Carbon-13 NMR spectroscopy and bacterial description
by Valerie Nash Hall

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology
Montana State University

Abstract:
There is very little knowledge of the pathogenicity of cereal grains and grasses by the bacterium, Xanthomonas campestris pathovar translucens. On a molecular level, interactions between pathogen and host and description of the pathogen itself have not been discussed. Natural abundance carbon-13 nuclear magnetic resonance spectroscopy, a technique affording description of intact organisms, was used to describe and distinguish between strains within the translucens pathovar. A unique, repeatable spectrum for each strain of the organism was obtained when using intact cells as the nmr sample. In addition, differences were noted between a normal parent and a transposon derived mutant which had become incapable of pathogenizing barley. This preliminary work was conducted to gain knowledge of the applicability of nmr spectroscopy to studies involving the plant/pathogen complex.

When using natural abundance carbon-13 nmr spectroscopy, the amount of nmr time needed for data accumulation of bacterial samples (8 hours) is likely to be prohibitory for routine use. To shorten the time needed for data collection, a method of carbon-13 enrichment of the natural abundance spectrum was devised. Carbon-13, in the form of uniformly labelled glucose, was incorporated into the various cellular components where the majority of the nmr resonances are observed (carbohydrate and fatty acid), and a spectrum was obtained in a relatively short time (1-2 hours).

CARBON-13 NMR SPECTROSCOPY

AND BACTERIAL DESCRIPTION

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APPROVAL

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Valerie Nash Hall

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Nov 30, 1983 — Date

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Signature Valerie N. Hall

Date 9-18-83

In dedication to my family:
my husband, Craig, and my son, Christen.

Vita

Valerie Nash Hall, daughter of William and Adelaide Nash, was born February 23, 1958, in Livingston, Montana. She received her secondary education in Livingston, and graudated from Park High School in May 1976. In March 1978, she entered Montana State University and graduated in March, 1982 with a Bachelor of Science in Agriculture. She entered graduate school at Montana State University, Department of Plant Pathology in March of 1982 and will complete degree requirements for a Master of Science degree in December 1983.

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Abstract

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When using natural abundance carbon-13 nmr spectroscopy, the amount of nmr time needed for data accumulation of bacterial samples (8 hours) is likely to be prohibitory for routine use. To shorten the time needed for data collection, a method of carbon-13 enrichment of the natural abundance spectrum was devised. Carbon-13, in the form of uniformly labelled glucose, was incorporated into the various cellular components where the majority of the nmr resonances are observed (carbohydrate and fatty acid), and a spectrum was obtained in a relatively short time (1-2 hours).

INTRODUCTION

It has traditionally been impossible to survey a system on a molecular level without separation of the whole into its minute parts. This is especially true when discussing agricultural research and the interactions between plants and the organisms parasitizing them.

There are many unanswered questions involving general plant and pathogen interactions: What are the mechanisms of pathogenicity? Why are otherwise indistinguishable pathogens able to infect different hosts; and what are the steps in disease generation where manipulation could favor the host over the pathogen? Current research techniques are often incapable of approaching these questions with the aegricorpus in mind: the sharing of biosynthetic materials and pathways by more than one individual organism.

These circumstances have influenced knowledge regarding the plant/pathogen complex causing Bacterial Leaf Streak-Black Chaff of cereal grains and grasses. There is little molecular information available of biosynthetic metabolism and structure of the causal agent *Xanthomonas campestris* pv. *translucens*.

Standard physiological tests have revealed no basis for differentiation of this bacterium from other xanthomonads. In addition, no molecular information distinguished each bacterial strain within the *translucens* pathovar. Traditionally, strains have been identified by host range testing, a cumbersome and time consuming technique. Although host range testing describes a very important aspect of the pathogen, it is self limiting, and directed toward only one trait of the organism.

The goal of this research was development of a technique of carbon-13 nmr spectroscopy of live bacterial cells. This technique was used for differentiation between strains of *Xanthomonas campestris* pv. *translucens* and will potentially be used for studies involving mechanisms of pathogenicity. Initial experimentation utilized the naturally abundant (1.1%) carbon-13. Analysis was not directed toward a certain molecule, but rather toward the entire spectrum of carbon atoms contained in the sample. In hopes of quickly gaining better resolution and a clearer picture of what was carried by the system, a method of carbon-13 enrichment was designed where the isotope would be incorporated into many significant areas of the cell.

LITERATURE REVIEW

There is a distinct need for a system of molecular examination of living organisms which allows one to look at the system in its intact form. Traditional biochemical analysis is often most successful when used to examine structural or metabolic components individually. When complex metabolic systems are taken apart, disruptions occur which are not common to the living system, and incorrect data may be obtained.

Nuclear magnetic resonance spectroscopy (nmr) is a technique allowing for description of intact organisms without known disruption of the living system. Nmr is a scientific tool which was theoretically devised in the mid 1940's. Since that time, the techniques and applications of nmr spectroscopy have expanded into a productive field of biochemistry.

A brief theoretical explanation of nuclear magnetic resonance spectroscopy by Shulman, (1983), is summarized: Nmr spectroscopy relies on the fact that atomic nuclei with an odd number of protons and neutrons have intrinsic magnetism that, in essence, makes each nucleus a "bar magnet." Included are the hydrogen-1(proton) nucleus, occurring in 99.98% of all hydrogen atoms in nature, the carbon-13 nucleus, which is the nucleus of only 1.1% of all carbon atoms, and the nucleus of phosphorus-31, which occurs in essentially all phosphorus atoms.

In nmr spectroscopy, two fields are applied to the sample. The first field is a strong magnetic field. It causes the nuclear "bar magnets" to orient themselves so that the dipole of each nucleus is aligned either with the field or against it. Alignment with the field is the state in which the nucleus stores less energy than it does when it is aligned against the field.

A second field is then applied in pulses. It consists of electromagnetic radiation in the radio frequency band of the energy spectrum. For any particular strength of the magnetic field in which the sample is placed, there is a particular frequency of electromagnetic radiation for which each quantum of radiation will carry precisely the right amount of energy to allow a certain type of atomic nucleus to "flip." When the electromagnetic radiation is no longer applied, the nuclei relax back to a state of alignment with the magnetic field. In doing so, these small "bar magnets" reradiate a measurable amount of energy and this is the basis for the nmr signal. The chemical environment of the atomic nuclei dictates the energy level necessary to "flip" a nucleus and, therefore, the amount of energy reradiated.

A spectrometer records the varying amounts of re-radiated energy. After a mathematical reinterpretation or "Fourier transform" of the data to move from a time domain to a frequency domain, the results are presented as a spectrum with one to many individual resonances, depending on the sample used. Each resonance represents a certain atomic nucleus and the larger the concentration of that nucleus in the sample, the more intense the resonance.

Proton nmr spectroscopy is a fast and useful tool for determining structures of pure compounds. However, its use in biological work is limited. The number of protons found in a biological sample is far too great to allow for an easily interpretable spectrum. A more useful and definitive nucleus for use with biological systems is carbon-13. However, this isotope has a low level of natural abundance and necessitates a longer data gathering time than needed for nuclei which are virtually 100% abundant, i.e. H-1 and P-31.

In light of this, carbon-13 nmr spectroscopy and its relation to biological systems is usually approached with use of a carbon-13 labelled compound. A given peak in an nmr spectrum can then be easily assigned to the labelled carbon atom at a particular position in a given molecule. The number of carbon-13 atoms at that position in a sample is far greater than one would predict from the

1.1% natural abundance of carbon-13. It is possible to follow the carbon-13 introduced as a label as it passes through various positions and various molecules.

Studies utilizing compounds labelled with carbon-13 include work by Shulman et al.(1978,1983), on glucose metabolism in Escherichia coli by following a labelled carbon number one on a glucose molecule. Bancroft et al. (1982,unpublished), monitored the conversion of aspartic acid to lysine with carbon number one labelled aspartic acid. The utilization and conversion of labelled glucose to volatile acids have been monitored by carbon-13 nmr (Rundquist et al.,1981). Direct observations of porphyrinogen biosynthesis in living systems were made using carbon-13 labelled substrates and glucose (Scott et al.,1979). Glucose metabolism has been observed in several living organisms, among them Treponema spp. (Ohsaka et al,1981). Dictyostelium discoideum (Hall, et.al.,1983, unpublished),Saccharomyces cerevisiae, (Bancroft et al., unpublished), rabbit erythrocytes, Escherichia coli and rat liver cells (Shulman,1978).

In addition to information gathered involving major metabolic processes, production of several antibiotics from labelled precursors has been followed in vitro with carbon-13 nmr. Included are tylosin, leucomycin and pathway intermediates of these produced by Streptomyces spp. (Omura et al,1977). Incorporation of acetate to minimycin, compounds related to the Krebs cycle and biosynthetic studies with the lancocidin group of antibiotics were monitored by Uramoto et al.(1978). Other synthetic pathways for antibiotics include donamycin and adreamycin (Shaw et al.,1979.) and malonomycin (Schipper et al.,1979). Also included are ranotycin (Snopes et al.,1979), caerulomycin (McInnes et al.,1979), chlorothricin (Masacaretti et al.,1979), streptothrycin (Gould et al.,1979), and bleomycin (Nakatani et al.,1980).

The kinetics of enzyme action have been elucidated in vivo using carbon-13 nmr spectroscopy. Some systems examined include: the binding of galactopyranocides to peanut agglutinin (Neurohr et al.,1981), bacterial and mammalian histidine decarboxylase (Johnson et al.,1977), dihydrofolate reductase systems in _Streptococcus faecium_ (Cocco et.al,1978) and alkaline phosphatase production in _Escherichia coli_ (Browne,et al.,1976).

In addition to metabolic studies, structural studies using labelled compounds have been undertaken. Several amino acids of the tobacco mosaic virus protein coat were observed by Hemminga et al.(1981), and chemical modification of the RNA of _Torula_ was studied directly with carbon-13 nmr spectroscopy (Chang et al.,1981). Studies involving membrane lipid structure (Smith, 1978) and the structure of capsular polysaccharides (Jennings et al., 1977) have been completed.

