Characterization of plasmids among the three species of *Gluconobacter*

by

Lori L. Brookman

Dissertation submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

IN

BIOLOGY

APPROVED:

G. William Claus, Chairman

Noel R. Krieg

Charles R. Rutherford

Eric A. Wong

Allan A. Yousten

July 1995

Blacksburg, VA

Key words: *Gluconobacter*, plasmids, limited oxidations, antimicrobial susceptibility, hybridizations, phenotype

ACKNOWLEDGMENTS

This dissertation is dedicated to the memory of my grandfather, James A. Kelbaugh.

I would like to first thank the members of my Graduate Advisory Committee, Drs. Noel R. Krieg, Charles L. Rutherford, Eric A. Wong, and Alan A. Yousten for their valuable input and help throughout my graduate career. I especially would like to thank my major advisor, Dr. G. William Claus, for all of his guidance, patience, time, and most importantly, his friendship.

I also must thank Loretta Albert, Sue Herbein, Kathy Pennington, and Bonnie Williams for providing assistance with the use of equipment, guidance in relation to my duties as a Graduate Teaching Assistant, and often shoulders to cry on. I thank Sue Rasmussen for all her assistance. I also thank the Department of Biology, in particular, Dr. Joe R. Cowles, for having faith in my abilities as a Graduate Student and supporting me monetarily during my years in Graduate School.

I have made so many invaluable friendships while at Virginia Tech and I would like to personally acknowledge the following: Khrys Duddleston, Laura Via, Andrew Arrage, Tad Seyler, Myron Beaty, Rob Atkinson, Rich Kirshner, Glen Carlyle, Joe Succic, Marcus Juker, Mary Alice Woodburn, Lana Nassif, Yuda Anriany, Suzanne Swartwood, Donna Jensen, Ross Zirkle, John Mayo, Cody Cain, Jamie Stanek, Twilla Eaton, Margarita Correa, Rob Lampe, and Karen Rippere.

Lastly, I would like to thank my parents, Gil and Kila Dawson, and my grandmother, Delsie "Dodie" Kelbaugh for their support and confidence in my abilities. Most importantly, I would like to thank my husband, Steve, for his love, kindness, and support, especially when times were tough and I needed someone to just be there. I also thank Sydney for his affection.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

DISSERTATION OVERVIEW

The genus *Gluconobacter* consists of acetic acid bacteria which have the ability to generate acidic products from their substrates, particularly acetic acid from ethanol. For this reason, the gluconobacters live in acidic, sugary environments such as flowers, honey bees, fruits, cider, vinegar, wine and beer. The gluconobacters carry out a strictly respiratory type of metabolism using only oxygen as a terminal electron acceptor. They do not completely oxidize a substrate to carbon dioxide. Instead, they partially oxidize the substrate using membrane-bound dehydrogenases and excrete the product into the surrounding growth medium. It is these limited oxidations that make the gluconobacters industrially useful.

Although much is known about the physiology of the limited oxidations in the gluconobacters, little is known of their genetics, particularly, their plasmids. The overall purpose of this dissertation was to determine if *Gluconobacter* plasmids correlate with oxidative capability and/or antibiotic resistance.

To achieve this goal, I first needed a way to screen strains of *Gluconobacter* for their ability to oxidize many different substrates. I developed an assay that used an unusual artificial electron acceptor, tetranitroblue tetrazolium (TNBT) and then tested the ability of six strains to oxidize 13 chemical compounds. Although most strains were able to oxidize the 13 compounds tested, they accomplished this with varying extents of oxidation. These differences were noted even with strains representing the same species. The development of this TNBT reduction assay and the results from its use are described in Chapter 1.

I next needed to determine the plasmid profiles of the gluconobacters. I selected twenty-two strains that represented the three species of *Gluconobacter*.

Plasmids were isolated using alkaline lysis, phenol-chloroform extraction, and lithium chloride-ethanol precipitation, then plasmids were separated according to size using agarose-gel electrophoresis. Plasmids were found in 18 of the 22 strains, and the plasmids ranged in size from 2 to > 54 kb. Many strains contained plasmids of similar size. These results are presented and discussed in Chapter 2.

Once I determined the gluconobacters' plasmid profiles, I next tested the susceptibility of these cultures to antimicrobial agents using agar diffusion assays. Most strains showed almost identical susceptibility to the antimicrobial agents tested, and no relationship was observed between plasmids and antimicrobial agent resistance. These results are presented and discussed in Chapter 3.

I also wished to compare the plasmid profiles given in Chapter 2 with the substrates oxidized by these strains. Only one possible correlation was found, and that was between the presence of plasmids and the ability to oxidize butanol. However, I chose not to study this further, because these plasmids had no known genetic markers for tracking plasmid transformations into *Gluconobacter* strains that lacked plasmids. This possible association between plasmids and butanol oxidation is presented and discussed in Chapter 3.

To further characterize *Gluconobacter* plasmids, I used hybridization analysis to determine plasmid relatedness between strains representing the three species. Isolated plasmids were non-radioactively labeled and used to probe plasmids from other strains of *Gluconobacter*. I found that more similarities existed between plasmids from strains of the same species, although, plasmids from strains of different species also hybridized with one another. The results of this hybridization analysis are given in Chapter 4.

Although I was unable to determine if genes responsible for limited oxidations or resistance to antimicrobial agents reside on *Gluconobacter* plasmids, I was able to learn more about these plasmids. For example, I was the first to determine which strains among the three *Gluconobacter* species contain plasmids. I also reported the sizes of most of these plasmids (except those over 54 kb). I was also the first to show *Gluconobacter* plasmid relatedness by using hybridization analyses. I also found strong evidence for genetic similarities of three strains of *G. oxydans.*

CHAPTER 1

Development and Use of a Tetrazolium Assay for Measuring Useful Biotransformations in *Gluconobacter* Strains

SUMMARY

The gluconobacters use membrane-bound dehydrogenases to rapidly carry out single-step oxidations of hydroxyl-containing organic compounds and convert them to products that are released into the surrounding medium. Available literature reports that many compounds are oxidized, but little is known of the oxidative diversity among individual strains within this genus. Here I describe an assay that was developed and used to quickly test the oxidative ability of non-growing cell suspensions. I used tetranitroblue tetrazolium (TNBT) as an artificial electron acceptor, because it is insoluble in its reduced form and binds tightly to the cell at its site of reduction. When reaction mixtures were centrifuged, both cells and reduced TNBT were removed, and the amount of TNBT reduced per mg cell protein was quantitatively determined at the end of one hour. This assay demonstrated a broad range of optima for pH (5.7 to 6.7), temperature (43 to 48^{o}C), and substrate concentration (2.5 to 7.5%). This TNBT reduction assay was used to test six gluconobacter strains (representing the three known species) for their ability to oxidize 13 compounds representing six chemical classes. Most strains oxidized all of the compounds tested, but the extent of oxidation varied greatly between strains (from 0.1 to 38 mmole of substrate oxidized per mg cell protein x 10^{-5}). Most importantly, different *Gluconobacter* strains representing the same species showed very different oxidative capabilities. I believe that this simple

TNBT reduction assay could be used as an important tool in strain selection for industrial oxidations by the gluconobacters.

INTRODUCTION

The genus *Gluconobacter* consists of Gram-negative rods that generate acetic acid from ethanol and survive in acidic environments having a pH as low as 3.6 (7, 20). They also generate acidic products from sugars, alcohols, and polyols, and they are isolated from flowers, honey bees, fruits, cider, vinegar, wine and beer (7,14). Although the genus contains no animal pathogens, a few strains reportedly cause pink disease in pineapples (5) and browning and rot in both apples and pears (VanKeer et al., 1981). Based upon differences in DNA-DNA homologies (17) and phenotypic tests (15), the genus is divided into three species: *Gluconobacter oxydans, G. frateurii,* and *G. asaii.* In addition, the gluconobacters carry out a strictly respiratory type of metabolism and use only oxygen as a terminal electron acceptor (7). More specifically, they do not completely oxidize their carbon and energy sources (substrates) to carbon dioxide (3), because they lack a complete tricarboxylic acid (TCA) cycle (10). Instead, the gluconobacters only partially oxidize these substrates and excrete the oxidation products into the surrounding medium.

The most important mechanisms for accomplishing these oxidative bioconversions are the membrane-bound NAD(P)-independent dehydrogenases (3,6,7,16,22). These membrane-bound enzymes are responsible for the rapid conversion of more than 90% of the substrate to the limited oxidation product (8,19), and these efficient biotransformations have industrial value. For example, the

gluconobacters' oxidation of sorbitol to sorbose is part of the Reichstein Process used for the manufacture of vitamin C (23).

In addition to their ability to rapidly and efficiently convert substrates to products, the gluconobacters also oxidize many different substrates. Edwards (Edwards, D.E. 1990. M.S. Thesis. Virginia Tech, Blacksburg) lists more than 100 substrates reportedly oxidized by different strains of gluconobacters. However, many of these studies use membrane fractions and colorimetric assays that have limited use for screening large numbers of strains or assessing the ability of a few strains to oxidize many different substrates. I wanted to develop a rapid assay that would easily measure many membrane-driven limited oxidations by many strains.

To avoid time consuming cell fractionation steps, it seemed desirable to use whole cells in this new assay. However, whole cells contain both membrane-bound and cytosolic dehydrogenases (2), and I desired an assay that would favor operation of only the membrane-bound dehydrogenases .

