhizobium japonicum*, and *Alcaligenes latus* (Partridge and Yates 1982; Stults *et al.* 1987; Doyle and Arp 1988).

Resting cells of heterotrophically-grown *Pseudomonas saccharophila* were able to take up nickel for a short period under conditions in which hydrogenase was not derepressed. When they were placed under an atmosphere conducive to hydrogenase derepression, the rate of short-term nickel uptake was not affected. Yates and Robson (1985) and Takakuwa (1987) did not find any difference in the incorporated $^{63}Ni^{2+}$ between Hup^+ and Hup^- strains of *A. chroococcum* and *Rhodobacter capsulatus*. In *Alcaligenes eutrophus* H16, the long-term uptake of $^{63}Ni^{2+}$ was markedly diminished when the appropriate autotrophic conditions were not provided (Kaltwasser and Frings 1980). In *Clostridium pasteurianum* and *Acetogenium kivui*, nickel uptake (3 to 30 min incubation) was stimulated by H_2 (Bryson and Drake 1988; Yang *et al.* 1989) most probably due to the involvement of hydrogenase. In the present study, the presence of preformed hydrogenase with or without exogenous H_2 did not affect the short-term uptake rate suggesting three possible interpretations: (a) the energy generated through the hydrogenase-catalyzed reaction is not

supporting $^{63}Ni^{2+}$ uptake; (b) $^{63}Ni^{2+}$ is not inserted into the preformed apohydrogenase if such apoprotein is present; and (c) $^{63}Ni^{2+}$ is not exchangeable with nonradioactive nickel which could have been tightly bound to hydrogenase.

The kinetic parameters for nickel uptake in *Pseudomonas saccharophila* suggest that the uptake system has a relatively low affinity for and a high rate of accumulation of nickel. The K_m value of 31.7 uM nickel is very similar to that of the wild-type strain of *Bradyrhizobium japonicum* with 26 uM (Stults *et al.* 1987). The present uptake system has a lower affinity for nickel compared to that of other N_2-fixing organisms such as *Rhodobacter capsulatus* with 5.5 uM and *Anabaena cylindrica* with 17 nM (Campbell and Smith 1986; Takakuwa 1987), and other non-N_2-fixing H_2-oxidizing organisms such as *Alcaligenes eutrophus* H16 with 17 uM, *Methanobacterium bryantii* with 3.1 uM, *Clostridium thermoaceticum* with 3.2 uM, and *Acetogenium kivui* with 2.3 uM (Jarell and Sprott 1982; Lohmeyer and Friedrich 1987; Lundie et al. 1988; Yang *et al.* 1989); it has a higher affinity than that of *Clostridium pasteurianum* with 85 uM and *Methanothrix concilii* with 91 uM (Bryson and Drake 1988; Baudet *et al.* 1988).

The present data may indicate the existence of two forms of nickel-uptake system in *P. saccharophila*. The major nickel-uptake system does not appear to be dependent on energy, either chemical or electrochemical, since sucrose, preformed hydrogenase plus exogenous H_2, and

metabolic inhibitors had no significant effect on uptake rate. Hence, the uptake system of *Pseudomonas saccharophila* resembles that of *Bradyrhizobium japonicum* (Stults *et al.* 1987), *Methanothrix concilii* (Baudet *et al.* 1988), and *Azotobacter chroococcum* (Partridge and Yates 1982).

One-hundred fold excess of monovalent cations had no effect on nickel uptake, and divalent metals such as Co^{2+}, Zn^{2+}, Mg^{2+}, and Mn^{2+} did not completely inhibit nickel uptake. Unlabelled Ni^{2+} gave the highest inhibition of $^{63}Ni^{2+}$ uptake. These results suggest that the major uptake system of *P. saccharophila* is relatively specific for nickel. Among the divalent metals other than $^{58}Ni^{2+}$, Co^{2+} gave the highest inhibition of nickel uptake. Both Co^{2+} and Ni^{2+} are transported by the same system that transports Mg^{2+} in *Enterobacter (Aerobacter) aerogenes*, *Bacillus megaterium*, *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus subtilis* (Webb 1970; Jasper and Silver 1977; Silver and Lusk 1987). Co^{2+} was the only metal found to appreciably inhibit nickel uptake in *Methanobacterium bryantii* (Jarell and Sprott 1982) and *M. concilii* (Baudet *et al.* 1988) but these nickel-uptake systems are not coupled to the Mg^{2+}-transport system. These observations therefore indicate that Co^{2+} uptake may not necessarily be part of the Mg^{2+}-transport system. The fact that nickel uptake in *P. saccharophila* was inhibited to a certain extent by Mg^{2+}, might suggest that it is part of the energy-dependent Mg^{2+}-transport system reported in other

organisms (Webb 1970; Jasper and Silver 1977; Takakuwa 1987; Lohmeyer and Friedrich 1987; Bryson and Drake 1988; Nies and Silver 1989). However, Mg^{2+} at concentration 100 times higher than $^{63}Ni^{2+}$ did not completely inhibit nickel uptake. Moreover, the nickel-uptake system of *Pseudomonas saccharophila* does not appear to be energy-dependent and a 40-fold excess of Mg^{2+} in combination with Ni^{2+} did not affect hydrogenase synthesis. These observations suggest that the major nickel-uptake system of *P. saccharophila* is not coupled to the Mg^{2+}-transport system.

The possible presence of a second minor nickel-uptake system which appears to be energy-dependent and to be coupled to the Mg^{2+}-transport system is based on the following observations: (a) DNP slightly inhibited nickel uptake, (b) DNP decreased exchange between radioactive and non-radioactive nickel, and (c) Mg^{2+} showed partial inhibition of nickel uptake.

About 58% of the $^{63}Ni^{2+}$ taken up was exchangeable with a 100-fold excess of nonradioactive nickel. Because the exchange reaction was inhibited partially by 2,4-dinitrophenol, the reaction is therefore energy-dependent to a certain extent. It is reasonable to assume that the remaining 42% of the $^{63}Ni^{2+}$ taken up is tightly bound to cellular components.

The $^{63}Ni^{2+}$ incorporated during growth under nitrogen-limited chemolithotrophic conditions was distributed with a lower percentage in the membrane fraction (27%) than in the

soluble fraction (67%). Nakamura *et al.* (1985) found 68% and 19% nickel distributed into particulate and soluble fractions, respectively, of autotrophically-grown (with urea as nitrogen source) cells of *Xanthobacter autotrophicus* strain Y38. They found hydrogenase residing in the membrane fraction and also reported the presence of nickel-stimulated urease activity but its location was not mentioned. Colbeau and Vignais (1983) found 50% of the incorporated $^{63}Ni^{2+}$ in the membrane proteins of *Rhodobacter capsulatus*. Similarly, Takakuwa (1987) found about 50% and 35% of $^{63}Ni^{2+}$ incorporated in the membrane and soluble fractions, respectively, of *R. capsulatus* and suggested that the presence of urease may account for the $^{63}Ni^{2+}$ found in the soluble fraction. In *Alcaligenes eutrophus* H16, approximately 80% of the radioactive nickel was located in the soluble fraction and 20% in the particulate fraction (Friedrich *et al.* 1982). In this organism, the soluble fraction contains the NAD-reducing hydrogenase and the membrane-bound hydrogenase resides in the particulate fraction. In the present study, it was expected that the membrane fraction would show higher $^{63}Ni^{2+}$ incorporation than the soluble fraction because hydrogenase activity was found in the former preparation (see Chapter 4). The fact that there was a relatively low percentage of $^{63}Ni^{2+}$ found in the membrane fraction could possibly be explained by the high level of urease in the soluble fraction. Urease is a known nickel enzyme (Mobley and Hausinger 1989).

6. A COUNTING METHOD FOR FREE-LIVING AEROBIC N_2-FIXING H_2-OXIDIZING BACTERIA

6.1. Introduction

In the N_2-fixation process, N_2 is converted to NH_3, with the concomitant production of H_2. Most N_2-fixing bacteria have the ability to utilize this H_2 as an energy source for growth and other metabolic activities. The benefits of recycling H_2 were proposed and subsequently demonstrated in some N_2-fixing bacteria (Dixon 1972; Robson and Postgate 1980). In this view, the occurrence of N_2-fixing H_2-oxidizing bacteria in natural systems, particularly H_2-rich sites such as legume fields (Conrad and Seiler 1979; Conrad and Seiler 1980), would be of considerable significance in terms of contribution to the N economy and conservation of H_2. However, reports on populations of N_2-fixing H_2-oxidizing bacteria in natural systems are few (De Bont and Leijten 1976; Watanabe *et al.* 1982; Gowda and Watanabe 1985; Dugnani *et al.* 1986).

For isolation and enumeration of N_2-fixing H_2-oxidizing bacteria, the N-deficient autotrophic medium with H_2, CO_2, N_2 as normalizing gas, and a small amount of O_2 is normally used (De Bont and Leijten 1976; Wiegel and Schlegel 1976; Aragno and Schlegel 1981; Knowles 1982a; Watanabe *et al.* 1982; Gowda and Watanabe 1983). However, some aerobic N_2-fixing H_2-oxidizing bacteria such as *Azospirillum brasilense*, *Azomonas agilis*, *Bacillus*

polymyxa, *Beijerinckia indica*, *Klebsiella pneumoniae*, *Azotobacter vinelandii*, and *Azotobacter chroococcum* do not grow chemolithotrophically (Bowien and Schlegel 1981; Malik and Schlegel 1981; Gowda and Watanabe 1983; Wong and Maier 1985). Furthermore, autotrophic methods of enumerating these bacteria involve problems in maintaining the desired atmosphere and monitoring growth and activity. Except for the cyanobacteria and other phototrophic bacteria, all known aerobic N_2-fixing H_2-oxidizing bacteria are basically heterotrophic (Aragno and Schlegel 1981; Bowien and Schlegel 1981), hence their isolation or enumeration may be simpler using a heterotrophic medium in which both nitrogenase and hydrogenase activity could be demonstrated. Although in general high concentrations of utilizable organic substrates repress formation of hydrogenase, relatively low levels would allow its expression (Maier *et al.* 1978; Barraquio and Knowles 1988).

This section of the thesis reports a most-probable-number (MPN) enumeration technique for aerobic N_2-fixing H_2-oxidizing bacteria that uses a heterotrophic semisolid medium in which both nitrogenase and uptake hydrogenase activities can be detected. The method was validated with pure cultures of diazotrophs and then employed to enumerate N_2-fixing H_2-oxidizing bacteria in some natural samples.

6.2. **Materials and methods**

6.2.1. *Bacterial strains*

The organisms used and their sources are shown in

Table 26. Except for *Pseudomonas saccharophila*, *Pseudomonas* strain 4B, *Pseudomonas stutzeri* (strain number not available), *Pseudomonas stutzeri* JM 300, *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas* G59, and the azotobacters, all were organisms isolated from the roots or rhizosphere of various plants. Three *Pseudomonas* strains, GR3-5, GR8-8, and GR12-2, were obtained from plants growing in the Canadian High Arctic (Lifshitz *et al.* 1986). *P. aeruginosa* served as negative control.

6.2.2. *Media*

The combined carbon source (sucrose, lactate and mannitol) medium as described by Rennie (1981) was used except for the following modifications: the vitamins were omitted (yeast extract was retained); 2.0 g L^{-1} Difco Noble agar was added; $Na_2FeEDTA$ was replaced with Sequestrene NaFe (13% Fe, Ciba Geigy Corp., Greensboro, N.C., U.S.A. 27400) at 0.062 g L^{-1}; and $NaMoO_4.2H_2O$ was reduced from 25 to 5.9 mg L^{-1}. It was later found that to obtain good growth of some organisms such as *P. stutzeri*, malate was added at a concentration of 2.5 g L^{-1}. The medium was prepared in 250-mL flasks and distributed aseptically into pre-sterilized cotton-plugged 14-mL serum bottles each receiving 4 mL (13 mm depth).

6.2.3. *Cultivation and assay of cultures for nitrogenase and uptake hydrogenase activities*

The pure cultures were grown in 50-mL flasks containing 10 mL liquid sucrose-NH_4Cl-minerals (Barraquio

TABLE 26. The organisms used in the study

Organism	Original source	Donor[a]
Pseudomonas aeruginosa ATCC 9027	infected ear	1
P. saccharophila ATCC 15946	mud from stagnant pond	2
P. stutzeri JM 300	soil	3
P. stutzeri (unnumbered strain)	soil	4
P. stutzeri CMT.9.A	*Sorghum* rhizosphere	5
P. diazotrophicus H8 ATCC 35402	wetland rice roots	6
Pseudomonas KLH 76	wetland rice roots	6
P. paucimobilis 5AJ & 29AJ	wetland rice rhizosphere	7
Pseudomonas DC	*Deschampsia caespitosa* roots	2
Pseudomonas 4B ATCC 43038	forest soil	2
Pseudomonas G59	soil percolated with nitrate	8
Pseudomonas GR3-5	*Eriophorum angustifolium* roots	9
Pseudomonas GR8-8	*Salix* sp. roots	9
Pseudomonas GR12-2	roots of unidentified grass	9
Azotobacter chroococcum MCD1	soil	10
A. vinelandii	soil	11
Azospirillum lipoferum ATCC 29707	wheat rhizosphere	12
A. lipoferum 34H	wetland rice roots	6
A. amazonense Y6 ATCC 35121	*Digitaria* roots	12
A. brasilense Sp7 ATCC 29145	*Digitaria* rhizosphere	12

[a] 1, J. Ingram, Department of Microbiology, Macdonald College, Ste. Anne de Bellevue, Quebec, Canada; 2, Y.-K. Chan, Agriculture Canada, Ottawa, Ontario, Canada; 3, C. A. Carlson, University of California, Davis, Calif., USA; 4, B. A. Bryan, University of Iowa, Iowa City, Iowa, USA; 5, D. Werner, Fachbereich Biologie, Philipps-Universität, Marburg, Federal Republic of Germany; 6, I. Watanabe, International Rice Research Institute, Los Banos, Philippines; 7, J. Balandreau, Centre de Pédologie, Centre National de la Recherche Scientifique, Nancy, France; 8, T. Aida, Ibaraki University, Ibaraki, Japan; 9, J. W. Kloepper, Allelix, Inc., Ontario, Canada; 10, K. Tibelius, Department of Microbiology, Macdonald College, Ste. Anne de Bellevue, Quebec, Canada; 11, Department of Microbiology, Macdonald College, Ste Anne de Bellevue; 12, J. Döbereiner, Empresa Brasileira de Pesquisa Agropecuaria, Rio de Janeiro, Brazil.

and Knowles 1988) or glucose-NH_4Cl-minerals or malate-NH_4Cl-minerals or nutrient broth, as appropriate for the organism, for 24 h at 30°C (25°C for arctic isolates) on a gyratory shaker (250 rpm). This culture was used to inoculate 10 mL of the same medium to a final concentration of 5% or 10%, which was then incubated for 12 h under the same conditions. The cells were harvested by centrifugation (10,000 x *g* for 10 min) and washed thrice with saline, then resuspended in the original volume. One-tenth mL of this suspension was inoculated into each of a series of the serum bottles containing the melted (55°C) semisolid medium (no malate added). The serum bottles were incubated for 3 days at 30°C (5 days at 20°C for arctic isolates) under stationary conditions, and then the cotton plugs were carefully replaced with sterilized butyl stoppers. First an experiment was carried out in which one set of cultures was used for C_2H_2 reduction and the other set for tritium (H^3H) uptake assays. For C_2H_2 reduction assay, 0.1 mL of cotton-filtered C_2H_2 (Liquid Carbonic Canada, Ltd) was injected into each serum bottle and then the bottles were incubated for 24 h at 30°C (20°C for arctic isolates) under stationary conditions. For H^3H uptake assay, 0.3 mL of H^3H (specific activity, 20.6 uCi per mL) was injected into each bottle, then the bottles were incubated similarly. In other experiments the cultures used for C_2H_2 reduction assay were then subjected to H^3H uptake assay. After 0.2 mL gas samples were taken for C_2H_4 analysis. 0.3 mL of H^3H was

injected. The bottles were incubated for another 24 h under the same conditions as above, after which they were vortexed vigorously to homogenize. Then 0.1 mL samples were taken and transferred into scintillation vials containing 4 mL of Beckman Ready-Solv MP scintillation liquid. Triplicate bottles were used throughout the experiment.