A relatively large amount of data has been gathered utilizing carbon-13 labelled compounds. In contrast, the use of natural abundance carbon-13 nmr for general description or characterization of intact systems has been used very little.

Soil organic fractions extracted in sequence with several solvents have been successfully elucidated (Sciacovelli et al.,1980) and natural abundance carbon-13 nmr of rat skin has been carried out by Yoshikawa and Oshaka (1981). Special carbon-13 nmr techniques were used for determination of protein content in single, viable horse bean and soy bean, bean and corn seeds. Significant seed to seed variations were found which can be used as a basis for rapid seed selection in plant breeding programs (Rutar et al., 1980).

Use of natural abundance carbon-13 nmr with microbial systems has been limited. However, one unusual experiment was carried out by Matsunaga et al.(1980). In this study, the fungus Penicillium ochro-chloran was grown in a glucose based medium and subsamples were taken periodically from two to twenty days. Natural abundance carbon-13 data were gathered and the resulting spectra showed a periodic rise and fall of selected cytoplasmic carbon compounds over the duration of the experiment. The age or growth stage of the fungus was characterized in this experiment, but no comparisons were made from one fungal species to another or between fungal isolates of the same species. To date, no reports have been made on bacterial description using natural abundance carbon-13 nmr nor have plant pathogens of any kind been studied.

The Organism

The bacterium chosen for this study was Xanthomonas campestris pv. translucens, the causal agent of Bacterial Leaf Streak of cereal grains and grasses, sometimes referred to as Black Chaff. The organism had been described as "Bacterium translucens" by Jones, et al., (1917) as follows: cylindrical rods, rounded at ends, solitary or in pairs, individual rods, 0.5 - 0.8 x 1 - 2.5 microns, motile by a single polar flagellum, aerobic, no spores, gram negative, and forming yellow colonies on peptone beef agar plates. In 1942 Hagborg revised the classification and amended the species description to include five closely related organisms which are distinguishable chiefly by differences in pathogenic capabilities on wheat, oats, barley and rye. Recently, this pathogen, assumed to be culturally, physiologically and biochemically indistinguishable from Xanthomonas campestris except by host reaction, has been named a pathovar of the Xanthomonas campestris group. (Dye et al.,1975, and Young, et al.,1978).

The bacterium infects leaves through stomata and spreads intracellularly (Jones, et al.,1917, and Bamberg, 1936). Bacterial invasion is confined only to the thin-wall parenchyma possessing intracellular space, i.e. mesophyll of leaf, chlorenchyma and ground tissue parenchyma of the leaf sheaths. In naturally infected leaves, no vascular bundle elements have been shown to be infected.

This disease is described as a widely occurring disease attacking leaves, leaf sheaths and glumes. Early infections are characterized by water-soaked lesions with bacterial exudates and later by persistent transparency following the death of the parts invaded (Kim, 1982).

The primary source of inoculum for this disease is infested seed. The bacterium penetrates the pericarp and infection of the plumule occurs through wounds or stomata on the coleoptile. It spreads rapidly through the tissues and finally reaches the enclosed foliage leaves. By elongation, the infected leaf carries the bacteria into the aerial parts of the seedling with water-soaked streaks on the primary leaf.

Optimum temperatures for the growth of this organism on agar have been reported to be 25 - 30 C, and optimum temperature for growth in the field appears to be 22 C. In addition, high relative humidity increases the incidence and severity of the disease (Kim,1982).

The disease is transmitted in the field by aphids, straw, wind and rain, and transmission via diseased plants coming in contact with healthy plants occurs only when the host is water congested. Field epidemiology studies in Montana using marked (antibiotic resistant) strains of Xanthomonas campestris pv. translucens indicated that this bacterium is capable of spreading 28 square meters from a single infection locus within 39 days. This experiment was run under dryland conditions (Hall et al.,1981).

Xanthomonas campestris pv. translucens has two unique aspects by which it differs from the other members of the Xanthomonas campestris group, suggesting that Xanthomonas campestris pv. translucens should be returned to its prior designation as the species Xanthomonas translucens instead of a pathovar of the species Xanthomonas campestris (Kim,1982). Included are the ability to nucleate ice formation which was first reported by Kim in 1982. It is not known if this factor affects the organism's ability to infect its host. However, a severe epidemic of bacterial leaf streak on wheat and barley caused by this xanthomonad in Idaho in 1981 may have been predisposed by the frost occurring between June 15 - 25. Subsequent epidemics occurred in this area (Wesenberg and Sunderman, USDA-ARS Aberdeen Research Station, University of Idaho, personal communication, 1981). This is comparable to findings by Panagopoulos and Crosse (1964), who determined that the development of blossom blight of pear caused by *Pseudomonas syringae* was predisposed by the ice nucleating ability of that organism. Although ice nucleation by *Xanthomonas campestris* pv. *translucens*. may be a predisposing factor to a disease epidemic, the actual mechanism of pathogenesis by this organism is not clearly understood. In addition, very few of the genetic aspects of *Xanthomonas campestris* pv. *translucens* have been elucidated.

Another unique facet of the organism is the constitutive production of a B-galactosidase which allows for several indicator dye assays as a means of identification. This enzyme is associated only with *Xanthomonas campestris* pv. *translucens* among plant pathogens. This and several of the other nutritional features of the bacterium have been identified and have become the basis for a selective medium for *Xanthomonas campestris* pv. *translucens* (Kim et al.,1982).

At the time of this study there was no biochemical method used to differentiate between subgroups within the pathovar *translucens*. The only reliable technique was host range testing. This system measures the ability of the isolate to infect five hosts: wheat, barley, oats, rye and sometimes triticale. The host range testing system in itself reveals nothing biochemically definitive about the organism, and in addition, is a very cumbersome and time consuming technique. Understanding this xanthomonad could possibly be enhanced by nmr studies. The ability to quickly distinguish between strains of *Xanthomonas campestris* pv. *translucens* by a repeatable nmr spectrum would be a valuable tool. In addition, information about the soluble carbon content of the cells may lead to the discovery of mechanisms ot pathogenicity. A study utilizing the *Xanthomonas campestris* pv. *translucens* organism with its many unknowns can provide a model for nmr analysis of other bacterial systems and the involvement of microbes in plant pathology and medical studies.

CHAPTER I

Differentiation of *Xanthomonas campestris* pv. *translucens* Strains by Natural Abundance Carbon-13 nmr Spectroscopy

Introduction

Several strains of *Xanthomonas campestris* pv. *translucens* were compared on the basis on their natural abundance carbon-13 nmr spectra. Included in Figure 1 are three of these: X-58, isolated from wheat growing in Lebanon, X-161, from a Montana barley field and X-162 isolated from western wheat grass (*Agropyron smithii*) in North Dakota. The strains were chosen for their varying host range and location of collection. A reference strain of *Pseudomonas syringae*, M76, was also used for comparison. Whole cells of these bacterial strains were used as nmr samples and were collected when they were in a rapidly growing state.

In addition to the study described above, two related experiments were carried out utilizing the same bacterial strains. The first was to question the influence of stage of growth on the repeatability of the nmr spectrum, and a second was to detect any differences caused by an alternate growing medium, i.e., a minimal or

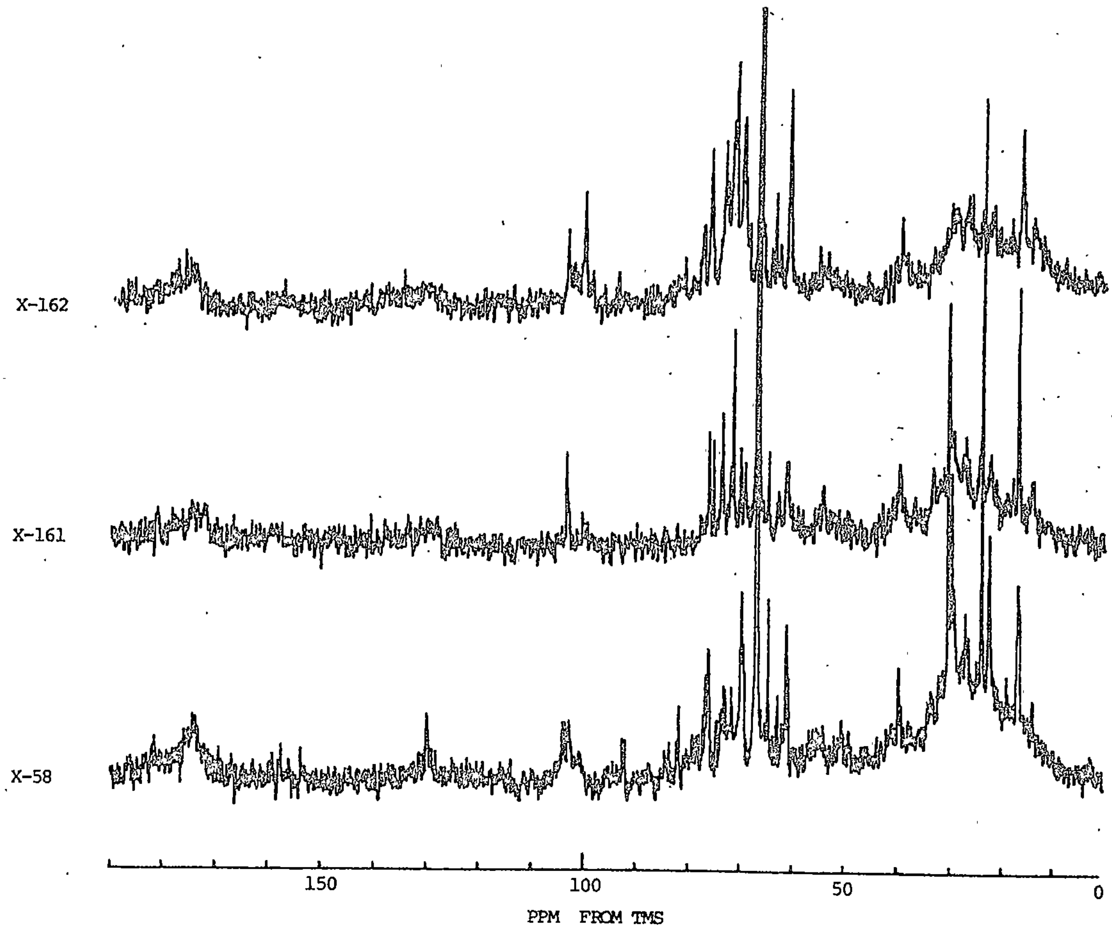


Figure 1. Natural abundance carbon-13 nmr spectra of three strains of _Xanthomonas campestris_ pv. _translucens_.

reduced nutrient medium.