For this type of assay, I could examine oxidation-product formation (4), but this seemed inappropriate for examining the ability to oxidize many different substrates. I also considered an oxygen-uptake assay, because limited oxidations by the gluconobacters are accomplished by plasma-membrane-bound dehydrogenases (1,2,13), and those enzymes depend upon membrane-bound electron-transport enzymes (16) where oxygen is used as the sole terminal electron acceptor (7). However, oxygen-uptake assays are time consuming, labor intensive, and require many oxygen electrodes or respirometer assemblies to simultaneously measure many strains and substrates. Alternatively, I chose a colorimetric assay that used a water-soluble tetrazolium salt rather than oxygen as a terminal electron acceptor. I reasoned that

multiple assays could be simultaneously performed in test tubes, that concentrated cell suspensions could be used to shorten reaction times, and that the extent of oxidation could be optically determined after removing the light-scattering cells and the reduced tetrazolium salt.

In 1965, Sedar and Burde (21) demonstrated that tetranitroblue tetrazolium (TNBT) turned from pale yellow (concentrated solutions) to black when converted from the oxidized to the reduced form. More importantly, they demonstrated that reduced TNBT is water insoluble and remains tightly bound to its site of reduction in eucaryotic cells and tissues. Hence, they used TNBT as a cytological stain to demonstrate that specific dehydrogenases occur in certain eucaryotic cell membranes.

In preliminary experiments, prior to the development of my assay, conditions that allowed for polar and peripheral intracytoplasmic membrane formation in gluconobacters were used (6), substrate and oxidized TNBT was added, and heavily stained polar and peripheral regions were microscopically observed (Andrews and Claus, unpublished observations). I reasoned that gluconobacter cells bind the black reduced TNBT to the plasma and intracytoplasmic membranes in the presence of suitable substrates and that centrifugation should remove both the cells and the reduced TNBT from a reaction mixture that contains a turbid cell suspension. Colorimetric analysis of the supernatant fluid should then allow me to quantitatively determine the amount of oxidized (non-reduced) TNBT remaining in the reaction mixture following substrate oxidation. From this determination, I should then be able to calculate the extent of substrate oxidation by these cells.

In this paper, I report the development of a TNBT assay that can quickly and quantitatively determine the extent of substrate oxidation by strains of *Gluconobacter*. Because of the unusual solubilities of this tetrazolium salt and the membrane-binding capacity of its reduced form, I propose that this assay primarily measures the activity of the membrane-bound dehydrogenases of the gluconobacters, even though whole cells are used in the assay. Although much is known of the substrate diversity of the gluconobacters as a group of bacteria, little is known regarding substrate utilization by individual strains of *Gluconobacter*. This report also demonstrates that different strains within the same species differ greatly in their ability to oxidize the same substrate. Thus, we believe that this assay may prove to be a valuable initial screening tool for those interested in determining which strains best carry out limited oxidations of industrially important substrates.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study were the same as those used in a previous DNA/DNA homology study (17) and tests of related phenotypic characteristics (15). *Gluconobacter oxydans* ATCC strain 621 was received from the American Type Culture Collection in January 1975, and *G. oxydans* IFO strain 3244 was received from Noberto Palleroni (Hoffman-LaRoche, Nutley, NJ) in 1977. *Gluconobacter frateurii* IFO strains 3254 and 3271 and *G. asaii* IFO strain 3297a were also gifts from Noberto Palleroni in 1977. *Gluconobacter asaii* ATCC strain 43781 was originally received in 1977 from K. G. Rohrback (Department of Plant Pathology, University of Hawaii at Manoa, Honolulu) and later deposited at the American Type Culture Collection. All strains were checked for purity, grown in a complex medium, suspended in a medium containing 15% glycerol, 1% yeast extract, and 1% peptone

then heat-sealed in glass vials and continuously maintained in liquid nitrogen Dewars until Jan. 1991 to April 1992. Working-stock cultures were prepared by growing the strains in broth containing (w/v) 5% glycerol, 1% yeast extract and 1% peptone until reaching approximately 0.9 OD_{620} and maintained until use by storing in 66% glycerol at -4°C. Cells used in the tetranitroblue tetrazolium (TNBT) experiments were grown in broth containing (w/v) 5% glycerol, 1% yeast extract and 1% peptone. Incubated cultures were shaken at 28°C until reaching approximately 0.9 OD_{620}. Cells were washed twice in 0.2 M succinate buffer at a pH of 5.2 and resuspended in this buffer unless otherwise noted. *Gluconobacter oxydans* ATCC strain 621 was used for the development of the tetranitroblue tetrazolium assay.

Optical properties of oxidized TNBT. The water solubility of oxidized TNBT varied greatly depending upon the commercial source, but we found that 5 mg of oxidized TNBT obtained from Sigma Chemical Co., St. Louis, MO, was readily dissolved in 10 ml of distilled water after just several hours of stirring at room temperature. An absorbance spectrum was prepared using 20 μg/ml of oxidized TNBT in distilled water and varying the wavelength from 200 to 800 nm using a Milton Roy Spectronic 1201 spectrophotometer and a distilled water blank. Maximum absorbance occurred at 277 nm and appeared as a single sharp peak without shoulders or other near-by peaks.

Initial TNBT reduction assay conditions. Reaction mixtures containing 5% (w/v or v/v) substrate, 20 μg/ml oxidized TNBT, and *G. oxydans* ATCC strain 621 cell suspensions as described above, were brought to a total volume of 4 ml with 0.2 M succinate buffer at pH 6.2 unless otherwise noted. Reaction mixtures lacking either cell suspension or substrate were also prepared for each experiment. All reaction

mixtures were incubated in a 48°C water bath for 60 min unless otherwise noted. Following incubation, all reaction mixtures were centrifuged (Beckman Model J2-21 centrifuge) at 12,100 x g at room temperature for 10 min to remove cells and reduced TNBT. The amount of oxidized TNBT remaining in each supernatant fluid was colorimetrically determined using a Milton Roy Spectronic 1201 spectrophotometer at 277 nm, and this absorbance was compared with a standard curve prepared with known quantities of oxidized TNBT. As little as 1 μg/ml of oxidized TNBT was detected in this way. The quantity of oxidized TNBT remaining at the end of each incubation was subtracted from the amount present in reaction mixtures lacking cells and substrate. This difference was presumed equal to the amount of TNBT reduced, and this value was used to determine the μg-quantity of TNBT reduced per milligram of cell protein. The amount of cell protein in these reaction mixtures was determined by the method of Lowry (11).

Varying assay conditions. Reaction mixtures contained varying quantities of cells (*G. oxydans* ATCC strain 621) and 0.2 M succinate buffer at a pH of 5.2 were incubated at 38°C. To determine the effect of pH on TNBT reduction, reaction mixtures were brought to a total volume of 4 ml with 0.2 M phosphate-succinate buffer separately adjusted to pH 4.2, 5.2, 5.7, 6.2, 6.7, 7.2, or 8.2. Succinate-phosphate buffer was used, because the buffering capacity of succinate buffer alone would not compensate for this range in pH values. Reaction mixtures were incubated at 38°C. To determine the effect of temperature on TNBT reduction, reaction mixtures were separately incubated at temperatures of 28, 38, 43, 48, or 53°C. To determine the effect of glycerol concentration on TNBT reduction, separate reaction mixtures were tested that contained 0, 2.5, 5.0, 7.5, and 10.0% glycerol (v/v). Otherwise, the contents and conditions of the assay were as described in the previous paragraph.

Experimental variations in the physical and chemical conditions of the assay allowed the determination of optimum values for pH, temperature, and concentrations of reduced TNBT, cells, and substrate. These optimum conditions are described in the Results section entitled *Recommended TNBT assay conditions*.

Rates of TNBT reduction. All reaction mixture components were preheated to 48^{o}C and added to reaction mixtures in the same concentrations as described in *Initial TNBT reduction assay conditions*, except that both glycerol and butanediol were separately used as substrates. Reaction mixtures were brought to a total volume of 40 ml with 0.2 M succinate buffer at a pH of 6.2 and prepared in duplicate. Samples containing 4 ml were removed from these reaction mixtures at 10 min intervals. The TNBT reduction in each reaction mixture was stopped with the addition of 8 μM of 2-heptyl hydroxyquinoline-N-oxide (Sigma Chemical Co., St. Louis, MO). Otherwise the conditions of the assay were as described above (*Initial TNBT reduction assay conditions*). Two separately prepared batches of cells were tested in this manner.

Determination of oxidative capabilities in strains of *Gluconobacter*. Strains were selected on the basis of which of the three species of *Gluconobacter* they represented (15). Substrates were selected based on their chemical group, the number of hydroxyl groups they contain, and upon the results of oxygen uptake studies using *G. oxydans* ATCC strain 621 (Edwards, D. E. 1990. M.S. Thesis. Virginia Tech, Blacksburg). The following substrates were purchased from Sigma Chemical Co., St. Louis, MO: β-D(-)-fructose, D(+)-mannose, sodium acetate, and ribitol (adonitol). Maltose and glycerol were purchased from Fisher Scientific, Atlanta, GA. D-raffinose pentahydrate, 1,3 butanediol, and (+/-) 2-butanol were purchased from Aldrich Chemical Co., Milwaukee, WI. Each strain and each substrate was tested under the

optimum conditions described in the Results (*Recommended TNBT assay procedures*) except cell suspensions were used at room temperature. Results of oxidative capabilities were reported as mmole of substrate oxidized per mg of cell protein based on two assumptions: (1) that one molecule of TNBT accepts two electrons to achieve its reduction; and (2) that the amount of TNBT reduced by a reaction mixture equals the amount of substrate oxidized.