6.2.4. *Analyses*

C_2H_4 was analyzed by FID gas chromatography as described previously (Tibelius and Knowles 1983). Radioactivity was determined using a Beckman LS 7500 liquid scintillation counter with ^{3}H-galactose as the standard.

6.2.5. *Environmental samples*

All the samples were taken between the months of June and September 1987. The plant samples, namely corn (hybrid Coop S259), wheat (*cv.* Frankenmuth), oat (*cv.* Laurent), barley (*cv.* Cadette), soybean (*cv.* Maple Arrow), and white bean (*cv.* Exrico 23), were obtained from the experimental plots of the Plant Science Department of Macdonald College. The plots were on a St. Bernard sandy loam and were either unfertilized or fertilized with ammonium nitrate. The litter samples were taken from under beech and pine in the Morgan Arboretum of McGill University, and the sediment samples came from a freshwater lake in Ontario, Lake St. George.

6.2.6. *Enumeration of N_2-fixing H_2-oxidizing bacteria in natural samples*

Samples were taken from the field and transported as soon as possible in plastic bags to the laboratory for processing. The lake sediment samples were kept overnight at 4°C. Samples of litters, roots, rhizosphere soils, and lake sediments were homogenized with saline for 20 min using a Virtis homogenizer. Soil from roots or nodules was sampled by shaking the material in 5 mL of saline. Ten-fold dilutions of the samples were made in saline and 0.1 mL of each dilution was inoculated into each serum bottle containing melted semisolid medium. Four serum bottles were inoculated for each dilution. All other conditions of incubation and assay were similar to those used for pure cultures. The cultures used for C_2H_2 reduction assay were also used for H^3H uptake assay. The cultures considered positive had C_2H_4 concentrations and ^3H uptake greater than those in uninoculated controls plus three times the control standard deviation. An MPN table for 4 tubes per dilution was constructed based on the formula of Halvorson and Ziegler (1933).

6.3. **Results and discussion**

6.3.1. *Pure cultures*

The semisolid heterotrophic medium with a small amount of yeast extract in combination with the MPN method is an established procedure to grow and enumerate aerobic diazotrophs (Knowles 1982a). In the present study, its use

was extended by incorporating the assay for uptake hydrogenase. The present technique combines two sensitive assay methods: the acetylene reduction and the O_2-dependent H^3H uptake. The semisolid medium allows the microaerophilic and aerobic diazotrophs to grow at the subsurface and surface of the medium, respectively. Thus if the O_2-sensitive nitrogenase is expressed under this condition, hydrogenase, which is often equally O_2-sensitive (Robson and Postgate 1980), may be expected to be expressed too. Furthermore, although the medium contains a relatively high level of the organic substrates, it is expected that the level decreases during growth to allow expression of hydrogenase.

All of the organisms listed in Table 26 showed nitrogenase activity, as well as hydrogenase activity in the presence and absence of 1 kPa C_2H_2, with the exception of *Pseudomonas aeruginosa*, *Pseudomonas paucimobilis* 29AJ, and *Pseudomonas* G59 which were negative for all three activities. Thus, all the diazotrophs that were reported previously to possess uptake hydrogenase (Bowien and Schlegel 1981; Malik and Schlegel 1981; Barraquio *et al.* 1983; Gowda and Watanabe 1983; Chan *et al.* 1986; Fu and Knowles 1986; Watanabe *et al.* 1987; Barraquio and Knowles 1988) exhibited positive activities. The results showed for the first time the presence of nitrogenase and hydrogenase activities in *Pseudomonas stutzeri* JM 300 and *Pseudomonas stutzeri* (unnumbered strain), and hydrogenase activity in

Pseudomonas stutzeri CMT.9.A (nitrogenase activity in this strain was reported by Krotzky and Werner (1987)), *Pseudomonas* strain 4B, *Pseudomonas paucimobilis* 5AJ, and the arctic *Pseudomonas* strains GR3-5, GR8-8, and GR12-2. All these pseudomonads except CMT.9.A and the three arctic isolates showed variable hydrogenase activities at least as high as, and some much higher than, the uninoculated control plus three times the control standard deviation. The inoculum size seemed to affect the activity, as large inocula gave well-developed pellicles that showed higher nitrogenase and hydrogenase activities. In earlier experiments in which a different medium was used, strains 4B and 5AJ had the same ^{3}H counts as the control (Barraquio and Knowles, Abstracts of the 87th Ann. Meeting of the Amer. Soc. Microbiol. p. 207, 1987). In the case of strains CMT.9.A, GR3-5, GR8-8, and GR12-2, several attempts to detect hydrogenase activity in the combined carbon source medium failed although nitrogenase activity was positive. Low hydrogenase activity was detected in these strains when malate was added to the medium and a large inoculum was used.

The fact that *Pseudomonas saccharophila* has hydrogenase which is expressed significantly only in the presence of H_2 (Barraquio and Knowles 1988) does not present a problem for the technique. The hydrogen evolved during N_2 fixation most likely induces the formation of the enzyme. Under autotrophic N_2-fixing conditions, exogenous

CO_2 and H_2 are absolutely necessary to support chemoautotrophic growth. Under heterotrophic N_2-fixing or non-N_2-fixing (in the presence of exogenous H_2) conditions, CO_2 is not needed for expression of hydrogenase (Tibelius and Knowles 1983; Barraquio and Knowles 1988).

Hydrogenase-positive organisms exhibited activity even in the presence of C_2H_2. C_2H_2 is a known inhibitor of uptake hydrogenase of some diazotrophs (Smith *et al.* 1976; Chan *et al.* 1980; Tibelius and Knowles 1983; Hyman and Arp 1987). Hydrogenases of some diazotrophs like *Pseudomonas saccharophila*, *Azospirillum amazonense*, *Azospirillum lipoferum*, *Derxia gummosa*, *Xanthobacter autotrophicus*, and *Bradyrhizobium japonicum* (bacteroids) are not very sensitive to C_2H_2 (Berndt and Wölfle 1978; Ruiz-Argüeso *et al.* 1979; Ravi Shankar *et al.* 1986; this thesis; Fu and Knowles 1988). One kPa C_2H_2 used in this study may exert some partial inhibitory effects on some C_2H_2-sensitive hydrogenases but it is much less than the 5 kPa used by Smith *et al.* (1976) and the 10 kPa used by Chan *et al.* (1980) who obtained a 51 and 81% inhibition, respectively.

6.3.2. *Environmental samples*

Examination of the natural samples showed that the technique can be used to enumerate and possibly isolate N_2-fixing H_2-oxidizing bacteria. The medium, however, could be modified by the addition of malate to include those organisms that do not utilize, or utilize weakly, the three original carbon sources. Nickel may also be included based

on the results presented in Chapter 5 of this thesis. The results of enumeration (Table 27) suggest that there were three types of natural systems in which (a) N_2-fixing bacteria were all H_2-oxidizing, (b) N_2-fixing bacteria were fewer than H_2-oxidizing bacteria, and (c) H_2-oxidizing bacteria were fewer than N_2-fixing bacteria. The first two systems may have a greater ability than the third to recover most of the hydrogen evolved during N_2 fixation. In the second system, it must be noted that some H_2-oxidizing bacteria in highly-diluted MPN cultures did not reduce C_2H_2. The medium could no doubt support a slight growth of non-N_2-fixing heterotrophs because of the small amount of yeast extract present. It may be concluded either that their hydrogenases are constitutive or that the 24-h exposure to H^3H might have stimulated hydrogenase expression.

The results showed that the soil attached to white bean nodules contained numbers of N_2-fixing H_2-oxidizing bacteria which were considerably higher than the other natural samples tested (Table 27). It should be noted, however, that the determinations were done only at one particular time thus making it difficult to draw a general observation. Enrichment of H_2-oxidizing bacteria in the rhizosphere of the nodules of hydrogen-evolving alfalfa, pigeon peas, and soybean was reported (LaFavre and Focht 1983; Cunningham *et al.* 1986; Wong *et al.* 1986). In the case of the alfalfa, all the isolates were negative for

TABLE 27. Populations of N_2-fixing H_2-oxidizing bacteria associated with some natural systems[a]

Sample[b]	MPN (10^4 bacteria/g dry wt) of the following bacteria:[c]		
	H_2ox	N_2fix	H_2ox N_2fix
Rhizosphere			
Corn[d] roots + RS	0	0	122
Wheat[d] roots + RS	0	107	32
Oat[d] roots + RS (140 kg of N/ha)	49	402	130
Barley[d] RS (0 kg of N/ha)	0	0	42
Barley[d] roots (0 kg of N/ha)	0	5	16
Barley[d] RS (150 kg of N/ha)	0	9	10
Barley[d] roots (150 kg of N/ha)	372	0	66
Soil from soybean[e] nodules	0	0	63
Soybean[e] roots + RS	11	7	13
White bean[f] + RS	563	0	37
Soil from white bean[f] nodules with:			
Mycorrhiza (0 P_2O_5)	0	0	2950
No mycorrhiza (0 P_2O_5)	0	0	1283
No mycorrhiza (35 kg of P_2O_5/ha)	579	189	361
No mycorrhiza (70 kg of P_2O_5/ha)	0	373	516
Soil			
Humisol	41	0	49
St. Bernard sandy loam	0	342	45
Maple forest brown soil	0	36	10
Litter			
Pine	0	69	7
Beech	3	118	4
Freshwater lake sediment			
Epilimnetic (3-m depth)	13	0	3
Hypolimnetic (14-m depth)	51	0	37

[a]Enumeration was done in semisolid combined carbon source medium without malate.

[b]RS, rhizosphere soil.

[c]H_2ox = H_2-oxidizing (not N_2-fixing); N_2fix = N_2-fixing (not H_2-oxidizing); H_2ox N_2fix = H_2-oxidizing N_2-fixing.

[d]Samples were taken from plants 60 days after planting.

[e]Samples were taken from plants 58 days after planting.

[f]Samples were taken from plants 65 days after planting.

C_2H_2 reduction (Cunningham *et al.* 1986) but for the others there was no indication whether they were N_2-fixing or not. N_2-fixing H_2-oxidizing bacteria were predominant in the histosphere of wetland rice (Watanabe *et al.* 1982). These observations suggest that the rhizosphere may be an important ecological niche for H_2 transformation.

7. GENERAL DISCUSSION AND CONCLUSIONS

7.1. Nitrogen fixation by *Pseudomonas saccharophila*

Pseudomonads are amongst the most ubiquitous and metabolically versatile organisms known (Section 2.1). No legitimate member, however, was reported to fix N_2 (Palleroni 1984). *P. saccharophila* is a well-characterized facultative chemolithotroph (Palleroni 1984) but its ability to fix N_2 was not critically examined. In this thesis, its ability to fix N_2 was demonstrated by C_2H_2 reduction and then confirmed by $^{15}N_2$ incorporation. *P. saccharophila* could fix N_2 under microaerobic conditions both heterotrophically and chemolithotrophically. Hence, it is the first (reported by Barraquio *et al.* 1986) approved species of *Pseudomonas* to exhibit such property (see also Section 2.1). The fact that it can fix N_2 under chemolithotrophic conditions suggests that H_2 via hydrogenase can provide ATP and reducing power for N_2 fixation and CO_2 fixation.

7.2. Hydrogen oxidation by *Pseudomonas saccharophila*

H_2 is evolved during N_2 fixation (see Section 2.2). Most diazotrophs are equipped with uptake hydrogenase to recycle this H_2 and such property has at least 3 beneficial effects (see Section 2.3). Theoretically, the presence of uptake hydrogenase will increase the efficiency of N_2 fixation. In this thesis, the presence of an active H_2-uptake system in *P. saccharophila* under both heterotrophi-

and chemolithotrophic conditions was demonstrated. The H_2 recycling in *Pseudomonas saccharophila* under heterotrophic conditions may be for protection of N_2 fixation against H_2 inhibition and /or production of ATP and/or reducing power because exogenous H_2 and uptake hydrogenase activity had no effect on O_2 sensitivity of nitrogenase. This could also be the effect of H_2 recycling in azospirilla (Pedrosa *et al.* 1982; Fu and Knowles 1988). NH_4^+-grown cultures of *P. saccharophila* showed hydrogenase only when H_2 was present in the headspace. Subsequently, H_2 was found to induce hydrogenase synthesis. Other inducible H_2-uptake systems have been reported in diazotrophs and non-diazotrophic hydrogen bacteria (Berndt and Wolfe 1978; Aragno and Schlegel 1978; Maier *et al.* 1979; Colbeau and Vignais 1983; Friedrich *et al.* 1984; De Vries *et al.* 1984; Van Berkum 1987; Doyle and Arp 1987; Prosser *et al.* 1988). It is conceivable that the H_2 evolved during N_2 fixation is responsible for stimulating hydrogenase expression as was thought for *Rhodobacter capsulatus* (Colbeau and Vignais 1983) and most probably for the inducible hydrogenase systems of other diazotrophs. NH_4^+ *per se*, therefore, does not inhibit hydrogenase expression.

Hydrogenase activity of *P. saccharophila* was tolerant to O_2. O_2-tolerant H_2-uptake systems (Aragno and Schlegel 1978; Fu and Knowles 1986; Wilde and Schlegel 1982) and O_2-sensitive ones (Maier *et al.* 1979; Ruiz-Argueso *et al.* 1979; Pedrosa *et al* 1982; Tibelius and Knowles 1983; Fu and

Knowles 1986) were reported. High concentrations of O_2, however, repressed formation of hydrogenase in *Pseudomonas saccharophila*. Synthesis of other hydrogenases are repressed by high O_2 level (Aragno and Schlegel 1978; Tibelius and Knowles 1983; Fu and Knowles 1986; Doyle and Arp 1987; Van Berkum 1987). Isolation of mutants that are tolerant to relatively high O_2 concentration (Merberg *et al.* 1983; Cangelosi and Wheelis 1984) suggests that O_2 regulation of hydrogenase formation is genetically linked. Sucrose at 15 mM repressed the synthesis of hydrogenase in *Pseudomonas saccharophila* but had no effect on the activity of preformed hydrogenase. There are H_2-uptake systems that are either repressible (Maier *et al.* 1979; Partridge *et al.* 1980; Doyle and Arp 1987) or not repressible (Graham *et al.* 1984; Van Berkum 1987; Van Berkum and Maier 1988) by organic substrates. The mechanism of hydrogenase repression by organic substrates is not yet clear and probably different mechanisms operate in O_2-tolerant and O_2-sensitive H_2-uptake systems. In *Bradyrhizobium japonicum*, cyclic AMP reversed hydrogenase repression by malate suggesting a catabolite repression phenomenon (Lim and Shanmugam 1979; McGetrick *et al.* 1985). However, cAMP had no effect on hydrogenase expression in *Alcaligenes eutrophus* and *Azotobacter chroococcum* (Friedrich 1982; Yates and Partridge 1984). In these organisms, the redox state of the cell was the factor that regulated hydrogenase synthesis (Friedrich 1982; Yates and Partridge).

Acidification of the medium and limiting O_2 rather than the organic substrate *per se* were claimed as responsible for decreased hydrogenase activity (Van Berkum 1987; Van Berkum and Maier 1988).

Hydrogenase activity of *Pseudomonas saccharophila* was found mainly in the membrane fraction. This agrees with the results of Podzuweit *et al.* (1983) and the general observation that aerobic diazotrophs have membrane-bound hydrogenase (Bowien and Schlegel 1981; Eisbrenner and Evans 1983). The absence of NAD-dependent soluble hydrogenase in *P. saccharophila* indicates that it has to generate NADH by reverse electron flow (Gottschalk 1986) when growing chemolithotrophically.