Materials and Methods

Bacteria were cultured in one liter units of Wilbrink's broth medium containing in grams per liter: sucrose 10, Bacto peptone 5 (Difco Laboratories) plus magnesium, potassium, and sodium salts. In addition, 100 mg cycloheximide (Sigma Chemical Company) were added after autoclaving for 20 minutes at 121 C and 20 lbs. pressure.

The cells were grown in vigorous shake culture at 28 C. Growth of bacterial populations was followed with turbidimetric readings every two hours using a Klett-Summersion colorimeter with a blue filter. When in late exponential growth phase, the cells were harvested by centrifugation at 8,000 x G for 10 minutes and were washed three times with a phosphate buffered saline solution (pH 6.8).

The resulting bacterial slurry was transferred to a clean 10 mm diameter nmr sample tube with a Pasteur pipette. An internal reference tube containing deuterium (the lock signal) and a dioxane reference was fitted inside the sample tube. Data was gathered on a Bruker WM-250 nmr spectrometer with an Oxford magnet. Carbon-13 nmr spectra were recorded at 62.83 MHz and were proton decoupled by broad band irradiation of the proton spectrum. Ninety degree pulses were applied one to ten times per second. Thirty thousand to 150,000 free induction decays were accumulated, exponentially multiplied with the line broadening factor of 4 Hz and Fourier transformed to give the resulting spectra (Figure 1). All spectra were recorded at least twice for the isolates shown and numerous others were recorded at least once.

Accuracy needed in sampling time. Bacterial cells were cultured as stated in the first experiment, however, the cells were harvested when in late stationary phase, 24 hours later than those in the previous experiment. The sample was prepared for use in the same manner as those tested in late log phase and the data gathered was compared to that accumulated for samples taken in the exponential phase of growth.

Effect of a reduced nutrient medium. Bacteria were grown in a reduced nutrient broth containing in grams per liter: glucose 2, Bacto peptone 1, magnesium, phosphorus and sodium salts and 100 mg cycloheximide. The culture was shaken vigorously at 28 C as before and the cells were harvested in late exponential growth phase as in the first experiment.

Results

Figure 1 shows the natural abundance carbon-13 nmr spectra of the three xanthomonads X-58, X-161 and X-162. There were common resonances, but others are unique from strain to strain (Figure 1, Table 1.). Table 1 lists the 14 most intense resonances for each spectrum. As the resonances were scaled to a common reference, comparisons between strains could be made. Included in Table 1 are the 14 most intense resonances of the reference strain of Pseudomonas syringae and Figure 2 presents the spectrum of this pseudomonad. Table 2 presents data from two individual experiments with strain X-58 as evidence of the repeatability of this technique.

Comparison of strain X-58 in exponential versus stationary phase is presented in Figure 3. Intensities displayed for this experiment cannot be compared to resonance intensities for the first experiment, as a slightly different reference concentration was used.

Growth of Xanthomonas campestris strain X-58 cells was not achieved in minimal medium + methionine + thiamine as postulated based on the organism's usual amino acid and co-factor requirements (Kim,1982). Strains other than X-58 of Xanthomonas campestris pv. translucens were also unable to grow in this medium. As a result, the reduced nutrient medium was developed which supported bacterial growth. Data accumulated from cell samples grown on the reduced nutrient medium varied in the carbohydrate region of the spectrum (60 ppm to 80 ppm) from the cell sample which was grown in Wilbrink's broth. (Figure 4.)

Table 1. Principal resonances in the natural abundance carbon-13 spectra of several strains of Xanthomonas and Pseudomonas (M-76).

Resonance Position Chemical Shift (PPM from TMS)	Resonance Intensity* X-58	X-161	X-162	M-76
103.2		1.15		
100.2			1.17	
76.2			1.66	2.40
76.0	1.43	1.40		
75.1		1.30		
73.4		1.44	1.79	
72.9			1.35	
72.4				2.58
72.1				2.27
71.9			2.02	
71.4				1.36
71.3		2.63	2.70	
70.0			1.99	2.01
69.7			1.90	
69.6	2.04			
64.5	1.98	1.24		
62.9				6.30
61.0			2.41	
60.9				5.53
60.8	1.72			
39.7				1.50
31.3				2.05
30.4	4.52	3.03		
30.3				2.20
30.2			1.21	
29.8	3.37			
29.4	2.12	1.47		
29.1	1.73			
27.7	1.38			
27.6		1.19		
27.2			1.26	
27.1	1.85	1.40		
26.6				1.67
26.5	1.57		1.22	
24.6				2.64

Table 1. cont.

Resonance Frequency (ppm)	X-58	X-161	X-162	M-76
24.4		1.17		
24.0	7.45	2.97		
22.5	2.71			
22.1		1.19		2.94
16.9		3.21		
16.8	2.16		1.90	2.52

* Resonance intensities of the different strains may be compared to one another because they have been scaled to a common reference.

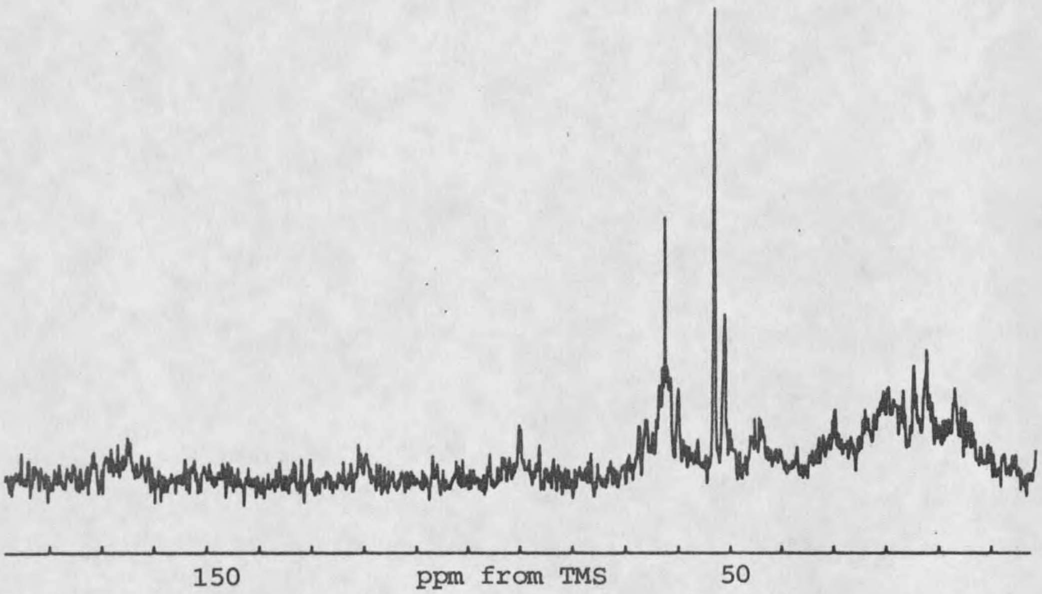


Figure 2. Natural abundance carbon-13 nmr spectrum of <u>Pseudomonas syringae</u> strain M76.

Table 2. Principle resonances of xanthomonad strain X-58; a comparison of two individual natural abundance carbon-13 experiments.

Resonance Position Chemical Shift (ppm from TMS)	Resonance Intensities	
	X-58 (no. 1)	X-58 (no. 2)
76.0	2.00	1.43
69.6	2.10	2.04
64.5	1.55	1.98
60.8	2.31	1.72
30.4	2.24	4.52
29.8	2.20	3.37
29.4	2.29	2.12
29.1	2.32	1.73
27.7	2.25	1.38
26.5	-	1.57
24.0	2.50	2.45
22.5	1.85	2.71
20.5	1.75	-
16.8	2.75	2.16