RESULTS

Preliminary observations. When turbid cell suspensions of *G. oxydans* ATCC strain 621 were added to a ceramic spot plate containing glycerol and oxidized tetranitroblue tetrazolium (TNBT), a black color was formed during 60 min incubation at room temperature. Controls lacking either glycerol or cell suspension showed no black color development. I suspected the black color was caused by TNBT reduction (glycerol oxidation) in these reaction mixtures. Since reduced TNBT is insoluble and reportedly remains tightly bound to its site of reduction within the cell (21), we reasoned that cells and reduced TNBT could easily be removed from reaction mixtures by centrifugation. When 4 ml reaction mixtures were incubated for 60 min and then centrifuged, the pellet was black, and the supernatant fluid was visibly clear. Subsequent experiments (data not shown) demonstrated that oxidized TNBT solutions absorbed maximally at 277 nm, and that Abs_{277} was directly proportional to the quantity of oxidized TNBT dissolved in water (correlation factor of 0.99).

I reasoned that the Abs_{277} of reaction mixture supernatant fluids following incubation and centrifugation represented the quantity of oxidized TNBT remaining in the reaction mixtures, and the amount of TNBT reduced should equal the difference

between the amount of oxidized TNBT at time zero and that remaining after 60 min incubation.

Cell concentration. Our first goal was to determine that TNBT reduction was caused only by cells acting on a substrate. No TNBT reduction (glycerol oxidation) was observed without glycerol (data not shown) or without cells (Figure 1). When cell concentrations varied (Figure 1), an increase in TNBT reduction (from 1.1 to 8.4 μg/ml) paralleled the increase in cell concentration from (0.7 to 3.3 mg protein per reaction mixture). These results demonstrate the absence of non-biological or endogenous oxidation by ATCC strain 621. They also show that cells, rather than some other reaction mixture component, caused the reduction of TNBT in these reaction mixtures. All subsequent assays were performed with reaction mixtures having cell concentrations of about 2 mg of protein.

Optimum pH. *Gluconobacter oxydans* ATCC strain 621 catalyzed TNBT reduction (glycerol oxidation) at pH 4.2, 5.2, and 7.2, but not at pH 8.2 (Figure 2A). Optimal TNBT reduction (glycerol oxidation) occurred at pH values between 5.7 and 6.7. Therefore, all subsequent assays were performed at pH 6.2.

Optimum temperature. *Gluconobacter oxydans* ATCC strain 621 accomplished the most rapid TNBT reduction (glycerol oxidation) between 43 and 48^{o}C, but also catalyzed significant glycerol oxidation at both lower and higher temperatures (Figure 2B). Therefore, all subsequent assays were performed at 48^{o}C.

Optimum substrate concentration. *Gluconobacter oxydans* ATCC strain 621 showed no significant differences in TNBT reduction (glycerol oxidation) at glycerol

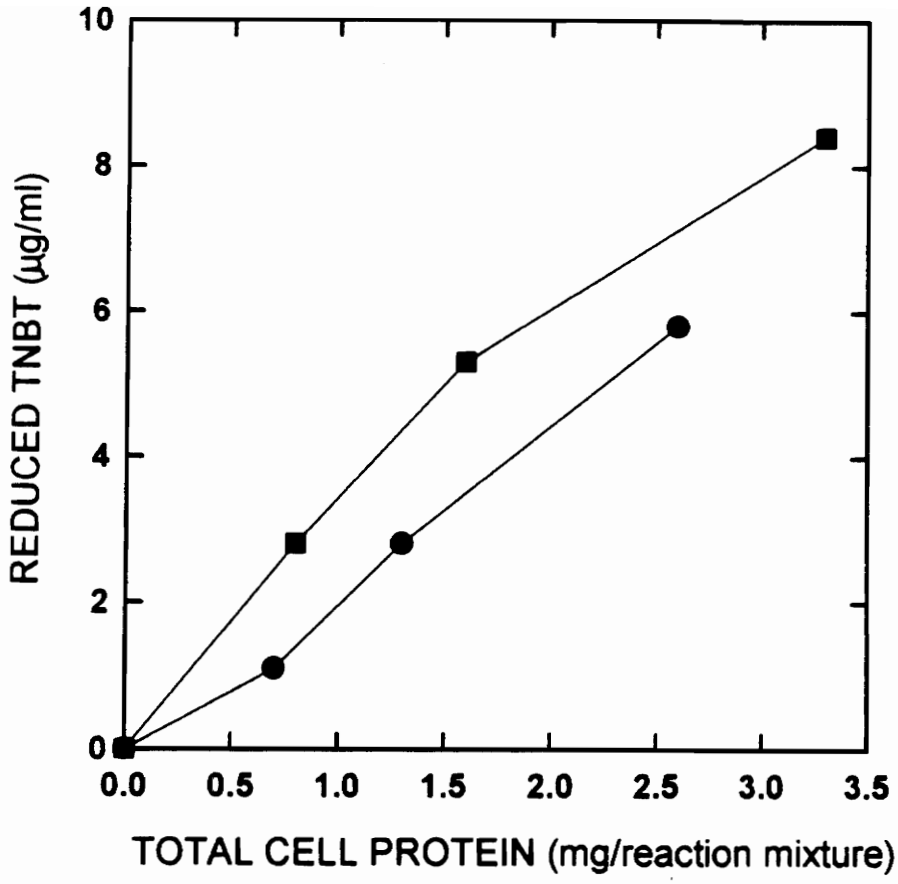
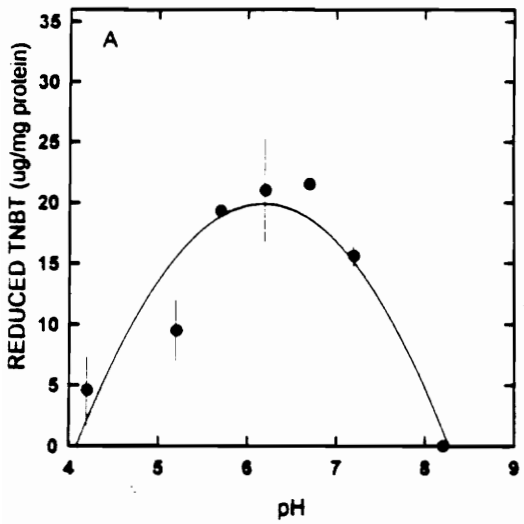
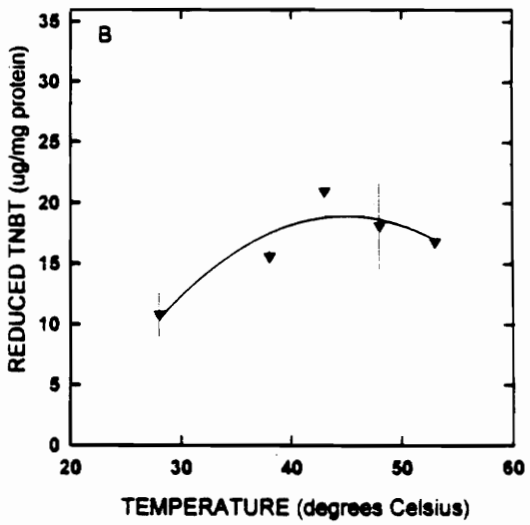
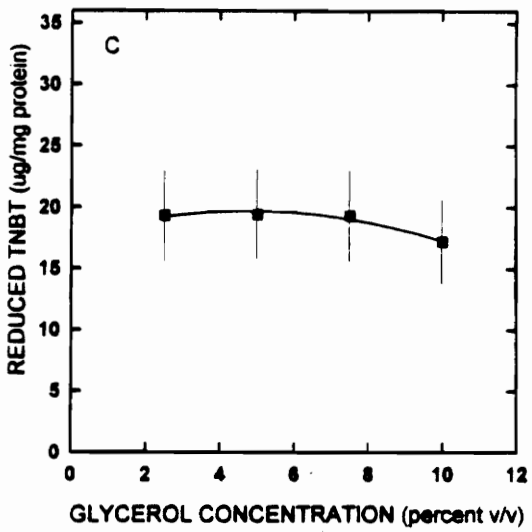


FIGURE 1. Effect of cell concentration on the extent of tetranitroblue tetrazolium (TNBT) reduction (glycerol oxidation). Reaction mixtures contained (in a total volume of 4 ml): 20 µg/ml of oxidized TNBT, 5% (v/v) of glycerol, and varying concentrations of *G. oxydans* ATCC strain 621 cells suspended in 0.2 M of succinate buffer (pH 5.2). Reaction mixtures were incubated for 60 min at 38^{o}C. The quantity of reduced TNBT and the protein concentration were determined as described in the Materials and Methods. Closed squares (▪) and closed circles (•) represent the data from two separately prepared cultures. Each data point represents the mean from two replicates.


A
REDUCED TNBT (ug/mg protein)
pH


B
REDUCED TNBT (ug/mg protein)
TEMPERATURE (degrees Celsius)


C
REDUCED TNBT (ug/mg protein)
GLYCEROL CONCENTRATION (percent v/v)

concentrations from 2.5 to 10.0 % (v/v) (Figure 2C). In subsequent assays, all substrates were added to reaction mixtures to a final concentration of 5.0 % (w/v or v/v).

Effect of incubation time. To determine if the 60 min incubation time prematurely limited the full extent of oxidation possible under these assay conditions, we examined oxidation rates of the two most rapidly oxidized compounds (glycerol and butanediol) at various times during the 60 min incubation. When room temperature cell suspensions (1.6 ml) of *G. oxydans* ATCC strain 621 were added to 3.4 ml of the reaction mixtures pre-warmed to 48^{o}C, we found a gradual increase in the rate of TNBT reduction during the first 20 min (data not shown). Alternatively, when all reaction mixture components were pre-warmed to 48^{o}C, the TNBT reduction rate appeared faster and more linear during these first 20 min (Figure 3). Little if any further TNBT reduction occurred after 30 min incubation. I concluded that, under the conditions reported here, the 60 min incubation time used in these and all subsequent experiments was about twice as long as needed to show full oxidation of the most rapidly converted compound tested.