7.3. Nickel utilization by *Pseudomonas saccharophila*

Nickel was known for its toxicity (reviewed by Babich and Stozky 1983) until Bartha and Ordal (1965) demonstrated that it was required for chemolithotrophic growth of *Alcaligenes eutrophus*. Now nickel is recognized as a very important metal in biological systems since it is a component of at least 4 enzymes (see Section 2.4). In this thesis, nickel stimulated growth, hydrogenase expression, and nitrogenase activity of *P. saccharophila* under N-limited chemolithotrophic conditions. The stimulation of nitrogenase activity by nickel is presumably through the enhancement of the H_2-uptake system. Reports on the effect of nickel on N_2 fixation are very few. In pure cultures, addition of nickel to heterotrophic semisolid cultures of

Azospirillum brasilense Sp7 did not have any effect on diazotrophic growth (Pedrosa and Yates 1983). In *Anabaena inaequalis*, photosynthesis and acetylene reduction were completely inhibited by 170 and 340 uM nickel, respectively (Stratton and Corke 1979), both relatively high concentrations. Nickel stimulated N_2 fixation in *Anabaena cylindrica* but the effect was neither on nitrogenase nor hydrogenase but on cyanophycin metabolism (Daday *et al.* 1988). In natural systems, nickel stimulated nitrogenase activity of saltmarsh sediments and soil and yield of soybean plants (Bertrand 1974; Bertrand and DeWolff 1974; Duxbury 1985). Stimulation of hydrogenase expression by nickel in *Pseudomonas saccharophila* was specific because other divalent metals and selenium were not as effective as nickel. Nickel-stimulated H_2-uptake systems were also reported in diazotrophs and non-diazotrophic hydrogen bacteria (Friedrich *et al.* 1981; Takakuwa and Wall 1981; Partridge and Yates 1982; Pedrosa and Yates 1983; Stults *et al.* 1984; Nakamura *et al.* 1985). The non-stimulatory effect of selenium on hydrogenase expression by *P. saccharophila* may indicate that the enzyme does not contain selenium in contrast to that of *Bradyrhizobium japonicum* (Boursier *et al.* 1988). Subsequently, nickel was found to be required for hydrogenase synthesis in *P. saccharophila*. The exact mechanism of its involvement, however, is not clear. Stults *et al.* (1986) showed that nickel regulates hydrogenase synthesis at the transcriptional level in *B. japonicum* but

Doyle and Arp (1988) proposed that nickel acts as a stabilizer of newly formed apohydrogenase in *Alcaligenes latus*. Increasing the concentration of nickel decreased the sensitivity of hydrogenase formation to O_2 in *Pseudomonas saccharophila*. This finding seems to be related to the proposal of Doyle and Arp (1988) on the stabilizing effect of nickel on apohydrogenase.

$^{63}Ni^{2+}$ was progressively incorporated under hydrogenase-derepression conditions suggesting that hydrogenase may act as a sink for nickel. However, the incorporated $^{63}Ni^{2+}$ was about 3 times higher in the soluble fraction than in the membrane preparation. This was unexpected because the hydrogenase of *P. saccharophila* is membrane-bound as shown in this thesis and by Podzuweit *et al.* (1983). The results may be explained by the localization of urease in the soluble fraction. Urease is a known nickel enzyme (Mobley and Hausinger 1989). This is the first report of urease activity in *P. saccharophila*. Urease activity was not detected in heterotrophic NH_4^+-grown cells of *P. saccharophila*, and presumably NH_4^+ repressed urease synthesis as reported in other organisms (Mobley and Hausinger 1989). It is possible that under N-limited chemolithotrophic conditions, hydrogenase and urease act as sinks for nickel.

Short-term nickel uptake by resting cells of *P. saccharophila* followed Michaelis-Menten kinetics with an apparent K_m of 31.7 uM and V_{max} of 3.8 nmol Ni^{2+} (mg

protein)$^{-1}$ min^{-1}. It appears to be independent of energy, chemical or electrochemical, because preformed hydrogenase plus H_2 in the headspace, sucrose, and metabolic inhibitors had no effect on nickel uptake rate. The nickel-uptake system of *Pseudomonas saccharophila*, therefore, is similar to that of *Bradyrhizobium japonicum* (Stults *et al.* 1987), *Methanothrix concilii* (Baudet *et al.* 1988), and *Azotobacter chroococcum* (Partridge and Yates 1982). It is relatively specific since no other cation other than Co^{2+} at concentration 100 times higher than $^{63}Ni^{2+}$, showed more than 50% inhibition. Mg^{2+} (500 uM) inhibited nickel uptake by only 39% and in combination with nickel did not affect hydrogenase synthesis. These observations plus the fact that nickel uptake does not appear to be dependent on energy suggest that the nickel-uptake system of *P. saccharophila* is not coupled to the Mg^{2+}-transport system reported in some other organisms (Webb 1970; Jasper and Silver 1977; Takakuwa 1987; Lohmeyer and Friedrich 1987; Bryson and Drake 1988; Nies and Silver 1989).

The present study does not preclude the existence of two forms of nickel-uptake system in *P. saccharophila*. The major uptake system is described above. The second minor uptake system appears to be energy-dependent and to be coupled to the Mg^{2+}-transport system.

7.4. A counting method for N_2-fixing H_2-oxidizing bacteria

The N-deficient autotrophic medium with H_2, CO_2, N_2 as normalizing gas, and a small amount of O_2 is usually

used for isolation and enumeration of N_2-fixing H_2-oxidizing bacteria (DeBont and Leijten 1976; Wiegel and Schlegel 1976; Aragno and Schlegel 1981; Knowles 1982; Watanabe *et al.* 1982; Gowda and Watanabe 1983). However, some aerobic N_2-fixing H_2-oxidizing bacteria do not grow chemolithotrophically (Pedrosa *et al.* 1980; Bowien and Schlegel 1981; Malik and Schlegel 1981; Gowda and Watanabe 1983; Wong and Maier 1985). In this thesis, an enumeration method to accommodate both heterotrophic and chemolithotrophic N_2-fixing H_2-oxidizing bacteria was developed. It employed a semisolid N-limited heterotrophic medium that allowed expression of both nitrogenase and uptake hydrogenase activities which were detected by the sensitive C_2H_2 reduction and H^3H uptake techniques, respectively. Pure culture studies resulted in the discovery of nitrogenase and uptake hydrogenase activities in *Pseudomonas stutzeri* JM 300, an approved species of *Pseudomonas*. Of the natural samples tested, the soil surrounding nodules of white beans showed relatively high numbers of N_2-fixing H_2-oxidizing bacteria. This finding is consistent with reports (Watanabe *et al.* 1982; LaFavre and Focht 1983; Cunningham *et al.* 1986; Wong *et al.* 1986) that the rhizosphere may be an important ecological niche for H_2 transformation.

In conclusion, the involvement of pseudomonads in many important ecological processes makes them one of the most important and commonly studied organisms. The discovery of N_2 fixation in *Pseudomonas saccharophila* and

recently in other strains and known species has ecological implications because of the ubiquity of pseudomonads. The presence of uptake hydrogenase in *P. saccharophila* under heterotrophic N_2-fixing conditions may help increase the efficiency of N_2 fixation. Its beneficial effect, however, may be more significant under chemolithotrophic N_2-fixing conditions. The stimulatory effect of nickel on hydrogenase expression and nitrogenase activity and the possible involvement of nickel in urease under N-limited chemolithotrophic conditions imply that ecosystems limited in N and organic C but rich in nickel, H_2 and CO_2 with relatively low O_2 concentration may be important ecological sites for H_2 and N_2 transformations.

8. REFERENCES

ADAMS, M. W. W., and HALL, D. O. 1979. Properties of the membrane-bound hydrogenase from the photosynthetic bacterium *Rhodospirillum rubrum*. Arch. Biochem. Biophys. **195**: 288-299.

ADAMS, M. W. W., and MORTENSON, L. E. 1984. The physical and catalytic properties of hydrogenase II of *Clostridium pasteurianum*. J. Biol. Chem. **259**: 7045-7055.

ADAMS, M. W. W., MORTENSON, L. E., and CHEN, J.-J. 1981. Hydrogenase. Biochim. Biophys. Acta **594**: 105-176.

AGGAG, M., and SCHLEGEL, H. G. 1974. Studies on a Gram-positive hydrogen bacterium, *Nocardia opaca* 1b. Arch. Microbiol. **100**: 25-39.

AGUILAR, O. M., YATES, M. G., and POSTGATE, J. R. 1985. The beneficial effect of hydrogenase in *Azotobacter chroococcum* under nitrogen-fixing, carbon-limiting conditions in continuous and batch cultures. J. Gen. Microbiol. **131**: 3141-3145.

ALBRACHT, S. P. J., GRAF, E. G., and THAUER, R. K. 1982. The EPR properties of nickel in hydrogenase from *Methanobacterium thermoautotrophicum*. FEBS Lett. **140**: 311-313.

ALEEM, M. I. H., ISHAQUE, M., and DONAWA, A. 1979. Phosphorylation coupled to O_2 and nitrate reduction in *Pseudomonas saccharophila* grown anaerobically with succinate and nitrate. Curr. Microbiol. **2**: 81-84.

ALMON, H., and BOGER, P. 1984. Nickel-dependent uptake-hydrogenase activity in the blue-green alga *Anabaena variabilis*. Z. Naturforsch. Biosci. **39**: 90-92.

ANDERSON, G. R. 1955. Nitrogen fixation by *Pseudomonas*-like soil bacteria. J. Bacteriol. **70**: 129-133.

ANDREWS, R. K., BLAKELEY, R. L., and ZERNER, B. 1988. Nickel in proteins and enzymes. *In* Metal ions in biological systems, vol. 23. *Edited by* H. Sigel and A. Sigel. Marcell Dekker, Inc., New York. pp. 165-284.

ANKEL-FUCHS, D., and THAUER, R. K. 1988. Nickel in biology: nickel as an essential trace element. *In* The bioorganic chemistry of nickel. *Edited by* J. R. Lancaster, Jr. VCH Publishers, New York. pp. 93-110.

ARAGNO, M., and SCHLEGEL, H. G. 1978. Physiological characterization of the hydrogen bacterium *Aquaspirillum autotrophicum*. Arch. Microbiol. **116:** 221-229.

ARAGNO, M., and SCHLEGEL, H. G. 1981. The hydrogen-oxidizing bacteria. *In* The prokaryotes, vol. 1. *Edited by* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel. Springer-Verlag, Berlin. pp. 865-893.

ARP, D. J., MCCOLLUM, L. C., and SEEFELDT, L. C. 1985. Molecular and immunological comparison of membrane-bound, H_2-oxidizing hydrogenases of *Bradyrhizobium japonicum*, *Alcaligenes eutrophus*, *Alcaligenes latus* and *Azotobacter vinelandii*. J. Bacteriol. **163:** 15-20.

BABICH, H., and STOTZKY, G. 1983. Toxicity of nickel to microbes: environmental aspects. Adv. Appl. Microbiol. **29:** 195-265.

BALDANI, J. I., BALDANI, V. L. D., SELDIN, L., and DOBEREINER, J. 1986. Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. Inter. J. Syst. Bacteriol. **36:** 86-93.

BALLY, R., THOMAS-BAUZON, D., HEULIN, T. H., BALANDREAU, J., RICHARD, C., and DE LEY, J. 1983. Determination of the most frequent N_2-fixing bacteria in a rice rhizosphere. Can. J. Microbiol. **29:** 881-887.

BARRAQUIO, W. L., DUMONT, A., and KNOWLES, R. 1980. Enumeration of free-living aerobic N_2-fixing H_2-oxidizing bacteria by using a heterotrophic semisolid medium and most-probable number technique. Appl. Environ. Microbiol. **54:** 1313-1317.

BARRAQUIO, W. L., and KNOWLES, R. 1988. Effect of hydrogen, sucrose, and oxygen on uptake hydrogenase in nitrogen-fixing and ammonium-grown *Pseudomonas saccharophila* ATCC 15946. J. Gen. Microbiol. **134:** 893-901.

BARRAQUIO, W. L., and KNOWLES, R. 1989. Beneficial effects of nickel on *Pseudomonas saccharophila* under nitrogen-limited chemolithotrophic conditions. Appl. Environ. Microbiol. **55:** 3197-3201.

BARRAQUIO, W. L., LADHA, J. K., and WATANABE, I. 1983. Isolation and identification of N2-fixing *Pseudomonas* associated with wetland rice. Can. J. Microbiol. **29:** 817-823.

BARRAQUIO, W.L., PADRE, B. C.,Jr., WATANABE, I., and KNOWLES, R. 1986. Nitrogen fixation by *Pseudomonas saccharophila* Doudoroff ATCC 15946. J. Gen. Microbiol. **132:** 237-241.

BARRAQUIO, W. L. and WATANABE, I. 1981. Occurrence of aerobic nitrogen-fixing bacteria in wetland and dryland plants. Soil Sci. Plt. Nutr. **27:** 121-125.

BARTHA, R., and ORDAL, E. J. 1965. Nickel-dependent chemolithotrophic growth of two *Hydrogenomonas* strains. J. Bacteriol. **89:** 1015-1019.

BAUDET, C., SPROTT, G. D., and PATEL, G. B. 1988. Adsorption and uptake of nickel in *Methanothrix concilii*. Arch. Microbiol. **150:** 338-342.

BAZYLINSKI, D. A., and BLAKEMORE, R. P. 1983. Nitrogen fixation (acetylene reduction) in *Aquaspirillum magnetotacticum*. Curr. Microbiol. **9:** 305-308.

BERNDT, H., and WOLFLE, D. 1978. Hydrogenase: its role as electron generating enzyme in the nitrogen-fixing hydrogen bacterium *Xanthobacter autotrophicus*. *In* Hydrogenases: their catalytic activity, structure, and function. *Edited by* H. G. Schlegel and F. Schneider. Goltze Verlag, Gottingen. pp. 327-351.

BERNSTEIN, D. E. 1944. Studies on the assimilation of dicarboxylic acids by *Pseudomonas saccharophila*. Arch. Biochem. 3: 445-458.

BERTRAND, D. 1974. Le nickel, oligo-élement dynamique pour les microorganismes fixateurs de l azote de l'air. C. R. Acad. Sc. Paris **278:** 2231-2235.

BERTRAND, D., and DEWOLFF, A. 1973. Importance du nickel comme oligo-élément pour les *Rhizobium* des nodosites des legumineuses. C. R. Acad. Sc. Paris **276:** 1855-1858.

BISHOP, P.E., PREMAKUMAR, R., JOERGER, R. D., JACOBSON, M. R., DALTON, D. A., CHISNELL, J. R., and WOLFINGER, E. D. 1988. Alternative nitrogen fixation systems in *Azotobacter vinelandii*. *In* Nitrogen fixation: hundred years after. *Edited by* H. Bothe, F. J. De Bruijn, and W. E. Newton. Gustav Fischer, Stuttgart. pp. 71-79.

BONAM,D., MCKENNA, M. C., STEPHENS, P. J., and LUDDEN, P. W. 1988. Nickel-deficient carbon monoxide dehydrogenase from *Rhodospirillum rubrum*: *In vivo* and *in vitro* activation by exogenous nickel. Proc. Natl. Acad. Sci. U.S.A. **85:** 31-35.

BONE, D. H. 1960. Localization of hydrogen activating enzymes in *Pseudomonas saccharophila*. Biochem. Biophys. Res. Comm. **3**: 211-214.

BOURSIER, P., HANUS, F. J., PAPEN, H., BECKER, M. M., RUSSELL, S. A., and EVANS, H. J. 1988. Selenium increases hydrogenase expression in autotrophically cultured *Bradyrhizobium japonicum* and is a constituent of the purified enzyme. J. Bacteriol. **170**: 5594-5600.

BOWIEN, B., and SCHLEGEL, H. G. 1981. Physiology and biochemistry of aerobic hydrogen-oxidizing bacteria. Ann. Rev. Microbiol. **35**: 405-452.

BREWIN, N. J., DE JONG, T. M., PHILLIPS, D. A., JOHNSTON, A. W. B. 1980. Cotransfer of determinants for hydrogenase activity and nodulation ability in *Rhizobium leguminosarum*. Nature (London), **288**: 77-79.