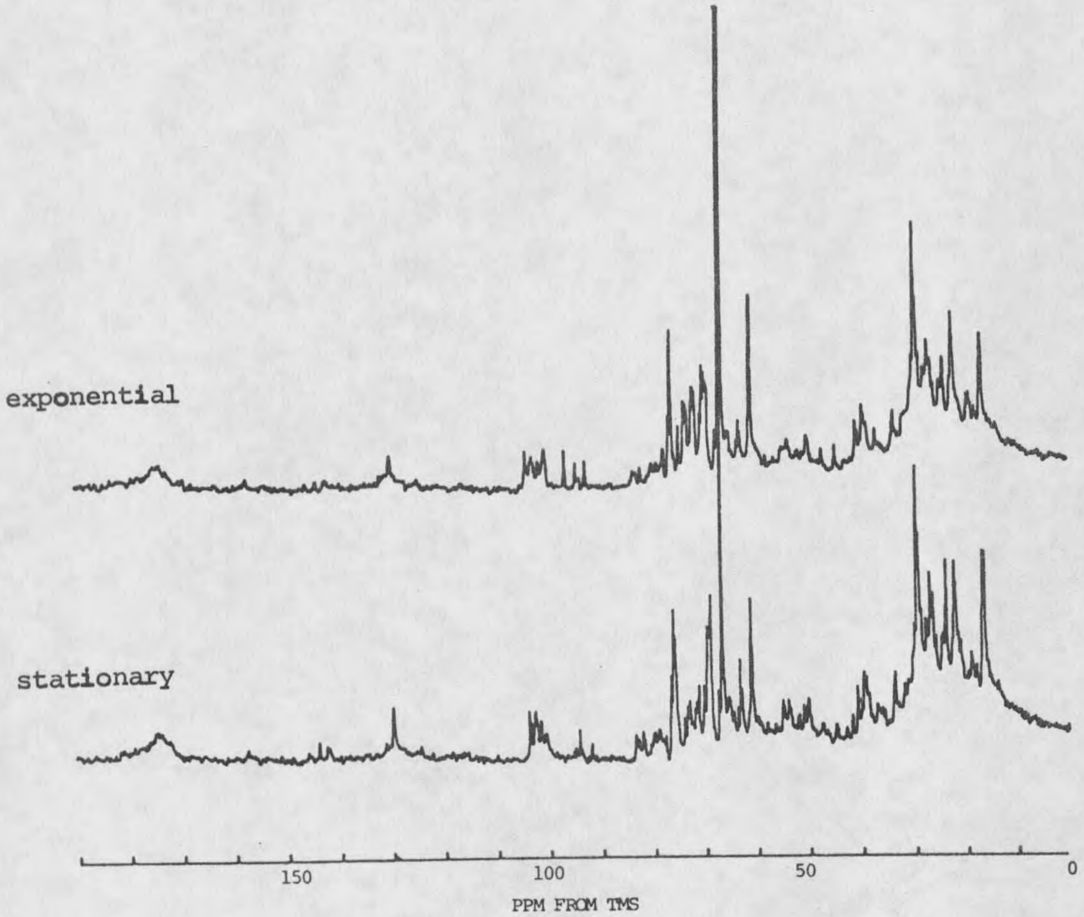


Figure 3. Comparison of exponential and stationary phases of growth of xanthomonad strain X-58 by natural abundance carbon-13 nmr spectroscopy.

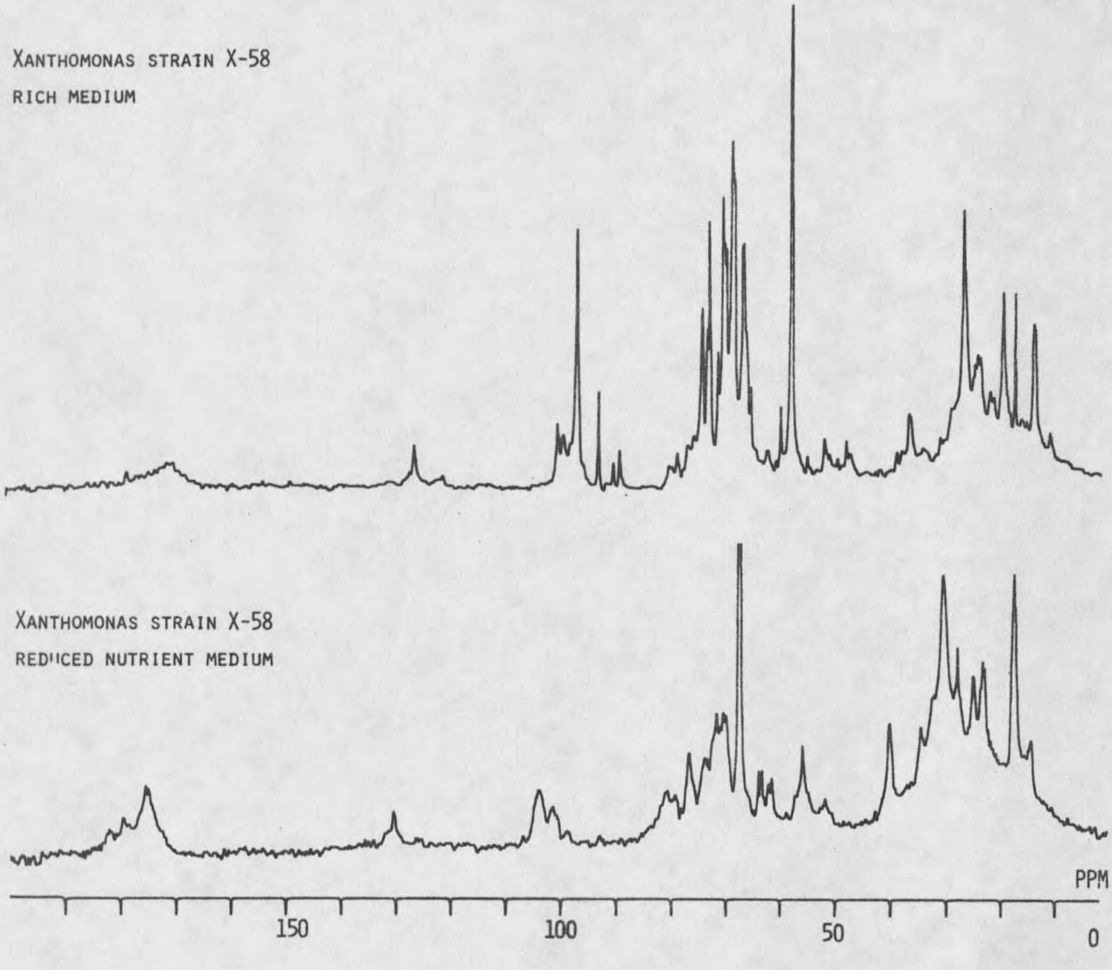


Figure 4. Comparison of carbon-13 natural abundance spectra of xanthomonad strain X-58 grown in rich and reduced nutrient media.

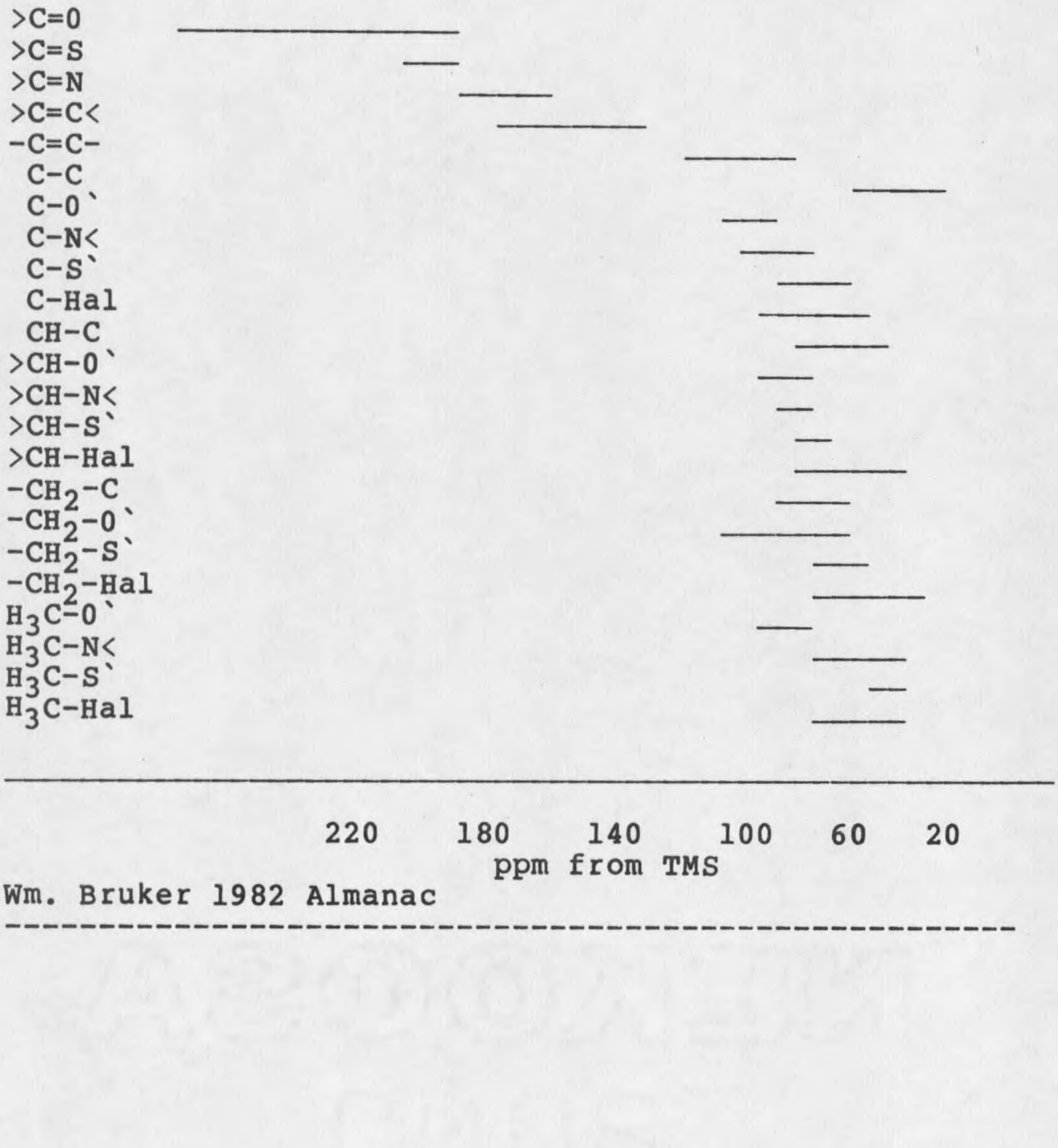
Discussion

The results of these experiments were a series of natural abundance carbon-13 spectra of living cell systems. Precise assignment of individual resonances to specific molecules would be a difficult task in a complex mixture such as this, and it was not attempted. However, general assignments can easily be made based on the tetramethylsilane (TMS) reference at 0 (Fig.5).

It is noteworthy that each bacterial strain tested singularly produced a unique spectrum. The repeatability of the technique was excellent based on resonance presence, and the spectral similarities between the bacterial samples of the pathovar are striking. This suggests that natural abundance carbon-13 nmr may provide a convenient, non-invasive method to characterize and classify bacterial systems.