Recommended procedure for the TNBT reduction assay. Suspend the pellet from 200 ml of a freshly prepared log phase *Gluconobacter* culture (~ 0.95 OD_{620}) in 60 ml of 0.2 M succinate buffer (pH 6.2), and wash twice in the same volume of this buffer. Prewarm the cell suspension to 48^{o}C just prior to use.

Prepare separate reaction mixtures in the following way. Add 0.2 g of solid substrate, and dissolve in 1.6 ml of 0.2 M succinate buffer (pH 6.2). Alternatively, add 0.2 ml of liquid substrate and dilute with 1.6 ml of the same succinate buffer. In this way, each reaction mixture will contain a final substrate concentration of 5% (w/v or

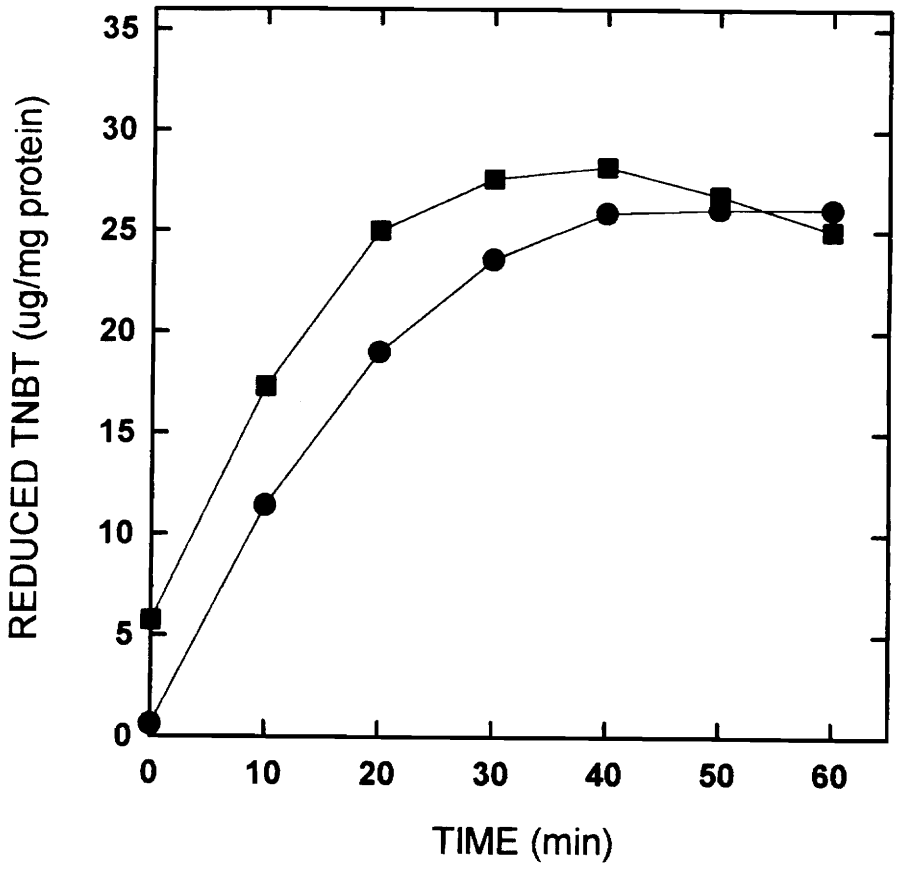


FIGURE 3. Rates of TNBT reduction by *G. oxydans* ATCC strain 621 oxidizing either glycerol (•) or butanediol (▪). Each reaction mixture contained 20 μg/ml of oxidized TNBT, 5 % (v/v) of substrate, and 1.6 ml of exponential phase cells (~ 2 mg of protein), and the volume of each was brought to 4 ml with 0.2 M of succinate buffer (pH 6.2). All reaction mixture components were preheated to 48°C, mixed with cell suspensions to start the reaction, then incubated at 48°C for the times indicated. Reactions were stopped by adding 8 μM 2-heptyl hydroxyquinoline-N-oxide (HQNO). Otherwise, assays were performed as described in the Materials and Methods. Data shown are the mean among two replicates each from two cell batches.

v/v). Next, add 0.4 ml of a stock solution containing 500 μg/ml of oxidized TNBT dissolved in distilled water. Then add 0.4 ml of 0.2 M succinate buffer (pH 6.2), and prewarm to 48°C. To begin the reaction, add 1.6 ml of the prewarmed cell suspension. These 4.0 ml reaction mixtures should contain about 0.5 mg cell protein per ml. Incubate the reaction mixtures at 48°C for 60 min.

Following incubation, centrifuge the entire reaction mixture at 12,100 x g for 10 min to remove cells and the reduced TNBT bound to these cells. Determine the Abs_{277} of the resulting supernatant fluid. Compare this value with a standard curve that shows the relationship between absorbance at 277 nm and concentration of oxidized TNBT. In addition, determine the Abs_{277} and oxidized TNBT concentration of supernatant fluids resulting from reaction mixtures lacking either substrate or cell suspension. These values must be taken into account so as to determine the difference between the initial concentration of oxidized TNBT and the amount remaining after 60 min incubation. The reaction mixtures lacking substrate should also be used to detect any endogenous respiration so that it can be subtracted from test values.

Oxidative capabilities of different *Gluconobacter* strains. The TNBT reduction assay was used to measure the ability of six *Gluconobacter* strains, representing three species, to oxidize thirteen different substrates following growth on glycerol (Figures 4 - 6). All strains were also tested for the ability to respire endogenously in the absence of a substrate. Only *Gluconobacter asaii* IFO strain 3297a showed endogenous respiration.

To facilitate the following description of oxidative abilities, we arbitrarily categorized the extent of oxidation into four groups (in mmole of substrate oxidized per

mg of cell protein x 10^{-5}): no oxidation; poor oxidation (from 0.1 to 4.9); moderate oxidation (from 5.0 to 14.9); and extensive oxidation (greater than 15).

Gluconobacter oxydans ATCC strain 621 demonstrated very different extents of oxidation among these thirteen substrates (Figure 4). This strain accomplished extensive oxidation of butanediol, butanol, ethanol and glycerol; moderate oxidation of cyclopentanol, glucose, mannose and sorbitol; and poor oxidation of fructose, maltose, raffinose, ribitol, and sodium acetate. On the other hand, *G. oxydans* IFO strain 3244 showed poor to moderate oxidation of all compounds tested except butanol, and this strain seemed to lack the constitutive ability to oxidize butanol.

Both strains of *Gluconobacter frateurii* (IFO 3271 and 3254) also showed a wide range of oxidative ability (Figure 5). Strain IFO 3271 extensively oxidized butanediol, butanol, ethanol, and glycerol, but moderately or poorly oxidized the other nine compounds. Strain IFO 3254 showed extensive to moderate oxidation of eight of these compounds, but it poorly oxidized cyclopentanol, maltose, sodium acetate, raffinose and ribitol.

Gluconobacter asaii ATCC strain 43781 demonstrated a moderate to poor oxidation of all tested compounds except ethanol which it oxidized extensively (Figure 6). When the large amount of endogenous activity of *G. asaii* IFO strain 3297a was subtracted from the experimentally determined values, this strain exhibited very poor if any oxidation of all compounds tested except ethanol which it oxidized moderately.

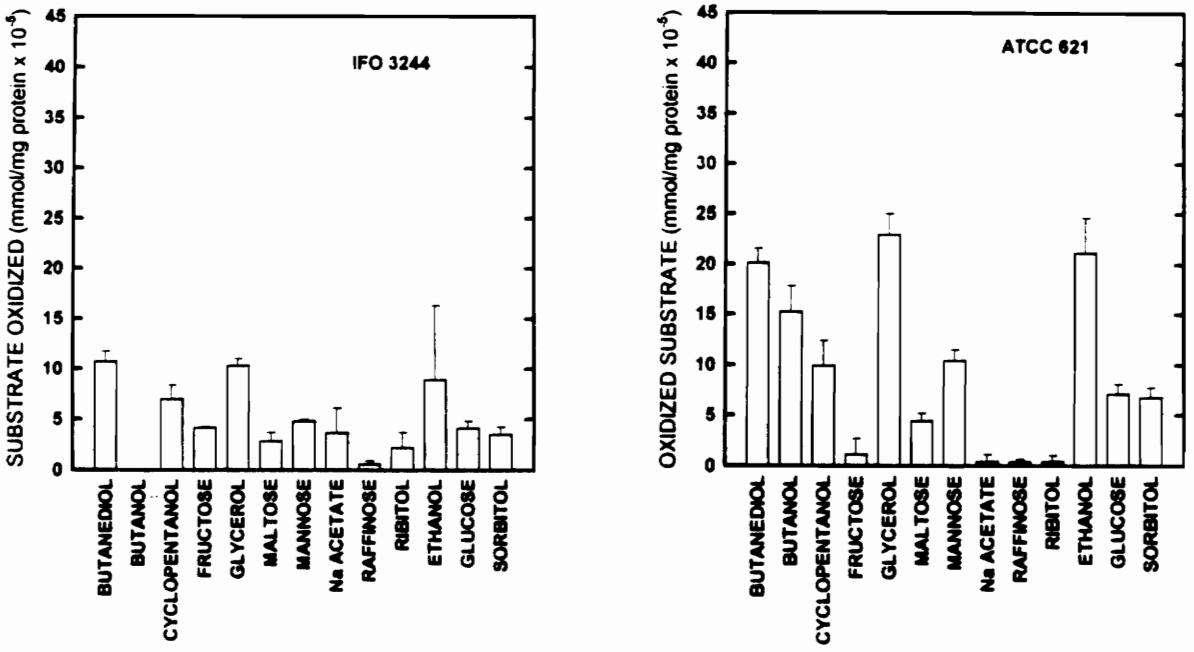


FIGURE 4. Extent of substrate oxidation by two strains of *G. oxydans.* Assays were performed using the TNBT reduction assay as described in the Materials and Methods. Vertical bars represent the standard deviation from the mean among two replicates each from three cell batches.