BRILL, W. J. 1979. Regulation of nitrogen fixation. *In* A treatise of dinitrogen fixation, sect I and II. *Edited by* R. W. F. Hardy, F. Bottomley, and R. C. Burns. John Wiley & Sons, New York. pp. 765-798.

BRYSON , M. F., and DRAKE, H. L. 1988. Energy-dependent transport of nickel by *Clostridium pasteurianum*. J. Bacteriol. **170**: 234-238.

BURNS, R. C., and BULEN, W. A. 1965. ATP-dependent hydrogen evolution by cell-free preparations of *Azotobacter vinelandii*. Biochim. Biophys. Acta **105**: 437-445.

BURR, T. J., and CEASER, A. 1984. Beneficial plant bacteria. Crit. Rev. Plant Sci. **2**: 1-20.

BURRIS, R. H. 1988. 100 years of discoveries in biological N_2 fixation. *In* Nitrogen fixation: hundred years after. *Edited by* H. Bothe, F. J. De Bruijn, and W. E. Newton. Gustav Fischer, Stuttgart. pp. 21-30.

BUSSE, H.-J., EL-BANNA, T., and AULING, G. 1989. Evaluation of different approaches for identification of xenobiotic-degrading pseudomonads. Appl. Environ. Microbiol. **55**: 1578-1583.

CAMMACK, R., FERNANDEZ, V. M., and SCHNEIDER, K. 1986. Activation and active sites of nickel-containing hydrogenases. Biochimie **68**: 85-91.

CAMMACK, R., FERNANDEZ, V. M., and SCHNEIDER, K. 1988. Nickel in hydrogenases from sulfate-reducing, photosynthetic, and hydrogen-oxidizing bacteria. *In* The bioinorganic chemistry of nickel. *Edited by* J. R. Lancaster, Jr. VCH Publishers, New York. pp. 167-190.

CAMPBELL, P. M., and SMITH, G. D. 1986. Transport and accumulation of nickel ions in the cyanobacterium *Anabaena cylindrica*. Arch. Biochem. Biophys. **244:** 470-477.

CANGELOSI, G. A., and WHEELIS, M. L. 1984. Regulation by molecular oxygen and organic substrates of hydrogenase synthesis in *Alcaligenes eutrophus*. J. Bacteriol. **159:** 138-144.

CANNON, F., BEYNON, J., BUCHANON-WOLLASTAN, V., BURGHOFF, R., CANNON, M., KWIATOWSKI, R., LAVER, G., and RUBIN, R. 1985. Progress in understanding organization and expression of *nif* genes in *Klebsiella*. *In* Nitrogen fixation research progress. *Edited by* H. J. Evans, P. J. Bottomley, and W. E. Newton. Nijhoff, Dordrecht. pp. 453-460.

CANTRELL, M. A., HAUGLAND, R. A., and EVANS, H. J. 1983. Construction of a *Rhizobium japonicum* gene bank and use in isolation of a hydrogen uptake gene. Proc. Natl. Acad. Sci. U.S.A. **80:** 181-185.

CHAN, Y.-K., NELSON, L. M., and KNOWLES, R. 1980. Hydrogen metabolism of *Azospirillum brasilense* in nitrogen-free medium. Can. J. Microbiol. **26:** 1126-1131.

CHAN, Y.-K., PAU, R., ROBSON, R. L., and MILLER, R. W. 1988. Expression of unconventional nitrogenase reactions in several nitrogen-fixing bacteria. *In* Nitrogen fixation: hundred years after. *Edited by* H. Bothe, F. J. De Bruijn, and W. E. Newton. Gustav Fischer, Stuttgart. p. 88.

CHAN, Y.-K., WHEATCROFT, R., and WATSON, R. J. 1986. Physiological and genetic characterization of a diazotrophic pseudomonad. J. Gen. Microbiol. **132:** 2277-2285.

CHATT, J. 1981. Towards new catalysts for nitrogen fixation. *In* Current perspectives in nitrogen fixation. *Edited by* A. H. Gibson and W. E. Newton. Elsevier North Holland, New York. pp. 15-21.

CHEN, P. -C., ALMON, H., and BOGER, P. 1986. Evidence for nitrogenase-catalyzed hydrogen-uptake in nitrogen-fixing filamentous blue-green algae. FEMS Microbiol. Lett. **37:** 45-49.

CHEN, J. S., and BLANCHARD, D. K. 1984. Purification and properties of the H_2-oxidizing (uptake) hydrogenase of the N_2-fixing anaerobe *Clostridium pasteurianum* W5. Biochem. Biophys. Res. Comm. **122:** 9-16.

CHEN, Y.-P., and YOCH, D. C. 1987. Regulation of two nickel-requiring (inducible and constitutive) hydrogenases and their coupling to nitrogenase in *Methylosinus trichosporium* OB3b. J. Bacteriol. **169:** 4778-4783.

CHISNELL, J. R., PREMAKUMAR, R., and BISHOP, P. E. 1988. Purification of a second alternative nitrogenase from a *nifHDK* deletion strain of *Azotobacter vinelandii*. J. Bacteriol. **170:** 27-33.

COLBEAU, A., CHABERT, J., VIGNAIS, P. M. 1983. Purification, molecular properties and localization in the membrane of the hydrogenase of *Rhodopseudomonas capsulata*. Biochim. Biophys. Acta **748:** 116-127.

COLBEAU, A., KELLEY, B. C., and VIGNAIS, P. M. 1980. Hydrogenase activity in *Rhodopseudomonas capsulata*: relationship with nitrogenase activity. J. Bacteriol. **144:** 141-148.

COLBEAU, A., and VIGNAIS, P. M. 1983. The membrane-bound hydrogenase of *Rhodopseudomonas capsulata* is inducible and contains nickel. Biochim. Biophys. Acta **748:** 128-138.

CONRAD, R. 1988. Biogeochemistry and ecophysiology of atmospheric CO and H_2. Adv. Microb. Ecol. **10:** 231-283.

CONRAD, R., and SEILER, W. 1979. Field measurements of hydrogen evolution by nitrogen-fixing legumes. Soil Biol. Biochem. **11:** 689-690.

CONRAD, R., and SEILER, W. 1980. Contribution of hydrogen production by nitrogen fixation to the global hydrogen budget. J. Geophys. Res. **85:** 5493-5498.

CUNNINGHAM, S. D., KAPULNIK, Y., and PHILLIPS, D. A. 1986. Distribution of hydrogen-metabolizing bacteria in alfalfa field soil. Appl. Environ. Microbiol. **52:** 1091-1095.

DADAY, A., MACKERRAS, A. H., and SMITH, G. D. 1985. The effect of nickel on hydrogen metabolism and nitrogen fixation in the cyanobacterium *Anabaena cylindrica*. J. Gen. Microbiol. **131**: 231-238.

DADAY, A., MACKERRAS, A. H., and SMITH, G. D. 1988. A role for nickel in cyanobacterial nitrogen fixation and growth via cyanophycin metabolism. J. Gen. Microbiol. **134**: 2659-2663.

DADAY, A., and SMITH, G. D. 1983. The effect of nickel on the hydrogen metabolism of the cyanobacterium *Anabaena cylindrica*. FEMS Microbiol. Lett. **20**: 327-330.

DALTON, H. 1980a. The cultivation of diazotrophic microorganisms. *In* Methods for evaluating biological nitrogen fixation. *Edited by* F. J. Bergersen. John Wiley & Sons, Ltd., Chichester. pp. 13-64.

DALTON, H. 1980b. Chemoautotrophic nitrogen fixation. *In* Nitrogen fixation. *Edited by* W. D. P. Stewart and J. R. Gallon. Acad. Press, London. pp. 177-195.

DALTON, H., and WHITTENBURY, R. 1976. The acetylene reduction technique as an assay for nitrogenase activity in the methane-oxidizing bacterium *Methylococcus capsulatus* strain Bath. Arch. Microbiol. **109**: 147-151.

DAVIS, J. B., COTY, V. F., and STANLEY, J. P. 1964. Atmospheric nitrogen fixation by methane-oxidizing bacteria. J. Bacteriol. **88**: 468-472.

DE BONT, J. A. M., and LEIJTEN, M. W. M. 1976. Nitrogen fixation by hydrogen-utilizing bacteria. Arch. Microbiol. **107**: 235-240.

DE LEY, J., and PARK, I. W. 1966. Molecular biological taxonomy of some free-living nitrogen fixing bacteria. Antonie van Leeuwenhoek J. Microbiol. Serol. **32**: 6-16.

DE VOS, P., and DE LEY, J. 1983. Intra- and intergeneric similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. Int. J. Syst. Bacteriol. **33**: 487-509.

DE VOS, P., KERSTERS, K., FALSEN, E., POT, B., GILLIS, M., SEGERS, P., and DE LEY, J. 1985. *Comamonas* Davis and Park 1962 gen. nov. nom. rev. emend., and *Comamonas terrigena* Hugh 1962 sp., nov., nom. rev. Int. J. Syst. Bacteriol. **35**: 443-453.

DE VOS, P. VAN LANDSCHOOT, A., SEGERS, P., TYTGAT, R., GILLIS, M., BAUWENS, M., ROSSAU, R., GOOR, M. POT, B., KERSTERS, K., LIZZARAGA, P., and DE LEY, J. 1989. Genotypic relationships and taxonomic localization of unclassified *Pseudomonas* and *Pseudomonas*-like strains by deoxyribonucleic acid:ribosomal ribonucleic acid hybridizations. Int. J. Syst. Bacteriol. **39:** 35-49.

DE VRIES, W., STAM, H., and STOUTHAMER, A. H. 1984. Hydrogen oxidation and nitrogen fixation in rhizobia, with special attention focused on strain ORS 571. Antonie van Leeuwenhoek **50:** 505-524.

DE WEGER, L. A., VAN BOXTEL, R., VAN DER BYRG, B., GRUTERS, R. A., GEELS, F. P., SCHIPPERS, B., and LUGTENBERG, B. 1986. Siderophores and outer membrane proteins of antagonistic, plant-growth-stimulating, root-colonizing *Pseudomonas* spp. J. Bacteriol. **165:** 585-594.

DIEKERT, G. B., GRAF, E. G., and THAUER, R. K. 1979. Nickel requirement for carbon monoxide dehydrogenase formation in *Clostridium pasteurianum*. Arch. Microbiol. **122:** 117-120.

DILWORTH, M. J., EADY, R. R., ROBSON, R. L., and MILLER, R. W. 1987. Ethane formation from acetylene as a potential test for vanadium nitrogenase *in vivo*. Nature (London), **327:** 167-168.

DINGLER, C., and OELZE, J. 1987. Superoxide dismutase and catalase in *Azotobacter vinelandii* grown in continuous culture at different dissolved oxygen concentrations. Arch. Microbiol. **147:** 291-294.

DIXON, R. 1988. Genetic regulation of nitrogen fixation. *In* The nitrogen and sulfur cycles. *Edited by* J. A. Cole and S. J. Ferguson. Cambridge Univ. Press, Cambridge. pp. 417-438.

DIXON, R. O. D. 1967. Hydrogen uptake and exchange by pea root nodules. Ann. Bot. **31:** 179-188.

DIXON, R. O. D. 1968. Hydrogenase in pea root nodule bacteroids. Arch. Microbiol. **62:** 272-283.

DIXON, R. O. D. 1972. Hydrogenase in legume root nodule bacteroids: occurrence and properties. Arch. Mikrobiol. **83:** 193-201.

DIXON, R. O. D., BLUNDEN, E. A. G., and SEARLE, J. W. 1981. Inter-cellular space and hydrogen diffusion in root nodules of pea *Pisum sativum* and lupine *Lupinus alba*. Plant Sci. Lett. **23:** 109-116.

DOBEREINER, J. 1988. Isolation and identification of root-associated diazotrophs. Plant Soil **110**: 207-212.

DONAWA, A. L., ISHAQUE, M., and ALEEM, M. I. H. 1971. Energy conversion in autotrophically-grown *Pseudomonas saccharophila*. Eur. J. Biochem. **21**: 292-300.

DONAWA, A. L., ISHAQUE, M., and ALEEM, M. I. H. 1973. CO_2 fixation and metabolic control in *Pseudomonas saccharophila*. Can. J. Microbiol. **19**: 1243-1250.

DOUDOROFFf, M. 1940. The oxidative assimilation of sugars and related substances by *Pseudomonas saccharophila*. Enzymologia **9**: 59-72.

DOYLE, C. M., and ARP, D. J. 1987. Regulation of H_2 oxidation activity and hydrogenase protein levels by H_2, O_2, and carbon substrates in *Alcaligenes latus*. J. Bacteriol. **169**: 4463-4468.

DOYLE, C. M., and ARP, D. J. 1988. Nickel affects expression of the nickel-containing hydrogenase of *Alcaligenes latus*. J. Bacteriol. **170**: 3891-3896.

DRAKE, H. L. 1982. Occurrence of nickel in carbon monoxide dehydrogenase from *Clostridium pasteurianum* and *Clostridium thermoaceticum*. J. Bacteriol. **149**: 561-566.

DRAKE, H. L. 1988. Biological transport of nickel. *In* Bioinorganic chemistry of nickel. *Edited by* J. R. Lancaster. VCH Publishers, New York. pp.

DUGNANI, L., WYRSCH, I., GANDOLLA, M., and ARAGNO, M. 1986. Biological oxidation of hydrogen in soils flushed with a mixture of hydrogen, carbon dioxide, oxygen, and nitrogen. FEMS Microbiol. Ecol. **38**: 347-351.

DUXBURY, T. 1985. Ecological aspects of heavy metal responses in microorganisms. Adv. Microb. Ecol. **8**: 185-235.

EADY, R. R. 1981. Regulation of nitrogenase activity. *In* Current perspectives in nitrogen fixation. *Edited by* A. H. Gibson and W. E. Newton. Aust. Acad. Sci., Canberra. pp. 172-181.

EADY, R. R., ROBSON, R. L., and SMITH, B. E. 1988. Alternative and conventional nitrogenases. *In* The nitrogen and sulfur cycles. *Edited by* J. A. Cole and S. J. Ferguson. Cambridge Univ. Press, Cambridge. pp. 363-382.

EBERZ, G., EITINGER, T., and FRIEDRICH, B. 1989. Genetic determinants of a nickel-specific transport system are part of the plasmid-encoded hydrogenase gene cluster in *Alcaligenes eutrophus*. J. Bacteriol. **171**: 1340-1345.

EGGSTEIN, M., and KREUTZ, F. H. 1955. Vergleichende Untersuchungen zur quantitativen Eiweissbestimmung im Liquor und eiweissarmen Lösungen. Klinische Wochenschrift **33**: 879-884.

EISBRENNER, G., and EVANS, H. J. 1982. Spectral evidence for a component involved in hydrogen metabolism of soybean nodule bacteroids. Plant Physiol. **70**: 1667-1672.

EISBRENNER, G., and EVANS, H. J. 1983. Aspects of hydrogen metabolism in nitrogen-fixing legumes and other plant-microbe associations. Annu. Rev. Plant Physiol. **34**: 105-136.

EISBRENNER, G., HICKOCK, R. E., and EVANS, H. J. 1982. Cytochrome patterns in *Rhizobium japonicum* cells grown under chemolithotrophic conditions. Arch. Microbiol. **132**: 230-235.

ELMERICH, C., GALIMAND, M., VIEILLE, C., DELORME, F., and DE ZAMAROCZY, M. 1988. Nitrogen fixation genes of *Azospirillum*. *In* Nitrogen fixation: hundred years after. *Edited by* H. Bothe, F. J. de Bruijn, and W. E. Newton. Gustav Fischer, Stuttgart. pp. 327-331.

EMERICH, D.W., RUIZ-ARGUESO, T., CHING, T. M., and EVANS, H. J. 1979. Hydrogen-dependent nitrogenase activity and ATP formation in *Rhizobium japonicum* bacteroids. J. Bacteriol. **137**: 153-160.

ENGELBERTS, K., SCHMIDT, E., and REINEKE, W. 1989. Degradation of o-toluate by *Pseudomonas* sp. strain WR 401. FEMS Microbiol. Lett. **59**: 35-38.