The limitations of the system must be known to avoid incorrect classification or diagnosis from the pathologist's viewpoint. Results of the two experiments completed using variable growing conditions indicated that growth phase had little effect on the presence of the resulting resonances. However, the intensities and therefore the concentrations of carbon containing metabolites were lower in the cells sampled in stationary phase. This indicated a possible utilization of pools of metabolites stored by the cells in exponential growth

Figure 5.General positions of carbon resonances



Wm. Bruker 1982 Almanac

phase.

According to their respective nmr spectra, the majority of differences between cells grown on a rich (Wilbrink's) medium and a reduced nutrient medium was in the region from 60 to 80 ppm. This corresponded to the carbohydrate region. It was concluded that the growing media affected soluble carbon containing cellular components. For taxonomic purposes, only one growing medium could be used to allow for consistent results.

One serious limitation of natural abundance carbon-13 nmr is the failure to detect compounds present in minute amounts (less than mM). However, in a bacterial characterization study where compounds are not assigned, the most serious limitation is the extensive time needed for data accumulation. For the bacterial data presented here, an average 8 hours of scanning time was used. In contrast, natural abundance work with fungi, yeast and algae (Abbott et al., unpublished) required very short data accumulation time. This is possibly due to the greater amount of soluble carbon containing molecules per unit of volume in fungi, yeast and algae. The ratio of structural cell wall material to soluble cytoplasmic contents is relatively high in bacterial cells. For example, a one gram sample of bacteria contains much more insoluble cell wall material than a comparable sample of fungi or algae. This large time requirement for bacterial data accumulation may be a limiting factor where spectrometer time is in great demand. The most logical step to alleviate the problem of extensive spectrometer time use was development of a technique to increase the carbon-13 content of the bacterial cells.

Table 3. Percentages of common resonances between strains of of *Xanthomonas* and *Pseudomonas*.

strain	X-58	X-161	X-162	M-76
X-58	93	27.2	7.6	3.7
X-161			7.6	3.7
X-162				12
M-76				

CHAPTER II

The Enrichment of Natural Abundance Spectra with Carbon-13 Labelled Glucose

Introduction

Natural abundance carbon-13 nmr spectra provided unique information about individual bacterial isolates, but from a practical point of view, the time required to collect the data from each sample may prohibit routine use. Increasing the carbon-13 content of the cells would decrease the time needed for data collection, and in addition, increase the resolution of the spectrum.

By mathematical manipulation, an increase in the carbon-13 concentration is not directly related to the resonance intensity. Instead, the relationship is exponential. A doubling of the carbon-13 content increases the clarity of data presentation four-fold, for example. As a result, a small increase in the isotopic level has the desired effect of decreasing the time needed for data accumulation.

Several questions arose with regard to the enrichment process. These included: 1) What was the best labelled carbon source to use and what was its availability? 2) What was the optimum level of label to add considering resolution enhancement versus cost? 3) What was the most suitable growing medium for maximum uptake of the labelled glucose? 4) At which growth phase would the cells incorporate and distribute the label most effectively, and how much time should be allowed for label incorporation? The first two questions were answered on the basis of cost and practicality. Solutions were found experimentally for the others.

Uniformly labelled glucose was chosen as the source of carbon-13 for enrichment. The rationale for this choice was the fact that the carbon atoms of glucose will eventually be channeled into every major cellular component synthesized by the system. Uniformly labelled glucose was readily available, although using large amounts of it would have been cost prohibitive at $1,000 to $1,500 per gram. Other less expensive labelled carbon sources, i.e. acetate, were not suitable for use by *Xanthomonas campestris* pv. *translucens*.

From a practical point of view, use of labelled glucose as the sole source of carbon would have defeated much of the purpose of enrichment. A reduction in spectrometer time, (estimated to cost $60.00 per hour) and therefore cost per sample, could be achieved if the amount of label added gave the greatest enrichment for the least possible cost. The amount chosen was 15 mg of uniformly labelled glucose per 100 ml of growing culture. As determined in the following experiments, this amount should have approximately doubled the carbon-13 content of the cells and, as a result, used one fourth the usual nmr scanning time. From a technical viewpoint, it seemed that it would have been convenient to use the labelled compound as the sole carbon source. This was not the case. The problem of carbon-carbon coupling arises when adjacent carbon atoms are both the carbon-13 isotope. The nmr signal from each one is influenced by the adjacent carbon to a degree where resonances intermediate in frequency appear which are not substantiated. The best resolution could be obtained if a single carbon atom on each molecule was labelled and on each molecule the label would appear in a different position. This is essentially the case in natural abundance carbon-13 spectroscopy, although the incidence of carbon-13 atoms is usually less than one per molecule.

Materials and Methods

Medium development. The first experimental data gathered was from the development of a glucose based medium which would support rapid growth of *Xanthomonas campestris* pv.*translucens* cultures and still allow for maximum synthesis of carbon containing cellular components by this bacterium. Several media were tried. The first was a minimal medium which contained in grams per liter: glucose 10, sodium, potassium and magnesium salts, 50 mg/l methionine and 50 mg/l of thiamine.(Davis, et al,1980).

The second minimal medium contained all the components of the first minimal salts medium plus 50 mg/l of glutamic acid, as suggested in Bergey's Manual of Determinative Bacteriology (1975). The pH was adjusted to 6.8 in both cases and the media were autoclaved at 121 C and 20 lbs. pressure for 15 minutes. Sidearm flasks containing 100 ml of media were used to allow for monitoring of growth of cultures. The amino acids and vitamins included in the media were added after autoclaving. After being dissolved in 5 ml of water, they were cold sterilized using autoclaved type GS 0.22 micron filters (Millipore Corporation). After autoclaving the broths, 10^6 cells of strains X-58, X-81S and X-33 of _Xanthomonas campestris_ pv. _translucens_ were taken from glucose based cultures and placed in each flask of autoclaved media. These cultures were shaken vigorously at 28 C for 7 days.

Two other media were tested for ability to support rapid growth of _Xanthomonas campestris_ pv._translucens_ Included was a glucose plus maltose medium containing in grams per liter: glucose 1, maltose 3, Bacto-peptone 5, sodium, potassium and magnesium salts and 100 mg of cycloheximide. Also included was the reduced nutrient medium discussed in Chapter 1 containing in grams per liter: glucose 2, Bacto-peptone 1, sodium, potassium and magnesium salts, and 100 mg/l of cycloheximide. Growth of the cultures in all of the above media was compared to the rapid growth on a rich medium (modified Wilbrink's) which contained in grams per liter: glucose 10, Bacto-peptone 5, sodium, magnesium and potassium salts and 100 mg of cycloheximide. The pH was again adjusted to 6.8 and the media autoclaved in the same manner as before. Media were inoculated with bacteria and incubated as described in the minimal salts procedure.

Growth curves. Using a Klett - Summerson spectrophotometer with a blue filter, growth of several of the bacterial cultures was monitored from the time of inoculation through the resulting growth phases. This information was necessary for setting up a standard procedure for label addition. The standard used for calibration was 5 ml of reduced nutrient medium in a 9 ml capped test tube. This standard was given a turbidity reading of 40 units where 1 unit is equivalent to 10^6 colony forming units per ml. The cultures grown in the sidearm flasks were read approximately every 2 hours and their turbidities were noted. Growth curves were recorded on semi-log paper. Samples from each of the flasks were plated on Wilbrink's agar at the end of growth determinations to detect any contamination.

Label addition. Depending on the availability, uniformly labelled glucose of either 87.5% or 93.0% isotopic purity was used for carbon-13 enrichment. Fifteen mg of uniformly labelled glucose was dissolved in deionized distilled water and cold sterilized as described previously. This sterilized glucose solution was placed directly into bacterial cultures growing in the reduced nutrient medium at different growth phases. As in the previous experiments, the bacteria were grown at 28 C and shaken vigorously. The cultures, grown in 100 ml amounts in sidearm flasks, were labelled in lag, late log or stationary phases. An attempt was also made to label the Xanthomonas campestris pv. translucens culture in late log phase and "chase" or force the label in by addition of 20 mg more of unlabelled glucose 20 minutes after the addition of the label.

A parallel control culture was grown for each of the labelling experiments, and unlabelled glucose in place of the carbon-13 labelled sugar was added in the same manner.

Cells were harvested for nmr experimentation by centrifugation at 8,000 X G for 10 minutes, then washed 3 times with a phosphate buffered saline solution. Comparisons were made of nmr spectra from one labelling method to the others, and between the labelled and the unlabelled control.

Results

Modified Wilbrink's broth, the rich medium to which all others were compared, supported growth of Xanthomonas campestris pv. translucens cultures quickly and heavily (Figure 6). The minimal salts medium plus methionine and thiamine failed to support growth of Xanthomonas campestris pv. translucens cultures. Addition of glutamic acid to the medium did not change this condition. Plating of 0 .1 ml of these inoculated media after 4 days indicated that live xanthomonads were present in both cases, however, their numbers were not increasing. Cultures grown in glucose plus maltose grew well, however, there was no distinction between the termination of utilization of one sugar and the beginning of utilization of the other. (Figure 6). Although the numbers were less when compared to the rich Wilbrink's medium (Figure 6), Xanthomonas campestris pv. translucens cells grew well in the reduced nutrient medium.

No indications were given by nmr spectra of any incorporation of labelled glucose in lag or log phase. The "chase" experiment in which the label was added late in log phase also showed no cellular incorporation of the carbon-13 glucose.