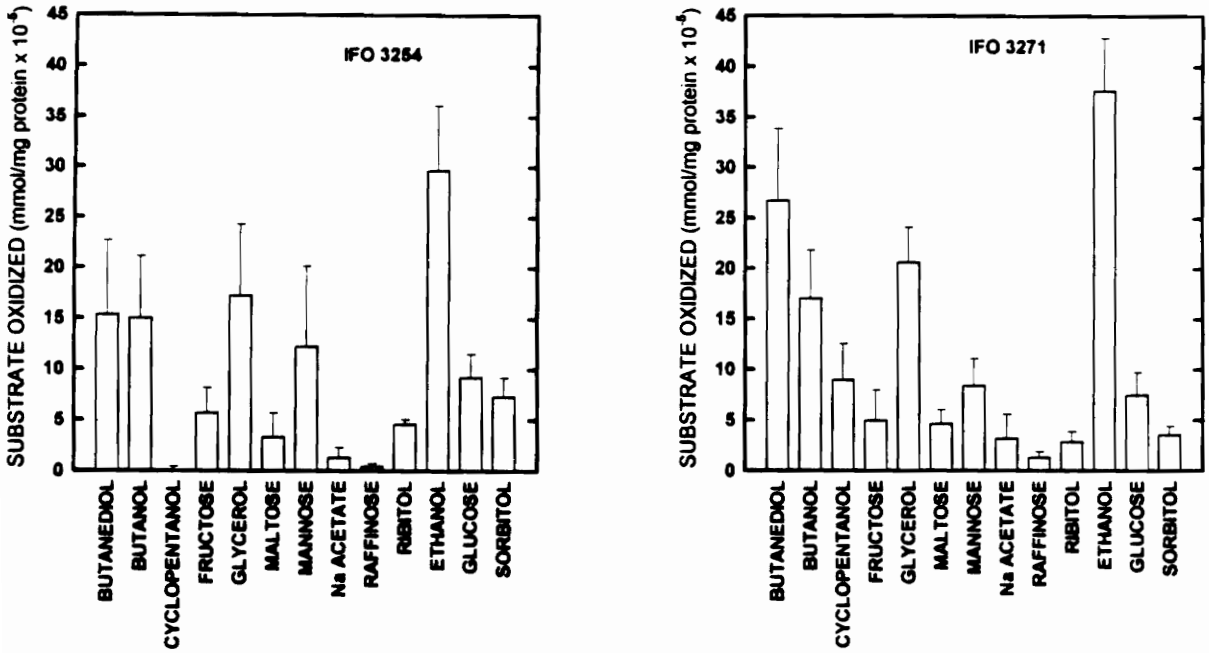


FIGURE 5. Extent of substrate oxidation by two strains of *G. frateurii*. Assays were performed using the TNBT reduction assay as described in the Materials and Methods. Vertical bars represent the standard deviation from the mean among two replicates each from three cell batches.

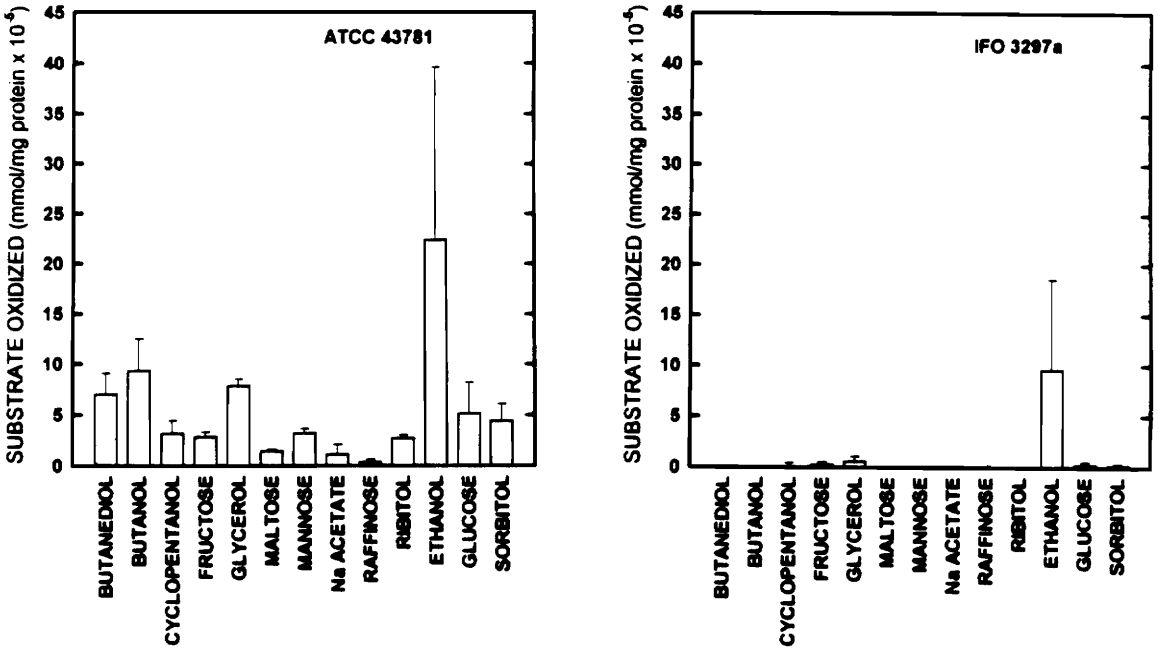


FIGURE 6. Extent of substrate oxidation by two strains of *G. asaii*. Assays were performed using the TNBT reduction assay as described in the Materials and Methods. Of all the gluconobacter strains tested in this study, only IFO 3297a exhibited endogenous respiration (oxidation in the absence of substrate); these endogenous values were subtracted from the data shown here. Vertical bars represent the standard deviation from the mean determined using all values among two replicates with three cell batches.

DISCUSSION

Optimum pH. The TNBT reduction assay optimum pH of 6.2 (Figure 2A) does not significantly differ from the optimum pH range (5.5 to 6.0) for gluconobacter growth (7). However, optimum pH for oxidative activity varies depending on the type of assay and the type of enzymes assayed. For example, oxygen uptake assays by whole cells oxidizing sorbitol demonstrate an optimum pH of 5.0 with only slight oxidation at pH 6.0 (23). By contrast, studies examining the gluconobacter dehydrogenases responsible for these oxidations suggest that the optimum pH for activity is dependent upon their location within the cell (2), (18). Arcus and Edson (2) report an optimum pH of 5.0 for mannitol and sorbitol oxidation using membrane fractions containing NAD(P)-independent dehydrogenases. However, their study shows that sorbitol and mannitol oxidation is greatest at a pH of 8.0 using soluble NAD(P)-dependent enzymes.

Olijve and Kok (18) used a different *Gluconobacter* strain and report that glucose oxidation by whole cells is accomplished solely by the membrane-bound dehydrogenases at pH values below 3.5 to 4.0. They believe that the pentose phosphate pathway is inhibited at these low pH values. When they used growth-limiting glucose concentrations, triphasic growth was observed at pH 5.5. From these results, they suggest that pH and glucose concentration play a key role in controlling whether glucose will be efficiently oxidized to gluconic acid by the membrane-bound dehydrogenases or transported into the cell and more thoroughly oxidized by the pentose phosphate pathway. Their findings suggest that the oxidations observed with our TNBT reduction assay at high substrate concentrations and a pH of 6.2 result from the gluconobacters' use of their membrane-bound dehydrogenases.

Optimum temperature. This TNBT assay's optimum temperature of 48°C for glycerol oxidation (Figure 2B) is very different from the 25 to 30°C optimum temperature for gluconobacter growth (7). However, oxygen uptake assays of sorbitol oxidation by whole cells demonstrate optimum activity at 44°C (23) and potassium ferricyanide assays of oxidations by an isolated membrane-bound polyol dehydrogenase show an optimum temperature of 38°C. All of these results suggest that the optimal temperature for membrane-bound dehydrogenase activity is significantly higher than that required for cell growth.

Optimum substrate concentration. My results show that concentrations of glycerol between 2.5 and 10% do not significantly affect the oxidations measured in the TNBT reduction assay (Figure 2C). White and Claus (23) found an optimum substrate concentration of 5 - 6 % (w/v) in whole-cell oxygen uptake studies using sorbitol as the substrate. Although they tested a range of 0 to 30 % (w/v) sorbitol, only a small decrease in sorbitol oxidation was observed with increasing sorbitol oxidations above 5-6%.

Suggested assay modifications. My results demonstrate a quantitative, simple, and rapid TNBT reduction assay using 4-ml volumes in test tubes. If one wished only to qualitatively detect oxidation of many substrates by many strains, one could proportionally reduce the reaction-mixture volumes and place them into depressions of a ceramic spot plate or the wells of a microtiter plate. After incubation, the spot-plate depressions or microtiter-plate wells could be scored for black color development. Blackening of the reaction mixture (reduction of the TNBT) will occur only where substrate oxidation occurs. Those strains or compounds could then be selected for further study.

It is conceivable that microtiter plates could also be used to quantitatively measure the extent of substrate oxidation. The volumes of reagents used in the recommended assay could be reduced, so that each reaction mixture would fit into one microtiter-plate well. After incubation, the entire plate could be centrifuged in a rotor equipped with a microtiter plate adapter to pellet the cells and reduced TNBT. A multiple pipettor might then be used to transfer supernatant fluids from the centrifuged microtiter-plate wells to a clean microtiter plate. The Abs_{277} of the optically clear solution in each well could then be automatically determined with a microtiter plate reader to quantitatively determine the loss of oxidized TNBT. On the other hand, I know of no commercially available microtiter plate reader equipped for measuring absorbance in the UV range. However, nitroblue tetrazolium might be substituted for TNBT. The maximum absorbance of reduced nitroblue tetrazolium occurs at 550 nm (12), and this could be measured by a microtiter plate reader following centrifugation to remove the cells. This modified procedure would conceivably provide a miniaturized, partially automated, and quantitative way to optically test many different turbid cell suspensions or substrates.