ENTNER, N., and DOUDOROFF, M. 1952. Glucose and gluconic acid oxidation of *Pseudomonas saccharophila*. J. Biol. Chem. **196**: 853-862.

EVANS, H. J., HARKER, A. R., PAPEN, H., RUSSELL, S. A., HANUS, F. J., and ZUBER, M. 1987. Physiology, biochemistry, and genetics of the uptake hydrogenase in rhizobia. Annu. Rev. Microbiol. **41**: 335-361.

FAUQUE, G., PECK, H. D., JR., MOURA, J. J. G., HUYNH, B. H., BERLIER, Y., DERVARTANIAN, D. V., TEIXEIRA, M., PRZYBYLA, A. E., LESPINAT, P. A., MOURA, I., and LEGALL, J. 1988. The three classes of hydrogenases from sulfate-reducing bacteria of the genus *Desulfovibrio*. FEMS Microbiol. Rev. **54:** 299-344.

FAUQUE, G., TEIXEIRA, M., MOURA, I., LESPINAT, P. A., XAVIER, A. V., DERVARTANIAN, D. V., PECK, H. D., JR., LEGALL, J., and MOURA, J. J. G. 1984. Purification, characterization and redox properties of hydrogenase from *Methanosarcina barkeri* (DSM 800). Eur. J. Biochem. **142:** 21-28.

FORD, C. M., TIBELIUS, K. H., YATES, M. G., ARP, D. J., and SEEFELDT, L. C. 1988. The identification and analysis of hydrogenase structural genes in *Azotobacter chroococcum*. *In* Nitrogen fixation: hundred years after. *Edited by* H. Bothe, F. J. de Bruijn, and W. E. Newton. Gustav Fischer, Stuttgart. pp. 275.

FRIEDRICH, B. 1989. Genetics of energy converting systems in aerobic chemolithotrophs. *In* Autotrophic bacteria. *Edited by* H. G. Schlegel and B. Bowien. Sci. Tech Publishers, Madison. pp. 415-430.

FRIEDRICH, B., FRIEDRICH, C. G., MEYER, M., and SCHLEGEL, H. G. 1984. Expression of hydrogenase in *Alcaligenes* spp. is altered by interspecific plasmid exchange. J. Bacteriol. **158:** 331-333.

FRIEDRICH, B., HEINE, E., FINCK, A., and FRIEDRICH, C. G. 1981. Nickel requirement for active hydrogenase formation in *Alcaligenes eutrophus*. J. Bacteriol. **145:** 1144-1149.

FRIEDRICH, B., KORTLUKE, C., HOGREFE, C., EBERZ, G., SILBER, B., and WARRELMANN, J. 1986. Genetics of hydrogenase from aerobic lithoautotrophic bacteria. Biochimie **68:** 133-145.

FRIEDRICH, C. G. 1982. Derepression of hydrogenase during limitation of electron donors and derepresion of ribulose bisphosphate carboxylase during carbon limitation of *Alcaligenes eutrophus*. J. Bacteriol. **149:** 203-210.

FU, H., and BURRIS, R. 1989. Ammonium inhibition of nitrogenase activity in *Herbaspirillum seropedicae*. J. Bacteriol. **171:** 3168-3175.

FU, C., and KNOWLES, R. 1986. Oxygen tolerance of uptake hydrogenase in *Azospirillum* spp. Can. J. Microbiol. **32:** 897-900.

FU, C., and KNOWLES, R. 1988. H_2 supports nitrogenase activity in carbon-starved *Azospirillum lipoferum* and *A. amazonense*. Can. J. Microbiol. **34**: 825-829.

FU, C., and KNOWLES, R. 1989. Intracellular location and O_2 sensitivity of uptake hydrogenase in *Azospirillum* spp. Appl. Environ. Microbiol. **55**: 2315-2319.

FUHRMANN, G. F., and ROTHSTEIN, A. 1968. The transport of Zn^{2+}, Co^{2+}, and Ni^{2+} into yeast cells. Biochim. Biophys. Acta **163**: 325-330.

GALLON, J. R., and CHAPLIN, A. E. 1987. An introduction to nitrogen fixation. Cassell Educational Ltd., London.

GILLIS, M., KERSTERS, K., HOSTE, B., JANSSENS, D., KROPPENSTEDT, R. M., STEPHAN, M. P., TEIXEIRA, K. R. S., DOBEREINER, J., and DE LEY, J. 1989. *Acetobacter diazotrophicus* sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugar cane. Int. J. Syst. Bacteriol. **39**: 361-364.

GOGOTOV, I. N. 1986. Hydrogenases of phototrophic microorganisms. Biochimie **68**: 181-187.

GOGOTOV, I. N., and SCHLEGEL, H. G. 1974. N_2-fixation by chemoautotrophic hydrogen bacteria. Arch. Microbiol. **97**: 359-362.

GOLOVACHEVA, R. S., and KALININSKAYA, T. A. 1968. *Pseudomonas ambigua* - a nitrogen-fixing bacterium from thermal springs. Microbiology **37**: 792-794.

GOTTSCHALK, G. 1986. Bacterial metabolism. Springer-Verlag, New York.

GOWDA, T. K. S., and WATANABE, I. 1983. Autoradiographic technique for screening chemolithotrophic hydrogen-utilizing bacteria. Can. J. Microbiol. **29**: 1081-1086.

GOWDA, T. K. S., and WATANABE, I. 1985. Hydrogen-supported N_2-fixation of *Pseudomonas* sp. and *Azospirillum lipoferum* under free-living condition and in association with rice seedlings. Can. J. Microbiol. **31**: 317-321.

GRAHAM, L. A., STULTS, L. W., and MAIER, R. J. 1984. Nitrogenase-hydrogenase relationships in *Rhizobium japonicum*. Arch. Microbiol. **140**: 243-246.

GREEN, M., and WILSON, P. W. 1953. Hydrogenase and nitrogenase in *Azotobacter*. J. Bacteriol. **65**: 511-517.

GUERINOT, M. L., WEST, P. A., LEE, J. V., and COLWELL, R. R. 1982. *Vibrio diazotrophicus* sp. nov., a marine nitrogen-fixing bacterium. Int. J. Syst. Bacteriol. **32**: 350-357.

GUERINOT, M. L., and PATRIQUIN, D. G. 1981. N_2-fixing vibrios isolated from the gastrointestinal tract of sea urchins. Can. J. Microbiol. **27**: 311-317.

HAAHTELA, K. 1985. Nitrogenase activity (acetylene reduction) in root-associated, cold-climate species of *Azospirillum*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* growing at various temperatures. FEMS Microbiol. Ecol. **31**: 211-214.

HAAHTELA, K., HELANDER, I., NURMIAHO-LASSILA, E. L., and SUNDMAN, V. 1983a. Morphological and physiological characteristics and lipopolysaccharide composition of N_2-fixing (C_2H_2-reducing) root-associated *Pseudomonas* sp. Can. J. Microbiol. **29**: 874-880.

HAAHTELA, K., KARI, K. and SUNDMAN, V. 1983b. Nitrogenase activity (acetylene reduction) of root-associated, cold-climate *Azospirillum*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* species during growth on various carbon sources and at various partial pressures of oxygen. Appl. Environ. Microbiol. **45**: 563-570.

HAAKER, H., DE KOK, A., and VEEGER, C. 1974. Regulation of dinitrogen fixation in intact *Azotobacter vinelandii*. Biochim. Biophys. Acta **357**: 344-357.

HAAKER, H., LAANE, C., and VEEGER, C. 1980. Dinitrogen fixation and the proton motive force. *In* Nitrogen fixation. *Edited by* W. D. P. Stewart and J. R. Gallon. Acad. Press, London. pp. 113-138.

HAAKER, H., and VEEGER, C. 1977. Involvement of the cytoplasmic membrane in nitrogen fixation by *Azotobacter vinelandii*. Eur. J. Biochem. **142**: 37-42.

HALVORSON, H. O., ZIEGLER, N. R. 1933. Application of statistics to problems in bacteriology: I. a means of determining bacterial population by the dilution method. J. Bacteriol. **25**: 101-121.

HAMMEL, K. E., CORNWELL, K. L., DIEKERT, G. B., and THAUER, R. K. 1984. Evidence for a nickel-containing carbon monoxide dehydrogenase in *Methanobrevibacter arboriphilicus*. J. Bacteriol. **157**: 975-978.

HARKER, A. R., XU, L.-S., HANUS, F. J., and EVANS, H. J. 1984. Some properties of the nickel-containing hydrogenase of chemolithotrophically grown *Rhizobium japonicum*. J. Bacteriol. **159:** 850-856.

HARTMANN, A., FU, H., and BURRIS, R. 1986. Regulation of nitrogenase activity by ammonium chloride in *Azospirillum* spp. J. Bacteriol. **165:** 864-000.

HARTMANN, A., and BURRIS, R. H. 1987. Regulation of nitrogenase activity by O_2 in *Azospirillum brasilense* and *Azospirillum lipoferum*. J. Bacteriol. **169:** 944-948.

HAUGLAND, R. A., CANTRELL, M. A., BEATY, J. S., HANUS, F. J., RUSSELL, S. A., and EVANS, H. J. 1984. Characterization of *Rhizobium japonicum* hydrogen uptake genes. J. Bacteriol. **159:** 1006-1112.

HAUSINGER, R. P. 1987. Nickel utilization by microorganisms. Microbiol. Rev. **51:** 22-42.

HERBERT, D., PHIPPS, P. J., and STRANGE, R. E. 1971. Chemical analysis of microbial cells. *In* Methods in microbiology, vol. 5B. *Edited by* J. R. Norris and D. W. Ribbons. Acad. Press, London. pp. 209-344.

HILL, S. 1971. Influence of oxygen concentration on the colony type of *Derxia gummosa* grown on nitrogen-free media. J. Gen. Microbiol. **67:** 77-83.

HILL, S. 1988. How is nitrogenase regulated by oxygen? FEMS Microbiol. Rev. **54:** 111-130.

HILL, S., and POSTGATE, J. R. 1969. Failure of putative nitrogen-fixing bacteria to fix nitrogen. J. Gen. Microbiol. **58:** 277-285.

HOCH, G. E., LITTLE, H.N., and BURRIS, R. H. 1957. Hydrogen evolution from soybean root nodules. Nature (London), **179:** 430-431.

HOOVER, T. R., ROBERTSON, A. D., CERNY, R. L., HAYES, R. N., IMPERIAL, J., SHAH, V. K., and LUDDEN, P. W. 1987. Identification of the V factor needed for synthesis of the iron-molybdenum cofactor of nitrogenase as homocitrate. Nature (London), **329:** 855-857.

HOM, S. S. M., GRAHAM, L. A., and MAIER, R. J. 1985. Isolation of genes (*nif/hup* cosmids) involved in hydrogenase and nitrogenase activities in *Rhizobium japonicum*. J. Bacteriol. **161:** 882-887.

HUYNH, B. H., CZECHOWSKI, M. H., KRUGER, H.-J., DERVARTANIAN, D. V., PECK, H. D., JR., and LEGALL, J. 1984. *Desulfovibrio vulgaris* hydrogenase: a non-heme iron enzyme lacking nickel that exhibits anomalous EPR and Mossbauer spectra. Proc. Natl. Acad. Sci. U.S.A. **81:** 3728-3732.

HWANG, J. C., CHEN, C. H., and BURRIS, R. H. 1973. Inhibition of nitrogenase-catalyzed reductions. Biochim. Biophys. Acta **292:** 256-270.

HYMAN, M. R., and ARP, D. J. 1987. Acetylene is an active-site directed, slow-binding, reversible inhibitor of *Azotobacter vinelandii* hydrogenase. Biochemistry **26:** 6447-6454.

INOUE, A., and HORIKOSHI, K. 1989. A *Pseudomonas* thrives in high concentrations of toluene. Nature **338:** 264-266.

ISHAQUE, M., DONAWA, A. L., and ALEEM, M. I. H. 1971a. Electron transport and coupled energy generation in *Pseudomonas saccharophila*. Can. J. Biochem. **49:** 1175-1182.

ISHAQUE, M., DONAWA, A. L., and ALEEM, M. I. H. 1971b. Oxidative phosphorylation in *Pseudomonas saccharophila* under autotrophic and heterotrophic growth conditions. Biochem. Biophys. Res. Comm. **44:** 245-251.

ISHAQUE, M., DONAWA, A. L., and ALEEM, M. I. H. 1973. Energy-coupling mechanisms under aerobic and anaerobic conditions in autotrophically grown *Pseudomonas saccharophila*. Arch. Biochem. Biophys. **150:** 570-579.

JARRELL, K. F., and SPROTT, G. D. 1982. Nickel transport in *Methanobacterium bryantii*. J. Bacteriol. **151:** 1195-1203.

JASPER, P., and SILVER, S. 1977. Magnesium transport in microorganisms. *In* Microorganisms and minerals. *Edited by* E. D. Weinberg. Marcel Dekker, New York. pp. 7-47.

JENNI, B., ISCH, C., and ARAGNO, M. 1988. Nitrogen fixation by new strains of *Pseudomonas pseudoflava* and related bacteria. J. Gen. Microbiol. **135:** 461-467.

JENSEN, V. 1979. Heterotrophic microorganisms. *In* Nitrogen fixation, vol. 1. *Edited by* W. J. Broughton. Clarendon Press, Oxford. pp. 30-56.

JOHNSON, J. L., and PALLERONI, N. J. 1989. Deoxyribonucleic acid similarities among *Pseudomonas* species. Int. J. Syst. Bacteriol. **39:** 230-235.

JONES, C. J. 1982. Bacterial respiration and photosynthesis. Amer. Soc. Microbiol., Washington D. C.

KALTWASSER, H., and FRINGS, W. 1980. Transport and metabolism of nickel in microorganisms. *In* Nickel in the environment. *Edited by* J. O. Nriagu. John Wiley & Sons, Inc., New York. pp. 463-491.

KAWASUMI, K., IGARASHI, Y., KODAMA, I., MINODA, Y. 1980. Isolation of strictly thermophilic and obligately autotrophic hydrogen bacteria. Agric. Biol. Chem. **44:** 1985-1986.

KENNEDY, C., BUCK, M., EVANS, D., HUMPHREY, R., JONES, R., RAMOS, J., ROBSON, R., SANTERO, E., TIBELIUS, K., TOUKDARIAN, A., WOODLEY, P., and YATES, M. G. 1987. The genetic analysis of nitrogen fixation, oxygen tolerance and hydrogen uptake in azotobacters. Phil. Trans. R. Soc. London **B317:** 159-171.

KENNEDY, C., and TOUKDARIAN, A. 1987. Genetics of azotobacters: applications to nitrogen fixation and related aspects of metabolism. Ann. Rev. Microbiol. **41:** 227-258.

KENNTEMISCH, T., DANNEBERG, G.,HUNDESHAGEN, B., and BOTHE, H. 1988. The occurrence of the alternative nitrogenase in the cyanobacterium *Anabaena variabilis*. FEMS Microbiol. Lett. **51:** 19-24.

KLEEBERGER, A., CASTORPH, H., and KLINGMULLER, W. 1983. The rhizosphere microflora of wheat and barley with special reference to Gram-negative bacteria. Arch. Microbiol. **136:** 306-311.

KLUCAS, R. V., HANUS, F. J., RUSSELL, S. A., and EVANS, H. J. 1983. Nickel: a micronutrient element for hydrogen-dependent growth of *Rhizobium japonicum* and for expression of urease activity in soybean leaves. Proc. Natl. Acad. Sci. U.S.A. **80:** 2253-2257.

KLUGKIST, J., and HAAKER, H. 1984. Inhibition of nitrogenase activity by ammonium chloride in *Azotobacter vinelandii*. J. Bacteriol. **157:** 148-151.

KNOWLES, R. 1977. The significance of asymbiotic dinitrogen fixation by bacteria. *In* A treatise on dinitrogen fixation, sect. IV: agronomy and ecology. *Edited by* R. W. F. Hardy and A. H. Gibson. John Wiley and Sons, New York. pp. 33-83.