When the sugar was added 5 hours into stationary phase growth, utilization and incorporation were noted. As growth was monitored, an increase of 4 - 5% in cell

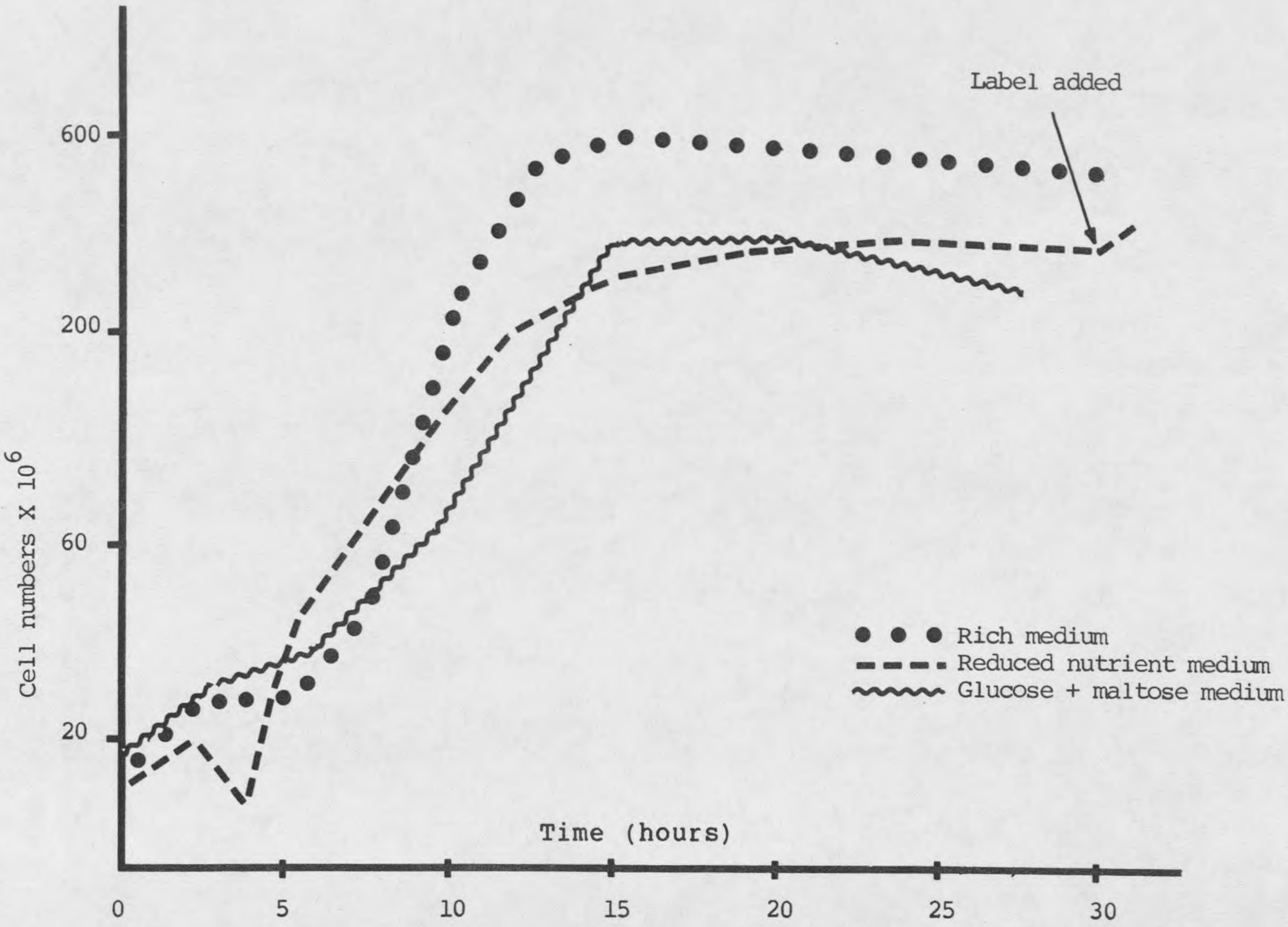


Figure 6. Comparison of growth on media of xanthomonad strain X-58.

number appeared within 45 minutes of glucose addition (Figure 6). No increase was noted after this rise and the number remained stable for 75 minutes at which time a slight decline was noted and the cells were harvested.

This stationary phase culture was used as an nmr sample, and resonances appeared after a compilation of a few hundred scans. After 2,000 scans had been accumulated, the resonances became notable (Figures 7 and 8), and increased with additional spectrometer time. Rather intricate characteristics were revealed after 50,000 scans of an enriched sample, and the resolution was considerably greater than displayed by a non-enriched spectrum (Figure 9).

Discussion

Experimental data provided answers for the third and fourth questions posed at the beginning of this chapter. Deriving a suitable growing medium compatible with carbon-13 enrichment proved to be the most difficult task involved in increasing carbon-13 content of *Xanthomonas campestris* pv. *translucens* cells. Several attempts made to culture the bacteria in minimal salts media plus amino acids were not successful. This was contrary to previous information indicating that the bacterium was capable of growing on a glucose based salts medium with methionine and thiamine (Kim, 1982). Glutamic acid was added to the above minimal medium as it had been noted as an essential

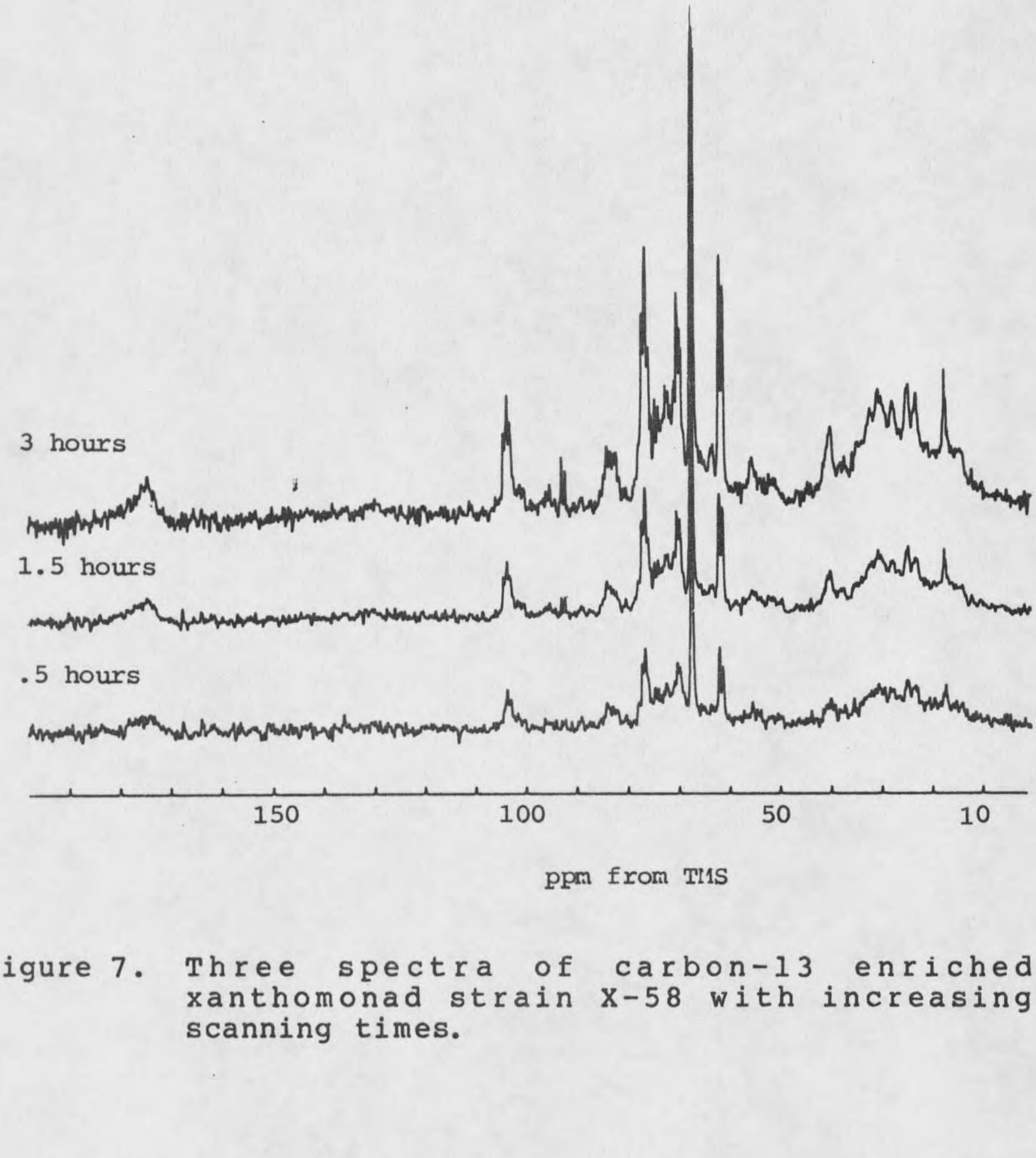


Figure 7. Three spectra of carbon-13 enriched xanthomonad strain X-58 with increasing scanning times.