Oxidative capabilities of different *Gluconobacter* strains. My use of the TNBT reduction assay with six gluconobacter strains and 13 substrates showed that there was as much variation in oxidative ability within one *Gluconobacter* species as there was between species. For example, *G. oxydans* IFO strain 3244 showed no butanol oxidation, whereas *G. oxydans* ATCC strain 621 demonstrated extensive butanol oxidation (Figure 4). Similarly, *G. frateurii* IFO strains 3271 and 3254 differ significantly in their ability to oxidize cyclopentanol (Figure 5). To my knowledge, this is the first time that oxidative properties have been compared between strains having known DNA/DNA homologies, and my data demonstrate that not all strains of one

Gluconobacter species will oxidize a particular substrate or oxidize it to the same extent. Therefore, one should not limit a search to strains within one species of *Gluconobacter* just because another strain in that species accomplished extensive oxidation of an important substrate.

My results also show that five of the six *Gluconobacter* strains tested showed some acetic acid oxidation (Figures 4 - 6). These results may at first appear to contradict the idea that the gluconobacters lack a complete oxidative tricarboxylic acid (TCA) cycle and, therefore, do not completely oxidize acetate to carbon dioxide (7). However, *G. oxydans* ATCC strain 621 is able to partially oxidize acetate by way of an incomplete TCA cycle that supports amino acid biosynthesis (9,10). Since I used whole cells in the TNBT reduction assay, it is conceivable that this slight acetate oxidation is only for the support of amino acid biosynthesis. Such biosynthesis would first require the uptake of acetate and subsequent oxidation by cytosolic TCA-cycle enzymes.

All *G. oxydans*, *G. frateurii*, and *G. asaii* stains tested in the TNBT reduction assay also poorly oxidized raffinose (less than 5 mmole of raffinose oxidized per mg cell protein x 10^{-5}). Edwards (Edwards, D. E. 1990. M.S. Thesis. Virginia Tech, Blacksburg) also observed only slight raffinose oxidation by *G. oxydans* ATCC strain 621 using membrane fractions in potassium ferricyanide reduction assays. Asai (3) reviews raffinose oxidation by the acetic acid bacteria and reports that some *Gluconobacter* strains oxidize raffinose and produce an acidic end product.

A distinct advantage of the TNBT reduction assay is the broad range of acceptable optima, such as pH, temperature, and especially substrate concentration. Such an advantage makes this assay forgiving, in that slight variations in assay conditions do not adversely affect one's results. Other advantages include: the value

of quantitatively measuring only limited oxidations by membrane-bound dehydrogenases; the ability to use whole cells as opposed to membrane fractions or purified enzymes; the potential to be miniaturized and/or used qualitatively; and the ability to perform the assay in a short amount of time. I believe that the TNBT reduction assay described in this report, or used with the suggested modifications, may become a valuable industrial tool for screening the ability of gluconobacter strains to oxidize important organic compounds.

ACKNOWLEDGMENTS

This study was supported in part by a Sigma Xi Grant-In-Aid of Research awarded to L. L. B. and by an unrestricted grant from Genencor International. I thank Norberto Palleroni for providing us with IFO strains from the culture collection of the Microbiology Department at Hoffman-LaRoche, Inc., Nutley, NJ. I also thank the following people who performed preliminary experiments in our laboratory that led to this investigation: Robert Holeman for chemical and optical properties of oxidized-TNBT; Mary Catherine Thomas for spot-plate demonstrations and early TNBT-reduction assays with G. oxydans ATCC strain 621; and Scott Andrews for microscopically demonstrating that reduced TNBT is tightly bound to G. oxydans cells and accumulates in regions occupied by the intercytoplasmic membranes.

REFERENCES

1. **Adachi, O., K. Tayama, E. Shinagawa, K. Matsushita and M. Ameyama.** 1978. Purification and characterization of particulate alcohol dehydrogenase from *Gluconobacter suboxydans*. Agric. Biol. Chem. (Tokyo) **42:** 2045-2056.
2. **Arcus, A.C. and N.L. Edson.** 1956. Polyol dehydrogenases 2. The polyol dehydrogenases of *Acetobacter suboxydans* and *Candida utilis*. Biochem. J. **64:** 385-394.
3. **Asai, T.** 1968. Acetic acid bacteria: Classification and biochemical activities. University of Tokyo Press, Tokyo.
4. **Batzing, B.L. and G.W. Claus.** 1971. Biphasic growth of *Acetobacter suboxydans* on a glycerol-limiting medium. J. Bacteriol. **108:** 592-595.
5. **Cho, J.J., A.C. Hayward and K.G. Rohrbach.** 1980. Nutritional requirements and biochemical activities of pineapple pink disease bacterial strains from Hawaii. Antonie van Leeuwenhoek J. Microbiol. Serol. **46:** 191-204.
6. **Claus, G.W., B.L. Batzing, C.A. Baker and E.M. Goebel.** 1975. Intracytoplasmic membrane formation and increased oxidation of glycerol during growth of *Gluconobacter oxydans*. J. Bacteriol. **123:** 1169-1183.
7. **De Ley, J. and J. Swings.** 1984. Genus II. *Gluconobacter* Asai 1935, 689, emend. mut. char. Asai, Iizuka and Komagata 1964, 100. In: Bergey's manual of systematic bacteriology, Volume 1 (Krieg, N.R., ed.), pp. 275-278, Williams and Wilkins, Baltimore.
8. **Feshami, B.A. and G.W. Claus.** 1991. A nonchromatographic assay for sorbitol in chemically-complex growth media containing sorbose. J. Indust. Microbiol. **7:** 57-61.

9. **Greenfield, S. and G.W. Claus.** 1969. Isocitrate dehydrogenase and glutamate synthesis in *Acetobacter suboxydans*. J. Bacteriol. **100:** 1264-1270.

10. **Greenfield, S. and G.W. Claus.** 1972. Nonfunctional tricarboxylic acid cycle and the mechanism of glutamate biosynthesis in *Acetobacter suboxydans*. J. Bacteriol. **112:** 1265-1301.

11. **Hanson, R.S. and J.A. Phillips.** 1981. Chemical composition. In: Manual of methods for general bacteriology (Gerhardt, P., R.G. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg and G.B. Phillips, ed.), pp. 328-364, American Society for Microbiology, Washington, D.C..

12. **Jones, K.M.** 1969. Artificial substrates and biochemical reagents. In: Data for biochemical research (Dawson, R.M.C., D.C. Elliott, W.H. Elliott and K.M. Jones, ed.), pp. 436-443, Oxford University Press, New York, NY.

13. **Kersters, K., W.A. Wood and J. De Ley.** 1965. Polyol dehydrogenases of *Gluconobacter oxydans*. Journal of Biological Chemistry. **240:** 965-974.

14. **Lambert, B., K. Kersters, F. Gossele, J. Swings and J. De Ley.** 1981. Gluconobacters from honey bees. Antonie van Leeuwenhoek J. Microbiol. Serol. **47:** 147-157.

15. **Mason, L.M. and G.W. Claus.** 1989. Phenotypic characteristics correlated with DNA-sequence similarities for three species of *Gluconobacter: G. oxydans* (Henneberg, 1897) DeLey 1961, *G. frateurii* sp. nov., and *G. asaii* sp. nov. Int. J. Syst. Bacteriol. **39:** 174-184.

16. **Matsushita, K., H. Toyama and O. Adachi.** 1994. Respiratory chains and bioenergetics of acetic acid bacteria. In: Advances in Microbial Physiology, Vol. 36 (Rose, A.H. and D.W. Tempest, ed.), pp. 247-301, Academic Press, San Diego, California.

17. **Micales, B.K., J.L. Johnson and G.W. Claus.** 1985. Deoxyribonucleic acid homologies among organisms in the genus *Gluconobacter*. Int. J. Syst. Bacteriol. **35:** 79-85.

18. **Olijve, W. and J.J. Kok.** 1979. Analysis of growth of *Gluconobacter oxydans* in glucose containing media. Arch. Microbiol. **121:** 283-290.

19. **Pronk, J.T., P.R. Levering, W. Olijve and J.P. Dijken.** 1989. Role of NADP-dependent and quinoprotein glucose dehydrogenases in gluconic acid production by *Gluconobacter oxydans*. Enzyme Microb. Technol. **11:** 160-164.

20. **Rainbow, C.** 1966. Nutrition and Metabolism of Acetic Acid Bacteria. Wallerstein Lab. Commun. **29:** 5-15.

21. **Sedar, A.W. and R.M. Burde.** 1965. The demonstration of the succinic dehydrogenase system in *Bacillus subtilis* using tetranitro-blue tetrazolium combined with techniques of electron microscopy. J. Cell Biol. **27:** 53-66.

22. **Shirashi, F., K. Kawakami, S. Kono, A. Tamura, S. Tsuruta and K. Kusunoki.** 1989. Characterization of production of free gluconic acid by *Gluconobacter suboxydans* adsorbed on ceramic honeycomb monolith. Biotechnol. Bioeng. **33:** 1413-1418.

23. **White, S.A. and G.W. Claus.** 1982. Effect of intracytoplasmic membrane development on oxidation of sorbitol and other polyols by *Gluconobacter oxydans*. J. Bacteriol. **150:** 934-943.