KNOWLES, R. 1982a. Free-living dinitrogen-fixing bacteria. *In* Methods of soil analysis, part 2, chemical and microbiological properties. *Edited by* A. L. Page, R. H. Miller, and D. R. Keeney. Amer. Soc. Agron. and Soil Sci. Soc. Amer. Inc., Madison, Wisconsin. pp. 1071–1092.

KNOWLES, R. 1982b. Denitrification. Microbiol. Rev. **46:** 43–70.

KOW, Y., W., and BURRIS, R. H. 1984. Purification and properties of membrane-bound hydrogenase from *Azotobacter vinelandii*. J. Bacteriol. **159:** 564–569.

KRIEG, N. R., and HOLT, J. G. 1984. Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore.

KROTZKY, A, and WERNER, D. 1987. Nitrogen-fixation in *Pseudomonas stutzeri*. Arch. Microbiol. **147:** 48–57.

KUHLA, J., and OELZE. J. 1988. Dependence of nitrogenase switch-off upon oxygen stress on the nitrogenase activity in *Azotobacter vinelandii*. J. Bacteriol. **170:** 5325–5329.

LAANE, C., HAAKER, H., and VEEGER, C. 1978. Involvement of the cytoplasmic membrane in nitrogen fixation by *Rhizobium leguminosarum* bacteroids. Eur. J. Biochem. **87:** 147–153.

LAANE, C., HAAKER, H., and VEEGER, C. 1979. On the efficiency of oxidative phosphorylation in membrane vesicles of *Azotobacter vinelandii* and of *Rhizobium leguminosarum* bacteroids. Eur. J. Biochem. **97:** 369–377.

LAANE, C., KRONE, W., KONINGS, W., HAAKER, H., and VEEGER, C. 1980. Short-term effect of ammonium chloride on nitrogen fixation by *Azotobacter vinelandii* and by bacteroids of *Rhizobium leguminosarum*. Eur. J. Biochem. **103:** 39–46.

LADHA, J. K., BARRAQUIO, W. L., and WATANABE, I. 1983. Isolation and identification of nitrogen-fixing *Enterobacter cloacae* and *Klebsiella planticola* associated with rice plants. Can. J. Microbiol. **29:** 1301–1308.

LAFAVRE, J. S., and FOCHT, D. D. 1983. Conservation in soil of H_2 liberated from N_2-fixation by Hup^- nodules. Appl. Environ. Microbiol. **46:** 304–311.

LALANDE, R., BISSONNETTE, N., COUTLEE,D., and ANTOUN, H. 1989. Identification of rhizobacteria from maize and determination of their plant-growth promoting potential. Plant Soil **115:** 7-11.

LAMBERT, G. R., CANTRELL, M. A., HANUS, F. J., RUSSELL, S. A., HADDAD, K. R., and EVANS, H. J. 1985a. Intra- and inter-species transfer and expression of *Rhizobium japonicum* hydrogen uptake genes and autotrophic growth capability. Proc. Natl. Acad. Sci. U.S.A. **82:** 3232-3236.

LAMBERT, G. R., HARKER, A. R., ZUBER, M., DALTON, D. A., HANUS, F. J., RUSSELL, S. A., and EVANS, H. J. 1985b. Characterization, significance, and transfer of hydrogen uptake genes from *Rhizobium japonicum*. *In* Nitrogen fixation research progress. *Edited by* H. J. Evans, P. J. Bottomley, and W. E. Newton. Martinus Nijhoff, Dordrecht. pp. 209-215.

LAMBERT, G. R., and SMITH, G. D. 1981. Hydrogen metabolism by filamentous cyanobacteria (blue green algae). Biol. Rev. **56:** 589-600.

LEE, S. B., and WILSON, P. W. 1943. Hydrogenase and nitrogen fixation by *Azotobacter*. J. Biol. Chem. **151:** 377-385.

LEE, S. B., WILSON, J. B., and WILSON, P. W. 1942. Mechanism of biological nitrogen fixation. X. Hydrogenase in cell-free extracts and intact cells of *Azotobacter*. J. Biol. Chem. **144:** 273-281.

LEGALL, J., LJUNGDAHL, P. O., MOURA, I., PECK, H. D., XAVIER, A. V., MOURA, J. J., TEIXEIRA, M., HUYNH, B. H., and DERVARTANIAN, D. V. 1982. The presence of redox-sensitive nickel in the periplasmic hydrogenase from *Desulfovibrio gigas*. Biochem. Biophys. Res. Comm. **106:** 610-616.

LEPO, J. E., HANUS, F. J., and EVANS, H. J. 1980. Chemoautotrophic growth of hydrogen-uptake positive strains of *Rhizobium japonicum*. J. Bacteriol. **141:** 664-670.

LIFSHITZ, R., KLOEPPER, J. W., SCHER, F. M., TIPPING, E. M., and LALIBERTE, M. 1986. Nitrogen-fixing pseudomonads isolated from roots of plants grown in the Canadian High Arctic. Can. J. Microbiol. **51:** 251-255.

LIM, S. T., and SHANMUGAM, K. T. 1979. Regulation of hydrogen utilization in *Rhizobium japonicum* by cyclic AMP. Biochim. Biophys. Acta **584:** 479-492.

LOHMEYER, M., and FRIEDRICH, C. G. 1987. Nickel transport in *Alcaligenes eutrophus*. Arch. Microbiol. **149:** 130-135.

LOWE, D. J., THORNELEY, R. N. F., and POSTGATE, J. R. 1984. The mechanism of substrate reduction by nitrogenase. *In* Advances in nitrogen fixation research. *Edited by* C. Veeger and W. E. Newton. Nijhoff/Junk, The Hague. pp. 133-138.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193:** 265-275.

LUNDIE, L. L., JR., YANG, H., HEINONEN, J. K., DEAN, S. I., and H. L. DRAKE. 1988. Energy-dependent high affinity transport of nickel by the acetogen *Clostridium thermoaceticum*. J. Bacteriol. **170:** 5705-5708.

MACKINTOSH, M. E. 1978. Nitrogen fixation by *Thiobacillus ferrooxidans*. J. Gen. Microbiol. **105:** 215-218.

MAIER, R. J. 1986. Biochemistry, regulation, and genetics of hydrogen oxidation in *Rhizobium*. CRC Crit. Rev. Biotechnol. **3:** 17-38.

MAIER, R. J., CAMPBELL, N. E. R., HANUS, F. J., SIMPSON, F.B., RUSSELL, S. A., and EVANS, H. J. 1978. Expression of hydrogenase activity in free-living *Rhizobium japonicum*. Proc. Natl. Acad. Sci. U.S.A. **75:** 3258-3262.

MAIER, R. J., HANUS, F. J., and EVANS, H. J. 1979. Regulation of hydrogenase in *Rhizobium japonicum*. J. Bacteriol. **137:** 824-829.

MAIER, R. J., and PROSSER, J. 1988. Hydrogen-mediated mannose uptake in *Azotobacter vinelandii*. J. Bacteriol. **170:** 1986-1989.

MALIK, K. A., and SCHLEGEL, H. G. 1981. Chemolithotropnic growth of bacteria able to grow under N_2-fixing conditions. FEMS Microbiol. Lett. **11:** 63-67.

MARKOWITS, A., KLEIN, H. P., and FISCHER, E. H. 1956. Purification, crystallization, and properties of the alpha-amylase of *Pseudomonas saccharophila*. Biochim. Biophys. Acta **19:** 267-273.

MARKWELL, M. A. K., HAAS, S. M., BIEBER, L. L., and TOLBERT, N. E. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:** 206-210.

MCCLUNG, C. R., PATRIQUIN, D. G., and DAVIS, R. E. 1983. *Campylobacter nitrofigilis*, sp. nov., a nitrogen-fixing bacterium associated with roots of *Spartina alterniflora* Loisel. Int. J. Syst. Bacteriol. **33:** 605-612.

MCFADDEN, B. A., TABITA, F. R., and KUEHN, G. D. 1975. Ribulose-diphosphate carboxylase from the hydrogen bacteria and *Rhodospirillum rubrum*. Methods Enzymol. **42:** 461-472.

MCGETRICK, A. M., GOULDING, C. F., MANIAN, S. S., and O'GARA, F. 1985. Catabolite repression and role of cyclic AMP in CO_2 fixation and H_2 metabolism in *Rhizobium* spp. J. Bacteriol. **163:** 1282-1284.

MEGANATHAN, R. 1979. Why has the "nitrogenase plasmid" snubbed the pseudomonads. J. Theor. Biol. **80:** 301-304.

MERBERG, D., O'HARA, E. B., and MAIER, R. J. 1983. Regulation of hydrogenase in *Rhizobium japonicum*: analysis of mutants altered in regulation by carbon substrates and oxygen. J. Bacteriol. **156:** 1236-1242.

MERGEAY, M., and GERITS, J. 1978. F'-plasmid transfer from *Escherichia coli* to *Pseudomonas fluorescens*. J. Bacteriol. **135:** 18-28.

MERRICK, M. J. 1988. Organization and regulation of nitrogen fixation genes in *Klebsiella* and *Azotobacter*. *In* Nitrogen fixation: hundred years after. *Edited by* H. Bothe, F. J. Bruijn, and W. E. Newton. Gustav Fischer, Stuttgart. pp. 293-302.

MINCHIN, F. R., SHEEHY, J. E., and WITTY, J. F. 1985. Factors limiting N_2 fixation by the legume-*Rhizobium* symbiosis. *In* Nitrogen fixation research progress. *Edited by* H. J. Evans, P. J. Bottomley, and W. E. Newton. Nijhoff, Dordrecht. pp. 285-291.

MOBLEY, H. L. T., and HAUSINGER, R. P. 1989. Microbial ureases: significance, regulation, and molecular characterization. Microbiol. Rev. **53:** 85-108.

MOHAN, P. M., PUDRA, M. P. P., and SASTRY, R. S. 1984. Nickel transport in nickel-resistant strains of *Neurospora crassa*. Curr. Microbiol. **10:** 125-128.

MORTENSON, L. E. 1978. The role of dihydrogen and hydrogenase in nitrogen fixation. Biochimie **60:** 219-224.

MOURA, J. J. G., MOURA, I., TEIXEIRA, M., XAVIER, A. V., FAUQUE, G., and LEGALL, J. 1988a. Nickel-containing hydrogenases. *In* Metal ions in biological systems. *Edited by* H. Sigel and A. Sigel. Marcel Dekker, Inc., New York. pp. 286-314.

MOURA, J. J. G., TEIXEIRA, M., MOURA, I., and LEGALL, J. 1988b. (Ni,Fe) Hydrogenases from sulfate-reducing bacteria: nickel catalytic and regulatory roles. *In* The bioinorganic chemistry of nickel. *Edited by* J. R. Lancaster, Jr. VCH Publishers, New York. pp. 167-190.

MULDER, E. G., and BROTONEGORO, S. 1974. Free-living heterotrophic nitrogen-fixing bacteria. *In* The biology of nitrogen fixation. *Edited by* A. Quispel. North-Holland Publishing Co., Amsterdam. pp. 38-85.

NAKAMURA, Y., SOMEYA, J., and SUZUKI, T. 1985. Nickel requirement of oxygen-resistant hydrogen bacterium *Xanthobacter autotrophicus* strain Y38. Agric. Biol. Chem. **49**: 1711-1718.

NELSON, L. M., and SALMINEN, S. O. 1982. Uptake hydrogenase activity and ATP formation in *Rhizobium leguminosarum* bacteroids. J. Bacteriol. **151**: 989-995.

NIES, D. H., and SILVER, S. 1989. Metal ion uptake by a plasmid-free metal-sensitive *Alcaligenes eutrophus* strain. J. Bacteriol. **171**: 4073-4075.

NISHIHARA, H., IGARASHI, Y., and KODAMA, T. 1989. Isolation of an obligately chemolithotrophic, halophilic and aerobic hydrogen-oxidizing bacterium from marine environment. Arch. Microbiol. **152**: 39-43.

NOKHAL, T.-H., and SCHLEGEL, H. G. 1980. The regulation of hydrogenase formation as a differentiating character of strains of *Paracoccus denitrificans*. Antonie van Leeuwenhoek J. Microbiol. **46**: 143-155.

NOVAK, P. D., and MAIER, R. J. 1987. Inhibition of hydrogenase synthesis by DNA gyrase inhibitors in *Bradyrhizobium japonicum*. J. Bacteriol. **169**: 2708-2712.

OAKLEY, C. J., and MURRELL, J. C. 1988. *nifH* genes in the obligate methane oxidizing bacteria. FEMS Microbiol. Lett. **49**: 53-57.

O'BRIAN, M. R., and MAIER, R. J. 1982. Electron transport components involved in hydrogen oxidation in free-living *Rhizobium japonicum*. J. Bacteriol. **152**: 422-430.

O'BRIAN, M. R., and MAIER, R. J. 1985a. Expression of cytochrome *o* in hydrogen uptake constitutive mutants of *Rhizobium japonicum*. J. Bacteriol. **161:** 507-514.

O'BRIAN, M. R., and MAIER, R. J. 1985b. Role of ubiquinone in hydrogen-dependent electron transport in *Rhizobium japonicum*. J. Bacteriol. **161:** 775-777.

O'BRIAN, M. R., and MAIER, R. J. 1988. Hydrogen metabolism in *Rhizobium*: energetics, regulation, enzymology, and genetics. Adv. Microb. Physiol. **29:** 1-52.

O'BRIAN, M. R., and MAIER, R. J. 1989. Molecular aspects of the energetics of nitrogen fixation in *Rhizobium*-legume symbioses. Biochim. Biophys. Acta **974:** 229-246.

ODYAMA, J. 1971. Simultaneous fixation of CO_2 and N_2 in the presence of H_2 and O_2 by a bacterium. Rep. Ferment. Res. Inst. **39:** 41-44.

ODYAMA, J. 1976. Bacterial culture by CO_2 or CO and N_2 as sole source of C and N. *In* Microbial production and utilization of gases. *Edited by* H. G. Schlegel, G. Gottschalk, and N. Pfennig. Erich Goltze, Gottingen. pp. 237-245.

PALLERONI, N. J. 1956. El metabolismo de los hidratos de carbono en *Pseudomonas saccharophila*. Ciencia e Investigacion **12:** 468-474.

PALLERONI, N. J. 1980. Isolation and properties of a new hydrogen bacterium related to *Pseudomonas saccharophila*. J. Gen. Microbiol. **117:** 155-161.

PALLERONI, N. J. 1984. Genus I. *Pseudomonas* Migula. *In* Bergey's manual of systematic bacteriology, vol. 1. *Edited by* N. R. Krieg and J. G. Holt. The Williams and Wilkins Co., Baltimore. pp 141-199.

PALLERONI, N. J., BALLARD, R. W., RALSTON, E., and DOUDOROFF, M. 1972. Deoxyribonucleic acid homologies among some *Pseudomonas* species. J. Bacteriol. **110:** 1-11.

PALLERONI, N. J., and DOUDOROFF, M. 1956. Mannose isomerase of *Pseudomonas saccharophila*. J. Biol. Chem. **218:** 535-548.

PALLERONI, N. J., and DOUDOROFF, M. 1965. Identity of *Pseudomomas saccharophila*. J. Bacteriol. **89:** 264.

PALLERONI, N. J., DOUDOROFF, M., STANIER, R. Y., SOLANES, R. E., and MANDEL, M. 1970. Taxonomy of the aerobic pseudomonads: the properties of the *Pseudomonas stutzeri* group. J. Gen. Microbiol. **60:** 215-231.

PALLERONI, N. J., KUNISAWA, R., CONTOPOULOU, R., and DOUDOROFF, M. 1973. Nucleic acid homologies in the genus *Pseudomonas*. Int. J. Syst. Bacteriol. **23:** 333-339.

PARTRIDGE, C. D. P., WALKER, C. C., YATES, M. G., and POSTGATE, J. R. 1980. The relationship between hydrogenase and nitrogenase in *Azotobacter chroococcum*: effect of nitrogen sources on hydrogenase activity. J. Gen. Microbiol. **119:** 313-319.