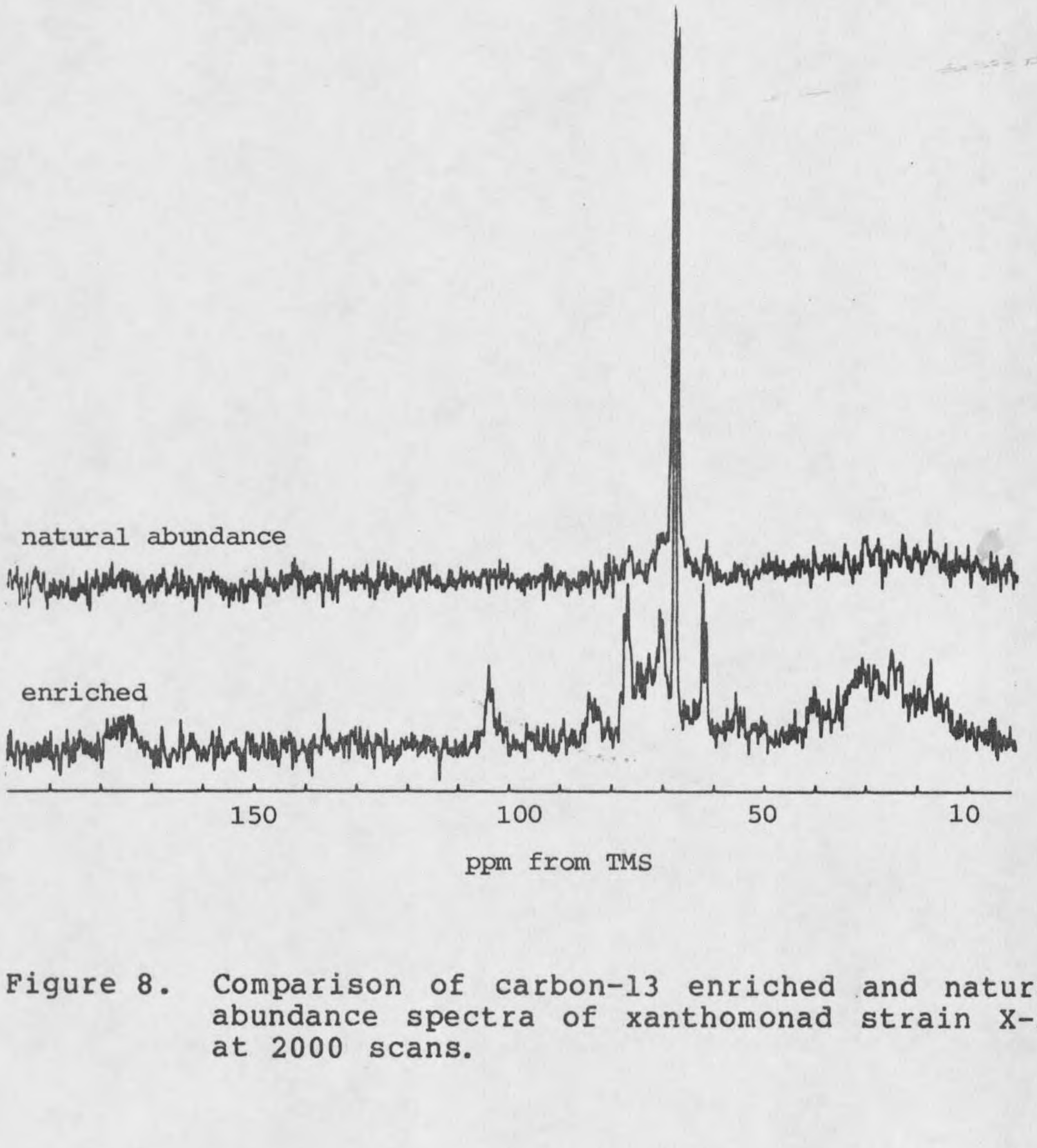


Figure 8. Comparison of carbon-13 enriched and natural abundance spectra of xanthomonad strain X-58 at 2000 scans.

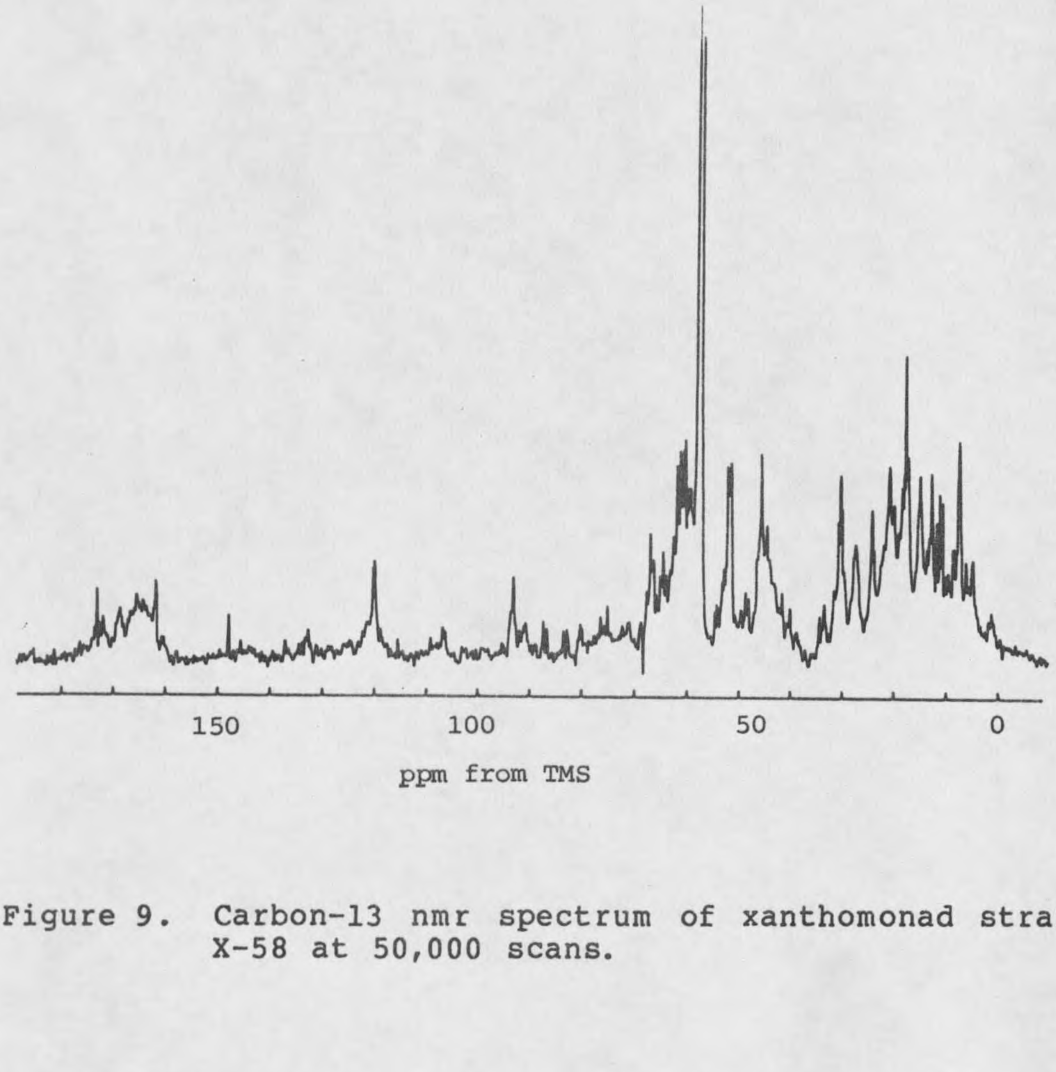


Figure 9. Carbon-13 nmr spectrum of xanthomonad strain X-58 at 50,000 scans.

amino acid for some xanthomonads. Again no growth occurred. Bacterial growth does occur when vitamin-free casamino acids are added at a level of 8 grams per liter (Kim,1982) This indicates that the limiting factor in the unsuccessful minimal media was an amino acid(s). The large amount of casamino acids (8 g/l) added was unsuitable for a growing medium to be used for labelled glucose uptake, however, as the organisms were capable of using any excess amino acids as a carbon source. This may have decreased the amount of glucose utilized by the organism.

No attempt was made to identify the limiting amino acids. The purpose of the growing medium was to provide a situation in which the organism was forced to synthesize as many cellular components as possible from constituents provided. As a result, the labelled carbons would be shunted into all the carbon containing cellular material, i.e. essentially all the cellular components. If all the amino acids were provided, no information would have been furnished by amino acid synthesis through the carbon-13 enrichment process.

The above statement is generally true, however, the vast amount of spectral information gathered from these cells was found in the areas of saturated hydrocarbons (15 - 30 ppm) and carbohydrates (60 - 80 ppm) when natural abundance data was gathered (Figure 1). Very little, if any, was found in the region reserved for amino acids (carboxyl group in the number 1 position at 170 to 185 ppm).

The optimal growth phase for glucose incorporation was one in which the majority of carbons taken into the system were labelled. When bacteria are growing in a rich (i.e. modified Wilbrink's) medium, only a fraction of the glucose was used to attain the maximum number of cells. A factor other than the carbon source was limiting in this case. When the amount of glucose was reduced, i.e. from 10 g/l to 2 g/l, a maximum percentage of the glucose was utilized and cell numbers were not greatly reduced.

In this situation, the labelled glucose was added and a high percentage of it was utilized by the cells. In a rich medium, only a percentage of the label was used. When adding a small amount of label and attempting only to double the carbon-13 content, maximum uptake was necessary.

The label was added when the glucose is the most limiting factor of growth. Glucose added in lag phase showed no incorporation and is possibly recycled during the following growth sequences. Much cellular recycled carbon is lost as CO_2 and thus the label was released into the atmosphere. The same case probably occured to a lesser extent when the label was added in log phase. If the cells had been harvested shortly after label addition, it is probable that less label would have been lost to CO_2. The problem of utilizing only a percentage of the labelled glucose again arose, as there was unlabelled glucose still available in the growing medium.

Maximum label uptake was recorded when carbon-13 labelled glucose was added late in the stationary phase of growth. At this point, utilization of available glucose was at a maximum provided glucose was a limiting factor. After addition of 15 mg of labelled glucose, the cell numbers quickly increased by approximately 5%. There were no other changes in the growing conditions. Once the increase in cell number abated, the Xanthomonas campestris pv. translucens cells were harvested. Nmr data was accumulated more quickly than in any other experiment and better resolution was provided. These were indications of an increase in the carbon-13 content of cellular material.

Chapter III

A Microbial Application

Introduction

The application of nmr to minute changes in microbial systems was tested by comparing data from xanthomonad strain X-58 and a transposon mutagenized isolate of X-58 provided by Kim (1982). The host range of strain X-58 includes barley, wheat, oats and rye, and the mutagenized strain proved to be pathogenic to wheat, oats and rye only. Carbon-13 nmr data on the two strains was gathered and compared for possible detection of gene products.