APPENDIX 1A

Absorbance spectrum of oxidized tetranitroblue tetrazolium

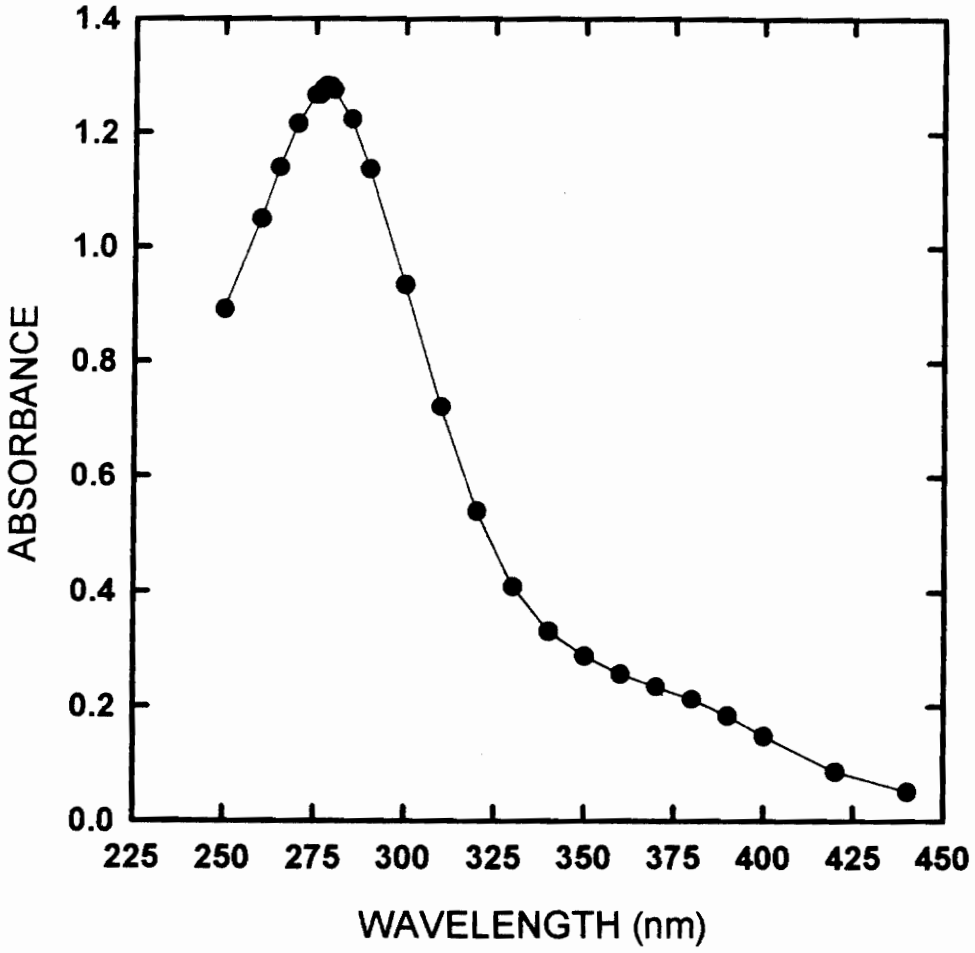


Figure A1. Absorbance spectrum of oxidized tetranitroblue tetrazolium (TNBT). A solution containing 20 μg/ml of oxidized TNBT in distilled water was measured against a distilled water blank at wavelengths between 200-800 nm with a Milton Roy Spectronic 1201 spectrophotometer. Maximum absorbance was found at 277 nm.

APPENDIX 1B

Relationship between oxidized tetranitroblue tetrazolium (TNBT) concentration and absorbance

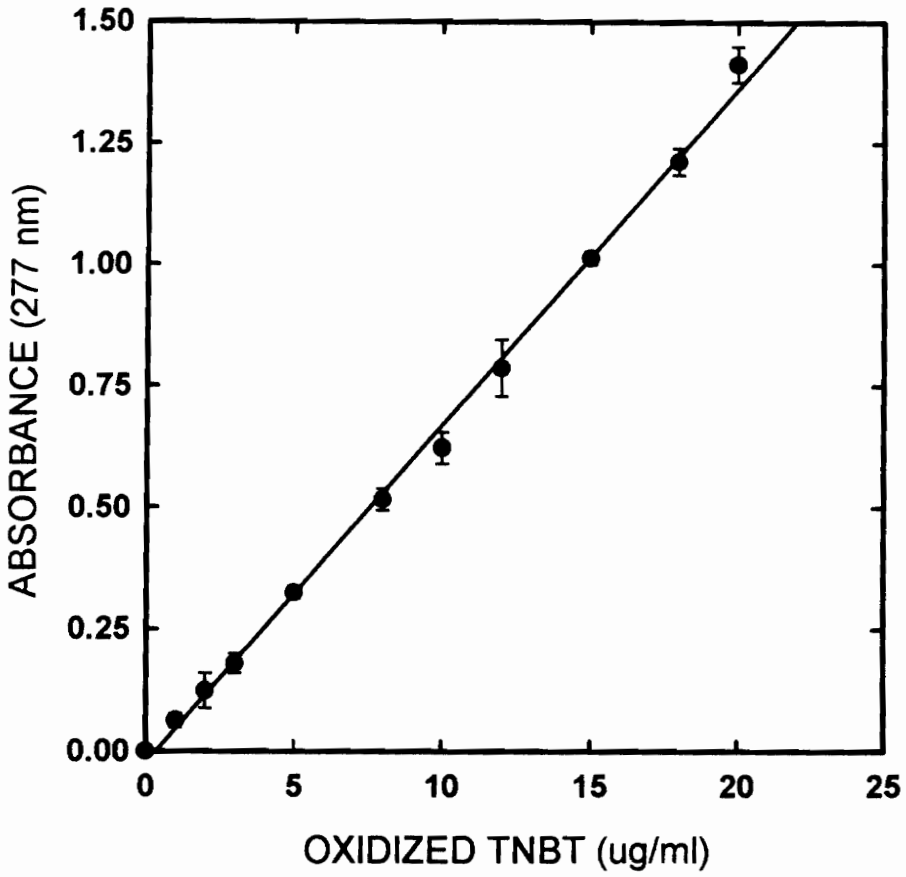


Figure B1. Relationship between oxidized TNBT concentration and absorbance at 277 nm. Oxidized TNBT was measured with a Milton Roy Spectronic 1201 spectrophotometer.

APPENDIX 1C

Effect of cell concentration on tetranitroblue tetrazolium (TNBT) reduction using optimal TNBT reduction assay conditions

The effect of cell concentration on the amount of tetranitroblue tetrazolium (TNBT) reduction was studied prior to the determination of all TNBT reduction optimal assay conditions (Chapter 1, Figure 1). For this reason, this experiment was repeated under optimal TNBT reduction assay conditions of pH 6.2, 48^{o} C, and 5% (w/v) glycerol concentration to determine if the changes in assay conditions affected the initial results. In addition, the effect of preheating the cell suspensions to 48^{o} C prior to beginning the 60-minute incubation was also determined. *Gluconobacter oxydans* ATCC strain 621 was used in these experiments. The rest of the assay conditions were as previously described in the Materials and Methods section of Chapter 1. The results of this experiment are shown in Figure C1. When different concentrations of cell protein was added to reaction mixtures, the extent of TNBT reduction differed very little whether the cell suspensions were preheated prior to incubation or just added at room temperature. However, the extent of glycerol oxidation did increase when optimal assay conditions were used as compared to the amount of glycerol oxidation that was observed in previous results (Chapter 1, Figure 1). These results confirm the use of the optimal assay conditions reported in Chapter 1 for the testing of *Gluconobacter* strains for their ability to oxidize various substrates.

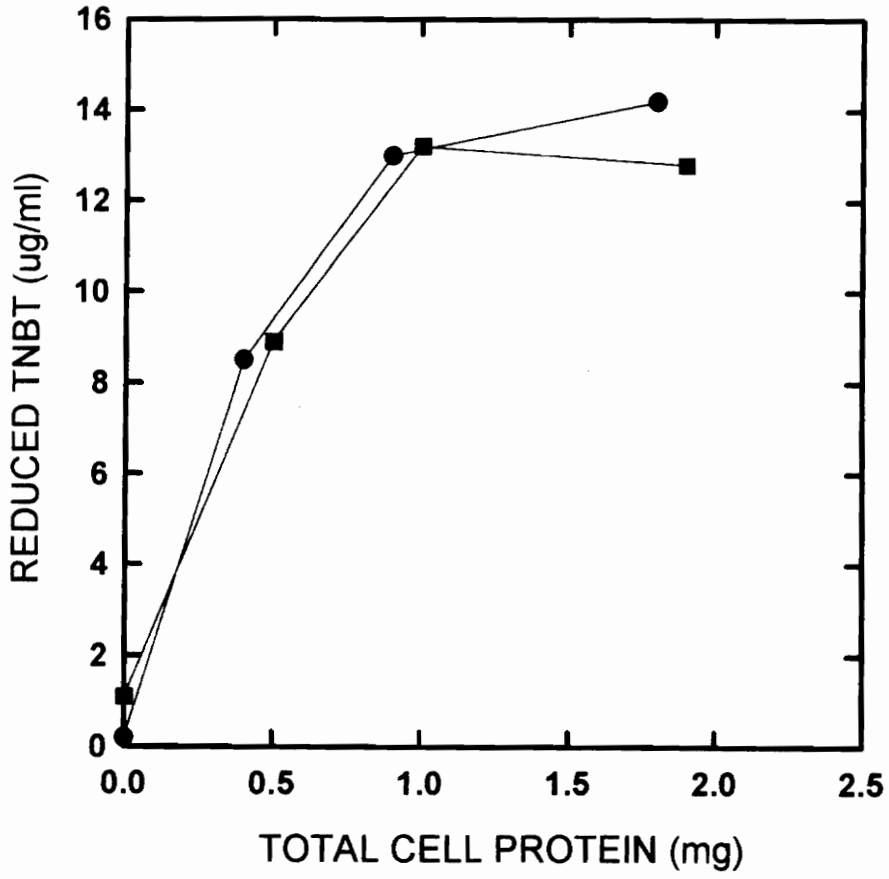


Figure C1. Effect of cell concentration on the extent of TNBT reduction (glycerol oxidation) after 60 min using optimal TNBT reduction assay conditions. Reaction mixtures contained (in a total volume of 4 ml): 20μg/ml oxidized TNBT, 5% (v/v) of glycerol, and varying concentrations of *G. oxydans* ATCC strain 621 cells suspended in 0.2 M succinate buffer (pH 6.2). Reaction mixtures were incubated for 60 min at 48°C. Closed squares represent reactions performed using cell suspensions that were preheated to 48°C prior to starting the reactions. Closed circles represent reactions performed with cell suspensions that were 25-30°C prior to starting the reactions. Each data point represents the mean of two replicates.