PARTRIDGE, C. D. P., and YATES, M. G. 1982. Effect of chelating agents on hydrogenase in *Azotobacter chroococcum*. Biochem. J. **204:** 339-344.

PAU, R. N. 1989. Nitrogenases without molybdenum. Trends Biochem. Sci. **14:** 183-186.

PAU, R. N., MITCHENALL, L. A., and ROBSON, R. L. 1989. Genetic evidence for an *Azotobacter vinelandii* nitrogenase lacking molybdenum and vanadium. J. Bacteriol. **171:** 124-129.

PAUL, E. A., and CLARK, F. E. 1989. Soil microbiology and biochemistry. Acad. Press, Inc., San Diego.

PAUL, E. A., and NEWTON, J. D. 1961. Studies of aerobic non-symbiotic nitrogen-fixing bacteria. Can. J. Microbiol. **7:** 7-13.

PEDERSON, D. M., DADAY, A., and SMITH, G. D. 1986. The use of nickel to probe the role of hydrogen metabolism in cyanobacterial nitrogen fixation. Biochimie **68:** 113-120.

PEDROSA, F. O. 1988. Physiology, biochemistry, and genetics of *Azospirillum* and other root-associated nitrogen-fixing bacteria. CRC Crit. Rev. Plant Sci. **6:** 345-384.

PEDROSA, F. O., DOBEREINER, J., and YATES, M. G. 1980. Hydrogen-dependent growth and autotrophic carbon dioxide fixation in *Derxia*. J. Gen. Microbiol. **119:** 547-551.

PEDROSA, F. O., STEPHAN, M., DOBEREINER, J., and YATES, M. G. 1982. Hydrogen-uptake hydrogenase activity in nitrogen-fixing *Azospirillum brasilense*. J. Gen. Microbiol. **128:** 161-166.

PEDROSA, F. O., and YATES, M. G. 1983. Effect of chelating agents and nickel ions on hydrogenase activity in *Azospirillum brasilense*, *A. lipoferum*, and *Derxia gummosa*. FEMS Microbiol. Lett. **17**: 101-106.

PHELPS, A. S., and WILSON, P. W. 1941. Occurrence of hydrogenase in nitrogen-fixing organisms. Proc. Soc. Exp. Biol. Med. **47**: 473-476.

PINKWART, M., SCHNEIDER, K., and SCHLEGEL, H. G. 1983. Purification and properties of the membrane-bound hydrogenase from N_2-fixing *Alcaligenes latus*. Biochim. Biophys. Acta **745**: 267-278.

PODZUWEIT, H. G., and ARP, D. J. 1987. Evidence for a branched respiratory system with two cytochrome oxidases in autotrophically grown *Alcaligenes latus*. Arch. Microbiol. **146**: 332-337.

PODZUWEIT, H. G., SCHNEIDER, K., and SCHLEGEL, H. G. 1983. Autotrophic growth and hydrogenase activity of *Pseudomonas saccharophila* strains. FEMS Microbiol. Lett. **19**: 169-173.

POPE, M. R., MURRELL, S. A., and LUDDEN, P. W. 1985. Covalent modification of the iron protein of nitrogenase from *Rhodospirillum rubrum* by adenine diphosphoribosylation of a specific arginine residue. Proc. Natl. Acad. Sci. U.S.A. **82**: 3173-3177.

POSSOT, O., HENRY, M., and SIBOLD, L. 1986. Distribution of DNA sequences homologous to *nifH* among archaebacteria. FEMS Microbiol. Lett. **34**: 173-177.

POSTGATE, J. 1974. Prerequisites for biological nitrogen fixation in free-living heterotrophic bacteria. *In* The biology of nitrogen fixation. *Edited by* A. Quispel. North Holland Publishing Co., Amsterdam. pp. 663-686.

POSTGATE, J. R. 1980. Nitrogen fixation in perspective. *In* Nitrogen fixation. *Edited by* W. D. P. Stewart and J. R. Gallon. Acad. Press, London. pp. 423-434.

POSTGATE, J. R. 1981. Microbiology of the free-living nitrogen-fixing bacteria, excluding cyanobacteria. *In* Current perspectives in nitrogen fixation. *Edited by* A. H. Gibson and W. E. Newton. Australian Acad. Sci., Canberra. pp. 217-228.

POSTGATE, J. R. 1982. The fundamentals of nitrogen fixation. University Press, Cambridge.

POSTGATE, J. R., and KENT, H. M. 1987. Qualitative evidence for expression of *Klebsiella pneumoniae nif* in *Pseudomonas putida*. J. Gen. Microbiol. **133**: 2563–2566.

POSTGATE, J., KENT, H. M., and ROBSON, R. L. 1986. DNA from diazotrophic *Desulfovibrio* strains is homologous to *Klebsiella pneumoniae* structural *nif* DNA and can be chromosomal or plasmid-borne. FEMS Microbiol. Lett. **33**: 159–163.

PRABHA, S., NAIR, S. K., DADARWAL, K. R., and TAURO, P. 1978. *Pseudomonas azotogensis* – an associated symbiont of Bajra (*Pennisetum americanum*). Plant Soil **49**: 657–659.

PREMAKUMAR, R., CHISNELL, J. R., and BISHOP, P. E. 1989. A comparison of the three dinitrogenase reductases expressed by *Azotobacter vinelandii*. Can. J. Microbiol. **35**: 344–348.

PROBST, I. 1980. Respiration in hydrogen bacteria. *In* Diversity of bacterial respiratory systems. *Edited by* C. J. Knowles. CRC Press Inc., Boca Raton. pp. 159–181.

PROCTOR, M. R., and WILSON, P. W. 1958. Nitrogen fixation by Gram-negative bacteria. Nature (London), **182**: 891.

PROSSER, J., GRAHAM, L., and MAIER, R. J. 1988. Hydrogen-mediated enhancement of hydrogenase expression in *Azotobacter vinelandii*. J. Bacteriol. **170**: 1990–1993.

QUIGLEY, M. M., and COLWELL, R. R. 1963. Properties of bacteria isolated from deep-sea sediments. J. Bacteriol. **95**: 211–220.

RAHMAN, M., WEINHARD, P. BALLY, R., HUBERT, J. L., and BALANDREAU, J. 1986. New nitrogen-fixing *Pseudomonas* species in the dominant rhizosphere bacterial population of rice. Fourth Int. Symp. Microb. Ecol., Ljubljana, Yugoslavia. p. 115.

RALSTON, E., PALLERONI, N. J., and DOUDOROFF, M. 1972. Deoxyribonucleic acid homologies of some so-called *Hydrogenomonas* species. J. Bacteriol. **109**: 465–466.

RANGA RAO, V. 1982. Effect of various nitrogenous compounds on depression of nitrogenase activity in cultured *Rhizobium* spp. J. Gen. Appl. Microbiol. **28**: 359–368.

RAVI SHANKAR, H. N., KENNEDY, I. R., and NEW, P. B. 1986. Autotrophic growth and nitrogen fixation in *Derxia gummosa*. J. Gen. Microbiol. **132**: 1797–1803.

RENNIE, R. J. 1981. A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. **27:** 8-14.

REPASKE, R., and REPASKE, A. C. 1976. Quantitative requirements for exponential growth of *Alcaligenes eutrophus*. Appl. Environ. Microbiol. **32:** 585-591.

RIEDER, R., CAMMACK, R., and HALL, D. O. 1984. Purification and properties of the soluble hydrogenase from *Desulfovibrio desulfuricans* (strain Norway 4). Eur. J. Biochem. **145:** 637-643.

ROBSON, R. L. 1979. Characterization of an oxygen-stable nitrogenase complex from *Azotobacter chroococcum*. Biochem. J. **181:** 569-575.

ROBSON, R. L. 1986. Nitrogen fixation in strains of *Azotobacter chroococcum* bearing deletions of a cluster of genes coding for nitrogenase. Arch. Microbiol. **146:** 74-79.

ROBSON, R. L., CHESSHYRE, J., WHEELER, K., JONES, R., WOODLEY, P., and POSTGATE, J. R. 1984. Genome size and complexity in *Azotobacter chroococcum*. J. Gen. Microbiol. **130:** 1603-1612.

ROBSON, R. L., and POSTGATE, J. R. 1980. Oxygen and hydrogen in biological nitrogen fixation. Annu. Rev. Microbiol. **34:** 183-207.

RUIZ-ARGUESO, T., EMERICH, D. W., and EVANS, H J. 1979. Characteristics of the H_2-oxidizing system in soybean nodule bacteroids. Arch. Microbiol. **121:** 199-206.

SALKINOJA-SALONEN, M. S., VAISANEN, E., and PETERSON, A. 1979. Involvement of plasmids in the bacterial degradation of lignin-derived compounds. *In* Plasmids of medical, environment and commercial importance. *Edited by* F. N. Timmis and A. Puhler. Elsevier/North Holland Biomedical Press, Amsterdam. pp. 301-314.

SALMINEN, S. O., and NELSON, L. M. 1984. Role of uptake hydrogenase in providing reductant for nitrogenase in *Rhizobium leguminosarum* bacteroids. Biochim. Biophys. Acta **764:** 132-137.

SAYAVEDRA-SOTO, L. A., POWELL, G. K., EVANS, H. J., and MORRIS, R. O. 1988. Nucleotide sequence of the genetic loci encoding subunits of *Bradyrhizobium japonicum* uptake hydrogenase. Proc. Natl. Acad. Sci. U.S.A. **85:** 8395-8399.

SCHINK, B., and ZEIKUS, J. G. 1984. Ecology of aerobic hydrogen-oxidizing bacteria in two freshwater lake ecosystems. Can. J. Microbiol. **30**: 260-265.

SCHIPPERS, B., BAKKER, A. W., and BAKKER, P. A. H. M. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Annu. Rev. Phytopathol. **25**: 339-358.

SCHLEGEL, H. G. 1976. The physiology of hydrogen bacteria. Antonie van Leewenhoek **42**: 181-201.

SCHLEGEL, H. G. 1989. Aerobic hydrogen-oxidizing (Knallgas) bacteria. *In* Autotrophic bacteria. *Edited by* H. G. Schlegel and B. Bowien. Sci. Tech Publishers, Madison. pp. 305-329.

SCHLEGEL, H. G., and EBERHARDT, U. 1972. Regulatory phenomena in the metabolism of knallgas bacteria. Adv. Microbiol. Physiol. **7**: 205-242.

SCHLESIER, M., and FRIEDRICH, B. 1981. *In vivo* inactivation of soluble hydrogenase of *Alcaligenes eutrophus*. Arch. Microbiol. **129**: 150-153.

SCHNEIDER, K., SCHLEGEL, H. G., and JOCHIM, K. 1984. Effect of nickel on activity and subunit and composition of purified hydrogenase from *Nocardia opaca* 1b. Eur. J. Biochem. **138**: 533-541.

SCOTT, R. A., WALLIN, S. A., CZECHOWSKI, M., DERVARTANIAN, D. V., LEGALL, J., PECK, H. D., JR., and MOURA, I. 1984. X-ray absorption spectroscopy of nickel in the hydrogenase of *Desulfovibrio gigas*. J. Amer. Chem. Soc. **106**: 6864-6865.

SEEFELDT, L. C., and D. J. ARP. 1986. Purification to homogeneity of *Azotobacter vinelandii* hydrogenase: a nickel and iron containing dimer. Biochimie **68**: 25-34.

SELDIN, L., VAN ELSAS, J. D., and PENIDO, E. G. C. 1984. *Bacillus azotofixans* sp. nov., a nitrogen-fixing species from Brazilian soils and grass roots. Int. J. Syst. Bacteriol. **34**: 451-456.

SHANMUGAM, K. T., and MORANDI, C. 1976. Amino acids as repressors of nitrogenase biosynthesis in *Klebsiella pneumoniae*. Biochim. Biophys. Acta **437**: 322-332.

SHIVAJI, S., RAO, N. S., SAISREE, L., SHETH, V., REDDY, G. S. N., and BHARGAVA, P. M. 1989. Isolation and identification of *Pseudomonas* spp. from Schirmacher Oasis, Antarctica. Appl. Environ. Microbiol. **55**: 767-770.

SILVER, S., and LUSK, J. E. 1987. Bacterial magnesium, manganese, and zinc transport. *In* Ion transport in prokaryotes. *Edited by* B. P. Rosen and S. Silver. Acad. Press, Inc., San Diego. pp. 165-180.

SIMPSON, F. B. 1987. The hydrogen reactions of nitrogenase. Physiol. Plantarum **69**: 187-190.

SIMPSON, F. B., and BURRIS, R. H. 1984. A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. Science **224**: 1095-1097.

SKAAR, H., RYSTAD, B., and JENSEN, A. 1974. The uptake of ^{63}Ni by the diatom *Phaeodactylum tricornutum*. Physiol. Planta **32**: 353-358.

SMITH, B. E., and LANG, G. 1974. Mossbauer spectroscopy of the nitrogenase proteins from *Klebsiella pneumoniae*. Structural assignment and mechanistic conclusions. Biochem. J. **137**: 169-180.

SMITH, L. A., HILL, S., and YATES, M. G. 1976. Inhibition by acetylene of conventional hydrogenase in nitrogen-fixing bacteria. Nature (London), **262**: 209-210.

SPRENT, J. 1979. The biology of nitrogen-fixing organisms. McGraw Hill, Maidenhead.

STACKEBRANDT, E., MURRAY, R. G. E., and TRUPER, H. G. 1988. *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives". Int. J. Syst. Bacteriol. **38**: 321-325.

STAM, H., STOUTHAMER, A. H., and VAN VERSEVELD, H. W. 1987. Hydrogen metabolism and energy costs of nitrogen fixation. FEMS Microbiol. Rev. **46**: 73-92.

STAM, H., VAN VERSEVELD, H. W., DE VRIES, W., and STOUTHAMER, A. H. 1984. Hydrogen oxidation and efficiency of nitrogen fixation in succinate-limited chemostat cultures of *Rhizobium* ORS 571. Arch. Microbiol. **139**: 53-60.

STANIER, R. Y., PALLERONI, N. J., and DOUDOROFF, M. 1966. The aerobic pseudomonads. A taxonomic study. J. Gen. Microbiol. **43**: 159-271.

STEPHENSON, M., and STICKLAND, L. H. 1931. Hydrogenase: a bacterial enzyme activating molecular hydrogen. I. The properties of the enzyme. Biochem. J. **25**: 205-214.

STOLP, H., and GADKARI, D. 1981. Nonpathogenic members of the genus *Pseudomonas*. *In* The prokaryotes, vol. 1. *Edited by* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel. Springer-Verlag, Berlin. pp. 719-741.

STRATTON, G. W., and CORKE, C. T. 1979. The effect of nickel on the growth, photosynthesis, and nitrogenase activity of *Anabaena inaequalis*. Can. J. Microbiol. **25**: 1094-1099.

STULTS, L. W., MALLICK, S., and MAIER, R. J. 1987. Nickel uptake in *Bradyrhizobium japonicum*. J. Bacteriol. **169**: 1398-1402.

STULTS, L. W., O'HARA, E. B., MAIER, R. J. 1984. Nickel is a component of hydrogenase in *Rhizobium japonicum*, J. Bacteriol. **159**: 153-158.

STULTS, L. W., SRAY, W. A., and MAIER, R. J. 1986. Regulation of hydrogenase biosynthesis by nickel in *Bradyrhizobium japonicum*. Arch. Microbiol. **146**: 280-283.

SYLVESTRE, M., MAILHIOT, K., AHMAD, D., and MASSE, R. 1989. Isolation and preliminary characterization of a 2-chlorobenzoate degrading *Pseudomonas*. Can. J. Microbiol. **35**: 439-443.

TABILLION, R., and KALTWASSER, H. 1977. Energieabhangige ^{63}Ni-Aufnahme bei *Alcaligenes eutrophus* Stamm H1 und H16. Arch. Microbiol. **113**: 145-151.

TABILLION, R., WEBER, F., and KALTWASSER, H. 1980. Nickel requirement for chemolithotrophic growth in hydrogen-oxidizing bacteria. Arch. Microbiol. **124**: 131-136.