Materials and Methods

Work by Kim (1982) produced the transposon mutagenized X-58 strain designated X-38-6. This putative mutant was prepared by mating *Xanthomonas campestris* pv.*translucens* strain X-58 with *Escherichia coli* strain 1830 carrying Mu bacteriophage and Tn5 which codes for resistance to kanamycin and to neomycin. This *Escherichia coli* was used as a transposon donor and the xanthomonad as the recipient. A mating time of 96 hours in nutrient broth was necessary for successful mutagenesis of the xanthomonad by the Tn5 plasmid from

Escherichia coli. A digestion and Southern Blot test (Southern, 1975) were not performed on this mutant to verify the transposon mutagenesis

The resulting xanthomonad mutants were tested for kanamycin and neomycin resistance transferred from the Tn5 plasmid. Antibiotic resistant xanthomonads were tested for virulence on barley, wheat, oats and rye under greenhouse conditions to detect a loss of genes for pathogenicity which had been replaced by genetic material coding for antibiotic resistance.

One such putative mutant, X-38-6, was compared to strain X-58 on the basis of its nmr spectrum. Cells of both the parent strain X-58 and the transposon mutagenized X-38-6 strain were grown in Wilbrink's broth and harvested in late log phase. The cells were washed with a phosphate buffered saline solution, and a one gram slurry was used as an nmr sample. The internal reference system previously described (Chapter 1) was again used. Eight hour natural abundance carbon-13 data accumulations were gathered for each and the resulting spectra compared.

Results

The transposon mutagenesis performed by Kim proved to be an involved procedure as optimum growing conditions for the two bacterial species were not compatible. However, 1200 antibiotic resistant xanthomonads were obtained. When tested for virulence on the four major cereal grain hosts, only one strain, X-38-6, proved to have lost its pathogenicity to barley, yet retained it on the three other hosts.

In this study, comparisons of natural abundance nmr spectra of the parent X-58 and mutant strain X-38-6 provided information that the carbon compounds contained in the two bacterial strains were not identical. Table 5 presents the principle resonances of each.

Discussion

As an application of carbon-13 nmr to cellular systems, the data presented here indicated that nmr spectroscopy was capable of distinguishing between bacterial strains assumed to differ genetically in very few loci. Although identification of the compounds responsible for differences between the spectra was not performed, it is possible that these compounds were involved in pathogenicity. The ability of this technique to distinguish between gene products in this manner indicated that carbon-13 nmr is a feasible diagnostic tool for agriculture.

Table 4.Comparison of most intense resonances Xanthomonas strain X-58 and mutant X-38-6

Resonance Frequency (ppm)	Strain X-58	X-38-6
76.0	*	
69.6	*	
64.5	*	
61.8		*
60.8	*	
40.0		*
39.7		*
31.3		*
30.4	*	*
29.8	*	*
29.4	*	
29.1	*	
27.7	*	*
27.6		*
27.1	*	*
26.5	*	
24.6		*
24.0	*	
22.5	*	*
22.1		*
20.5		*
16.8	*	*

*Resonance intensities cannot be compared as a different internal reference was used for each strain.

SUMMARY AND CONCLUSIONS

Nmr spectroscopy was originally designed to elucidate the structures of chemical compounds. Approximately 40 years after its conception, this scientific tool has provided considerably more molecular information than ever intended, and the possibilities for research with this technique are still expanding rapidly.

Nmr is a system which takes advantage of the inherent magnetism of certain atomic nuclei. These nuclei are aligned in a large magnetic field, then pulsed with electromagnetic energy which forces them into a position 180 degrees out of alignment with the first field. As the nuclei realign with the first field, they re-radiate the energy which was absorbed during the 180 degree flip. A spectrometer records these energy levels, which are unique for each nuclear environment.

Several atomic nuclei are suitable for nmr studies. The most common ones include the proton, a useful tool for structural determinations, phosphorus-31, nitrogen-15, and carbon-13.

With the development of carbon-13 nmr, applications to biology were provided. Most of the biological work with carbon-13 nmr involves metabolism of a labelled compound. The molecule can be labelled in one position with carbon-13 and then followed through a series of pathway intermediates. Labelling allows the atom at a certain position to very prominent in relation to the naturally abundant carbon-13 (about 1% of the total). Much of the experimental groundwork has been completed and more involved experimentation is now possible.

The characterization of many components of a system by detection of molecules containing the naturally abundant carbon-13 is a technique still in the formative stages. Detection of unique resonances between isolates of the plant pathogenic bacterium, Xanthomonas campestris within the pathovar translucens, supplies some credibility for the intricacies of the data provided by the system. A surprising homeostasis and repeatibility of resonance position is shown despite alterations in growing conditions.

Natural abundance carbon-13 nmr and bacterial systems is most limited by the need for of extended data gathering times. Eight hours of scanning time is necessary to provide clear spectra.

In an effort to shorten the data accumulation time, the method of increasing the carbon-13 content in all areas of the cell simultaneously was devised. This "increased abundance" of carbon-13 could give greater resolution of the data presented, in addition to shortening the experimentation time. A growing medium enabling high cellular incorporation of the carbon-13 source, uniformly labelled glucose was devised. In addition, growth phases were studied in relation to optimum label uptake.

The resulting method indicated that 15 mg of the uniformly labelled glucose per 100 ml of growing culture would greatly decrease the data accumulation time. At this level the cost increase was approximately $15.00 per sample, with a minimum reduction in spectrometer time of 75%. In addition, if the label was added in the stationary phase of growth, optimal uptake was achieved. This carbon-13 enrichment method also furnished greatly increased resolution of the spectrum.

The purpose of gathering molecular information about live cells or live systems is to learn as much as possible about the host, pathogen and the interactions between the two. Varied applications of this work are possible and the potential uses in agriculture are many. Along with detection of system interaction, control of these can be possibly be elucidated with the molecular information provided. Exposure of a large system (plant, pathogen or combination) to agricultural chemicals for disease control, growth stimulation or inhibition can furnish a considerable amount of information that is not possible with other research techniques. There is potential for studies with natural abundance carbon-13 nmr and also with labelled compounds. In addition to carbon 13 nmr, information gathered from other nuclei available for nmr experimentation (i.e. P-31 and N-15) is notable.

The advantage of nmr spectroscopy in agriculture is the allowance for discoveries of mechanisms of a living system. This technique will potentially expand the knowledge of individual living organisms, and the interactions occuring between organisms functioning as a single metabolic unit.

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APPENDIX

APPENDIX

Potential Uses of Carbon-13 Nmr in Agriculture

Natural abundance and "increased abundance" carbon-13 nmr have offered a surprising amount of information about microbial systems. The clarity and reliability of data presentation in cellular systems allows one to formulate ideas for further biological and agricultural applications of nmr. The fact that natural abundance carbon-13 nmr provides quite intricate spectra in bacterial, fungal (Matsunaga et al.,1980) and algal systems (Abbott et al., 1983, unpublished) presents several options for this technique.

The limitations of presently used nmr spectrometers should be elucidated before attempting to describe any potential uses. As with any scientific tool, the "signal to noise" ratio is important in nmr spectroscopy. The carbon containing cellular components which are present in the largest quantities will appear first and produce the largest amount of data. A longer data accumulation time or an effective increase in the carbon-13 content will allow metabolites contained in lesser amounts to appear. However, it is nearly impossible to detect carbon compounds of less than millimolar quantities. Although the actual per hour cost for some nmr systems is a limiting factor, competition for machine time is also a hindrance. Carbon-13 enrichment and the resulting faster data accumulation can alleviate this problem.

In this research project, molecular identification and assignment of the carbon resonances detected in bacterial cells was not attempted beyond general area identification. Exact identification of a mixture this complex would have been difficult. General area identification may provide important information in species identification, however. In addition, extensive catalogues of known spectra are available for comparison.

General species identification is an obvious application for biological nmr spectroscopy. Complex identification is also possible, as in the strain differentiation at the pathovar level of this project. This may aid in the diagnosis and forecasting of plant or animal disease. Interactions between host and parasite or host and pathogen could be detected by nmr spectral data gathered at different disease cycles. An interesting possibility is the combination of nmr imaging (or tomography) used in medicine with molecular identification spectroscopy. For example, entire plants might be visually and molecularly assessed at the same time. The biochemical mechanisms of flowering preceed the visual aspects and nmr analysis may reveal substantial information regarding the biochemical changes occurring.

The effects of chemical substances on plants and pathogens and methods of disease control might be observed with natural abundance carbon-13 spectroscopy. The nature of biochemical resistance to disease may be detected when placing the pathogen and host together and monitoring any chemical changes of the dual system.

Aside from natural abundance or "increased abundance" (in the case of carbon-13 enrichment) nmr spectroscopy, a number of possibilities exists as to the metabolism and action of labelled compounds. The mode of action of pesticides could be measured in vivo. Systemic activity of fungicides, for example, in plants can be detected with some creative labelling of the fungicide. Different plant sections and plant growth stages could be tested for the intact fungicide. Loss of its structure could be correlated to a loss of the carbon-carbon coupling created in the labelling technique.

An assay for the potential danger of agricultural chemicals might be developed. Using labelled pesticides, etc. in combination with cellular material (i.e. cloned skin cells in the case of human exposure), the potential to activate or inactivate chemical compounds could be assayed. Conversely, the effect on the target organism could be assessed with natural abundance spectroscopy.

Detection of unique metabolic pathway intermediates for host, pathogen or a combination of the two may possibly be part of the information gathered when feeding a labelled substrate or precursor. This information may lead to development of absolutely selective pesticides, growth promoters, etc. for a system.

One of the potential uses of nmr in agriculture is differentiation of isogenic organisms, both microbial, and on a larger scale, such as isogenic barley lines. Subtle differences detected may be related to pathogenicity, disease resistance or other factors of interest to the researcher.

Recent capabilities of nmr to handle larger and larger sample sizes (i.e. a human body) increase the research potential of this tool. Many agricultural applications of nmr will undoubtedly be confronted in the near future, recognizing the unique advantage of this technique as analysis of the intact system.