TABITA, F. R., CARUSO, P., and WHITMAN, W. 1978. Facile assay of enzymes unique to the Calvin cycle in intact cells, with special reference to ribulose 1,5-bisphosphate carboxylase. Anal. Biochem. **84**: 462-472.

TAKAKUWA, S. 1987. Nickel uptake in *Rhodopseudomonas capsulata*. Arch. Microbiol. **149**: 57-61.

TAKAKUWA, S., and WALL, J. D. 1981. Enhancement of hydrogenase activity in *Rhodopseudomonas capsulata* by nickel. FEMS Microbiol. Lett. **12**: 359-363.

TAMAOKA, J., HA, D.-M., and KOMAGATA, K. 1987. Reclassification of *Pseudomonas acidovorans* den Dooren de Jong 1926 and *Pseudomonas testosteroni* Marcus and Talalay 1956 as *Comamonas acidovorans* comb. nov. and *Comamonas testosteroni* comb. nov., with an emended description of the genus *Comamonas*. Int. J. Syst. Bacteriol. **37**: 52-59.

TEIXEIRA, M., FAUQUE, G., MOURA, I., LESPINAT, P. A., BERLIER, Y., PRICKRIL, B., PECK, H. D., JR., XAVIER, A. V., LEGALL, J., and MOURA, J. J. G. 1987. Nickel-(iron-sulfur)-selenium-containing hydrogenases from *Desulfovibrio baculatus* (DSM 1743). Eur. J. Biochem. **167**: 47-58.

TEIXEIRA, M., MOURA, I., FAUQUE, G., CZECHOWSKI, M., BERLIER, U., LESPINAT, P. A., LEGALL, J., XAVIER, A. V., and MOURA, J. J. G. 1986. Redox properties and activity studies on a nickel-containing hydrogenase isolated from a halophilic sulfate reducer *Desulfovibrio salexigens*. Biochimie **68**: 75-84.

TEIXEIRA, M., MOURA, I., XAVIER, A. V., HUYNH, B. H., DERVARTANIAN, D. V., PECK, H. D., JR., LEGALL, J., and MOURA, J. J. G. 1985. Electron paramagnetic resonance studies on the mechanism of activation and the catalytic cycle of the nickel-containing hydrogenase from *Desulfovibrio gigas*. J. Biol. Chem. **260**: 8942-8950.

THIMMAIAH, S. K., SHARMA, N. D., and LODHA, M. L. 1986. Regulation of uptake hydrogenase expression in mungbean (*Vigna radiata* L) *Rhizobium* strain S-24. Effect of carbon and nitrogen sources. Ind. J. Exptl. Biol. **24**: 242-245.

THOMAS-BAUZON, D., WEINHARD, P., VILLECOURT, P., and BALANDREAU, J. 1982. The spermosphere model. I. Its use in growing, counting and isolating N_2-fixing bacteria from the rhizosphere of rice. Can. J. Microbiol. **18**: 922-928.

THORNELEY, R. N. F., and EADY, R. R. 1973. Nitrogenase of *Klebsiella pneumoniae*: evidence for an adenosine triphosphate induced association of the iron-sulphur protein. Biochem. J. **133**: 405-408.

THORNELEY, R. N. F., and WILLISON, K. R. 1974. Nitrogenase of *Klebsiella pneumoniae*: inhibition of acetylene reduction by magnesium ion explained by the formation of an inactive dimagnesium-adenosine triphosphate complex. Biochem. J. **139**: 211-214.

TIBELIUS, K. H., and KNOWLES, R. 1983. Effect of hydrogen and oxygen on uptake hydrogenase activity in nitrogen-fixing and ammonium-grown *Azospirillum brasilense*. Can. J. Microbiol. **29**: 1119-1125.

TIBELIUS, K. H., and KNOWLES, R. 1984. Hydrogenase activity in *Azospirillum brasilense* is inhibited by nitrite, nitric oxide, carbon monoxide, and acetylene. J. Bacteriol. **160**: 103-106.

TIBELIUS, K. H., ROBSON, R. L., and YATES, M. G. 1987. Cloning and characterization of hydrogenase genes from *Azotobacter chroococcum*. Mol. Gen. Genet. **206**: 285-290.

TIBELIUS, K. H., and YATES, M. G. 1989. Complementation of *Azotobacter chroococcum* hydrogenase mutants by cloned *hup* determinants from *Bradyrhizobium japonicum* and *Azotobacter chroococcum*. FEMS Microbiol. Lett. **65**: 53-58.

TUBB, R. S., and POSTGATE, J. R. 1973. Control of nitrogenase synthesis in *Klebsiella pneumoniae*. J. Gen. Microbiol. **79**: 103-117.

VAN BAALEN, C., and O'DONNELL, R. 1978. Isolation of a nickel-dependent blue-green alga. J. Gen. Microbiol. **105**: 351-353.

VAN BERKUM, P. 1987. Expression of uptake hydrogenase and hydrogen oxidation during heterotrophic growth of *Bradyrhizobium japonicum*. J. Bacteriol. **169**: 4565-4569.

VAN BERKUM, P., and MAIER, R. J. 1988. Lack of carbon substrate repression of uptake hydrogenase activity in *Bradyrhizobium japonicum* SR. J. Bacteriol. **170**: 1962-1964.

VAN DER WERF, A. N., and YATES, M. G. 1978. Hydrogenase from nitrogen-fixing *Azotobacter chroococcum*. *In* Hydrogenases: their catalytic activity, structure and function. *Edited by* H. G. Schlegel and K. Schneider. Erich Goltze KG, Gottingen. pp. 307-326.

VAN DER ZWAAN, J. W., ALBRACHT, S. P. J., FONTIJN, R. D., and SLATER, E. C. 1985. Monovalent nickel in hydrogenase from *Chromatium vinosum*. FEBS Lett. **179**: 271-277.

VEEGER, C., LAANE, C., SCHERINGS, G., MATZ, L., HAAKER, H., and VAN ZEELAND-WOLBERS, L. 1980. Membrane energization and nitrogen fixation in *Azotobacter vinelandii* and *Rhizobium leguminosarum*. *In* Nitrogen fixation, vol. 1. *Edited by* W. E. Newton and W. H. Orme-Johnson. University Park Press, Baltimore. pp. 111-138.

VIGNAIS, P. M., COLBEAU, A., WILLISON, J. C., and JOUANNEAU, Y. 1985. Hydrogenase, nitrogenase, and hydrogen metabolism in the photosynthetic bacteria. Adv. Microb. Physiol. **26:** 155-234.

VIGNAIS, P. M., HENRY, M. F., SIM, E., and HELL, D. B. 1981. The electron transport system and hydrogenase of *Paracoccus denitrificans*. Curr. Topics Bioenerg. **12:** 115-196.

VITERI, S. E., and SCHMIDT, E. L. 1989. Chemoautotrophy as a strategy in the ecology of indigenous soil bradyrhizobia. Soil Biol. Biochem. **21:** 461-463.

VOETS, J. P., and DEBACHER, J. 1956. *Pseudomonas azotogensis* nov. sp., a new free-living nitrogen-fixing bacterium. Naturwissenschaften **43:** 40-41.

WALKER, C. C., and YATES, M. G. 1978. The hydrogen cycle in nitrogen-fixing *Azotobacter chroococcum*. Biochimie **60:** 225-231.

WANG, Z.-C., and WATT, G. D. 1984. H_2-uptake activity of the MoFe protein component of *Azotobacter vinelandii* nitrogenase. Proc. Natl. Acad. Sci. U.S.A. **81:** 376-379.

WATANABE, I., and BARRAQUIO, W. L. 1979. Low levels of fixed nitrogen required for isolation of free-living N_2-fixing organisms from rice roots. Nature (London), **277:** 565-566.

WATANABE, I., BARRAQUIO, W. L., and DAROY, M. L. G. 1982. Predominance of hydrogen utilizing bacteria among N_2-fixing bacteria in wetland rice roots. Can. J. Microbiol. **28:** 1051-1054.

WATANABE, I., SO, R., LADHA, J. K., KATAYAMA-FUJIMURA, Y., and KURAISHI, H. 1987. A new nitrogen-fixing species of pseudomonad: *Pseudomonas diazotrophicus* sp. nov. isolated from the roots of wetland rice. Can. J. Microbiol. **33:** 670-678.

WEATHERBURN, M. W. 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. **39:** 971-974.

WEBB, M. 1970. Interrelationships between the utilization of magnesium and the uptake of other bivalent cations by bacteria. Biochim. Biophys. Acta **222:** 428-439.

WELCH, R.M. 1981. The biological significance of nickel. J. Plant Nutr. **3:** 345-356.

WHITTENBURY, R., PHILLIPPS, K. C., and WILKINSON, J. F. 1970. Enrichment, isolation and some properties of methane-utilizing bacteria. J. Gen. Microbiol. **61:** 205-218.

WIEGEL, J., and SCHLEGEL, H.G. 1976. Enrichment and isolation of nitrogen-fixing hydrogen bacteria. Arch. Microbiol. **107:** 139-142.

WILDE, E., and SCHLEGEL, H. G. 1982. Oxygen tolerance of strictly aerobic hydrogen bacteria. Antonie van Leeuwenhoek J. Microbiol. **48:** 131-143.

WILHELM, E., BATTINO, R., and WILCOCK, R. J. 1977. Low-pressure solubility of gases in liquid water. Chem. Rev. **77:** 219-262.

WILLECKE, K., GRIES, E. -M., and OEHR, P. 1973. Coupled transport of citrate and magnesium in *Bacillus subtilis*. J. Biol. Chem. **248:** 807-814.

WILLEMS, A., BUSSE, J., GOOR, M. POT, B., FALSEN, E., JANTZEN, E., HOSSE, B., GILLIS, M., KERSTERS, K., AULING, G., and DE LEY, J. 1989. *Hydrogenophaga*, a new genus of hydrogen-oxidizing bacteria that includes *Hydrogenophaga flava* comb. nov. (formerly *Pseudomonas flava*), *Hydrogenophaga palleroni* (formerly *Pseudomonas palleroni*), *Hydrogenophaga pseudoflava* (formerly *Pseudomonas pseudoflava* and "*Pseudomonas carboxydoflava*"), and *Hydrogenophaga taeniospiralis* (formerly *Pseudomonas taeniospiralis*). Int. J. Syst. Bacteriol. **39:** 319-333.

WILSON, P. W., HULL, J. F., and BURRIS, R. H. 1942. Competition between free and combined nitrogen in nutrition of *Azotobacter*. Proc. Natl. Acad. Sci. U.S.A. **29:** 289-294.

WILSON, J. B., LEE, S. B., and WILSON, P. W. 1942. Mechanism of biological nitrogen fixation. IX. Properties of hydrogenase in *Azotobacter*. J. Biol. Chem. **144:** 265-271.

WOESE, C. R. 1987. Bacterial evolution. Microbiol. Rev. **51:** 221-271.

WOESE, C. R., BLANZ, P., and HAHN, C. 1984. What isn't a pseudomonad: the importance of nomenclature in bacterial classification. Syst. Appl. Microbiol. **5:** 179-195.

WONG, T. -Y., and MAIER, R. J. 1984. Hydrogen-oxidizing electron transport components in nitrogen-fixing *Azotobacter vinelandii*. J. Bacteriol. **159:** 348-352.

WONG, T.-Y., and MAIER, R. J. 1985. H_2-dependent mixotrophic growth of N_2-fixing *Azotobacter vinelandii*. J. Bacteriol. **63:** 528-533.

WONG, T.-Y., GRAHAM, L., O'HARA, E., and MAIER, R. J. 1986. Enrichment for hydrogen-oxidizing *Acinetobacter* spp. in the rhizosphere of hydrogen-evolving soybean root nodules. Appl. Environ. Microbiol. **52:** 1008-1013.

WOOD, P. M. 1988. Chemolithotrophy. *In* Bacterial energy transduction. *Edited by* C. Anthony. Acad. Press, London. pp. 183-230.

WORSEY, M. J., and WILLIAMS, P. A. 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J. Bacteriol. **124:** 7-13.

YANG, H., DANIEL, S. L., HSU, T., and DRAKE, H. L. 1989. Nickel transport by the thermophilic acetogen *Acetogenium kivui*. Appl. Environ. Microbiol. **55:** 1078-1081.

YATES, M. G. 1972. Electron transport to nitrogenase in *Azotobacter chroococcum*: azotobacter flavodoxin hydroquinone as an electron donor. FEBS Lett. **27:** 63-67.

YATES, M. G. 1977. Physiological aspects of nitrogen fixation. *In* Recent developments in nitrogen fixation. *Edited by* W. E. Newton, J. R. Postgate, and C. Rodriguez-Barrueco. Acad. Press, London. pp. 219-270.

YATES, M. G. 1988. The role of oxygen and hydrogen in nitrogen fixation. *In* The nitrogen and sulfur cycles. *Edited by* J. A. Cole and S. J. Ferguson. Cambridge Univ. Press, Cambridge. pp. 363-382.

YATES, M. G., and CAMPBELL, F. O. 1989. The effect of nutrient limitation on the competition between an H_2-uptake hydrogenase positive (Hup$^+$) recombinant strain of *Azotobacter chroococcum* and the Hup$^-$ mutant parent in mixed population. J. Gen. Microbiol. **135:** 221-225.

YATES, M. G., and PARTRIDGE, C. D. P. 1984. Regulation of synthesis of H_2-uptake hydrogenase in *Azotobacter chroococcum*: correlation with the NAD:NADH ratio. *In* Advances in nitrogen fixation research. *Edited by* C. Veeger and W. E. Newton. Nijhoff/Junk, The Hague. pp. 245.

YATES, M. G., and ROBSON, R. L. 1985. Mutants of *Azotobacter chroococcum* defective in hydrogenase activity. J. Gen. Microbiol. **131**: 1459-1466.

YATES, M. G., and WALKER, C. C. 1980. Hydrogenase activity and hydrogen evolution by nitrogenase in nitrogen-fixing *Azotobacter chroococcum*. *In* Nitrogen fixation, vol. 1. *Edited by* W. E. Newton and W. H. Orme-Johnson. University Park Press, Baltimore. pp. 95-109.

YOCH, D. C. 1974. Electron transport carriers involved in nitrogen fixation by the coliform, *Klebsiella pneumoniae*. J. Gen. Microbiol. **83**: 153-164.

YOCH, D. C. 1979. Electron-transport systems coupled to nitrogenase. *In* A treatise on dinitrogen fixation. Sect. I and II: inorganic and physical chemistry and biochemistry. *Edited by* R. W. F. Hardy, F. Bottomley, and R. C. Burns. John Wiley and Sons, New York. pp. 605-652.

YOCH, D. C., BENEMANN, J. R., VALENTINE, R. C., and ARNON, D. I. 1969. The electron transport system in nitrogen fixation by *Azotobacter*. II. Isolation and function of a new type of ferredoxin. Proc. Natl. Acad. Sci. U.S.A. **64**: 1404-1410.

YOU, C. B., LI, X., WANG, Y., QIU, Y., MO, X., and ZHANG, Y. 1983. Associative dinitrogen fixation of *Alcaligenes faecalis* with rice plants. Biol. Nitrogen Fixation, Sydney, Australia **11**: 92-103.

YOUNG, H. L., aand KLEIN, H. P. 1967. Effects of valine on protein synthesis and turnover in *Pseudomonas saccharophila* under "non gratuitous" inducing conditions. J. Bacteriol. **93**: 830-834.

YOUNGMAN, R. J. 1984. Oxygen activation: is the hydroxyl radical always biologically relevant? Trends Biochem. Sci. **9**: 280-282.

ZOLG, W., and OTTOW, J. C. G. 1975. *Pseudomonas glathei* sp. nov., a new nitrogen-scavenging rod isolated from acid lateritic relicts in Germany. Z. Allg. Mikrobiol. **15**: 287-299.

ZUBER, M., HARKER, A. R., SULTANA, M. A., and EVANS, H. J. 1986. Cloning and expression of *Bradyrhizobium japonicum* uptake hydrogenase structrual genes in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **83**: 7668-7672.