Chapter 2

Characterization of Plasmids from the Three Species of *Gluconobacter*

SUMMARY

The genus *Gluconobacter* consists of acetic acid bacteria that oxidize many different organic compounds. These rapid limited oxidations have great value to chemical and pharmaceutical industries, however, little is known about the genetics of the gluconobacters and the plasmids contained in these bacteria. Plasmids within the acetic acid bacteria may contain at least part of the genetic information necessary for some enzymatic processes that make these bacteria industrially important. For this reason, I wished to learn more about the plasmids within the genus *Gluconobacter* and how these plasmids are distributed among the three species within this genus. I examined 22 strains of *Gluconobacter* for the type and size of plasmids present. Plasmids were isolated by alkaline lysis, phenol-chloroform extraction, and lithium chloride-ethanol precipitation. Size estimations were made using known molecular weight standards that were run on each individual agarose gel containing *Gluconobacter* plasmid preparations. Plasmids were isolated from 18 of the 22 strains tested and ranged in size from 2 to > 54 kb. Some strains contained as many as 9 plasmids and some only one. Plasmids were not detected in four strains. Several strains contained plasmids of similar size. These similarities in size support the suggestion that plasmids have been transferred between strains of *Gluconobacter* during the evolution of these bacteria. This investigation is the first to compare plasmid occurrence among the three species of *Gluconobacter* and is an important first step in the characterization of these plasmids.

INTRODUCTION

Many types of bacteria, belonging to vastly diverse metabolic groups are known to contain plasmids. These bacteria include Gram-negative aerobic rods, Gram-negative facultatively anaerobic rods, Gram-negative cocci, Gram-positive rods, endospore-forming bacteria, and phototrophic bacteria (18). Plasmids vary greatly in size ranging from 2 kb to larger than 300 kb (7). Plasmids are usually circular molecules of double-stranded DNA that replicate autonomously, are stably inherited, posses the ability to transfer to different bacterial cells, and are independent of the host cell chromosome (see page 1, Ref. 6, 7). However, not all plasmids share these basic characteristics. For example, some plasmids can become integrated into the host cell chromosome and replicate along with it (7), and some *Streptomyces* and *Borrelia* plasmids are linear (1, 8, 9).

Characterization of plasmids in bacteria is important for two reasons. First, plasmids are important research tools for genetic analysis, because they are often used as vectors for transfer and characterization of genetic elements (see page 2, Ref. 6). Second, bacterial plasmids contain important genes, such as those responsible for antibiotic resistance, heavy metal resistance, metabolism of diverse compounds, nitrogen fixation, toxin production, gas vacuole formation, and conjugal properties (see page 1, Ref. 6, 7, 18). Although plasmids are often classified according to these phenotypic characteristics, many plasmids isolated from various bacteria are said to be "cryptic", because they encode no known phenotypic characteristic(s).

Plasmids in the acetic acid bacteria have only recently been investigated. Acetic acid bacteria are Gram-negative, rod-shaped bacteria that generate acidic products from substrate oxidation, in particular, acetic acid from ethanol (1, 16). Until

recently, all plasmids isolated from strains of acetic acid bacteria remained cryptic. Several investigators now suggest that the genetic information carried on some plasmids are associated with ethanol and glucose oxidation, acetic acid resistance, and cellulose biosynthesis (14, 15, 20).

Acetic acid bacteria are industrially important because of their use in vinegar production and sorbitol oxidation. Since plasmids may be involved in these processes, further characterization of plasmids from many strains of *Acetobacter* and *Gluconobacter* has become increasingly more important.

Several studies report the presence of plasmids in strains of both genera of acetic acid bacteria, the acetobacters and the gluconobacters. In 1983, Micales (12) screened the four *Gluconobacter* type strains (*G. oxydans* ATCC strain 19357, *G. oxydans* subsp. *melangenes* ATCC strain 33447, *G. oxydans* subsp. *suboxydans* ATCC strain 33448, and *G. sphaericus* (*G. oxydans)* IFO strain 12467) for the presence of plasmids. Plasmids were detected in all four strains and ranged in size from 1.8 to 117.7 megadaltons (2.7 to 178.3 kb). *G. oxydans* ATCC strain 19357 contained six plasmids, while *G. oxydans* subsp. *suboxydans* ATCC strain 33448 contained only two plasmids. The remaining two strains each contained five plasmids.

In addition to Micales' (12) report, McKibben (11) more extensively examined the three species of *Gluconobacter* for the presence of plasmids. She reported that 6 of 8 strains of *G. oxydans* contain plasmids and these plasmids ranged in size from 2.7 kb to 260 kb. All 3 strains of *G. frateurii* tested contained plasmids whereas, 2 of the 3 strains of *G. asaii* contained plasmids. The plasmids isolated from the latter two strains ranged in size from 3.9 kb to 100 kb.

Plasmids from strains of *Acetobacter* have also been studied. Valla et al. (19) reported several plasmids isolated from strains of *A. xylinum*. Both 44 kb and 64 kb

plasmids were observed in these strains. In addition, two large plasmids ranging between 200 to 300 kb in size were also observed. A 16 kb plasmid was also detected in some strains.

An extensive analysis of plasmids from 87 strains of acetic acid bacteria showed that these plasmids vary in size and in number (2). Of the 33 *Acetobacter* strains tested, 27 were found to contain plasmids, and most contained more than one. Plasmids from *Acetobacter* strains ranged in size from about 1 to 17 megadaltons (1.5 to 25.8 kb). Of the 36 *Gluconobacter* strains tested, 23 contained plasmids. *Gluconobacter* plasmids were generally larger than those found in *Acetobacter* strains, and *Gluconobacter* plasmids ranged from about 2 to larger than 17 megadaltons (3 to 25.8 kb). In contrast to *Acetobacter* strains, several *Gluconobacter* strains contained only one plasmid.

Several hybrid shuttle vectors have been constructed using plasmids from strains of *Acetobacter* or *Gluconobacter* combined with plasmids from *E. coli* (4, 5, 14). However, these chimeric plasmids are better developed and characterized in the acetobacters. For example, acetic acid fermentation was improved in a strain of *A. aceti* by cloning the aldehyde dehydrogenase gene from another *Acetobacter* strain into an *Acetobacter-E. coli* hybrid plasmid and then transforming the *A. aceti* strain with this plasmid (3).

The purpose of my investigation was to further characterize the plasmids in strains of *Gluconobacter* representing the three species *G. oxydans*, *G. frateurii*, and *G. asaii*. I hope that these characterizations may lead to a better understanding of the genetics of the gluconobacters and further the development of plasmid vector systems for use in these bacteria. Although many strains used in my investigation were also tested by Fukaya et al. (2), their plasmid DNA isolation procedures differed greatly from

the procedures reported here. Although my plasmid isolation procedure was similar to that of McKibben (11), our results differ for a number of strains. In addition, several strains reported in my investigation were not previously analyzed. My report also appears to be the first one to consider plasmid distribution and association within the three species of *Gluconobacter*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study were the same as those used in a previous DNA/DNA homology study (13) and tests of related phenotypic characteristics (10). *Gluconobacter oxydans* ATCC strains 621, 9937, 14960, 19357, and 23771 were received from the American Type Culture Collection, and *G. oxydans* IFO strains 3244, 3293, 3294, & 12528 and ATCC strain 23652 were received from Noberto Palleroni (Hoffman-LaRoche, Nutley, NJ). *Gluconobacter oxydans* IFO strain 12467 was received from the Institute of Fermentation (Osaka, Japan) and *G. oxydans* CSIRO strain B1507 was received from Division of food Resources, Commonwealth Scientific and Industrial Research Organization (Ryde, Australia). *Gluconobacter frateurii* IFO strains 3254, 3264, 3268, 3270, 3271, 3272, and 3286 were also received from Noberto Palleroni. *Gluconobacter asaii* IFO strain 3276 was received from the Institute of Fermentation and *G. asaii* IFO strain 3297 was a gift from Noberto Palleroni. *G. asaii* ATCC strain 43781 was originally received from K. G. Rohrback (Department of Plant Pathology, University of Hawaii at Manoa, Honolulu) and later deposited at the American Type Culture Collection. All strains were checked for purity, grown in a complex medium, suspended in a medium containing (w/v) 15% glycerol, 1% yeast extract, and 1% peptone then heat-sealed in glass vials and continuously maintained in liquid nitrogen

Dewars. Working-stock cultures were prepared by growing the strains in broth containing (w/v) 5% glycerol, 1% yeast extract and 1% peptone until reaching approximately 0.9 OD_{620} and maintained until use by storing in 66% glycerol at -4°C. Cells used for plasmid isolations were grown in 50 ml of broth containing (w/v) 5% glycerol, 1% yeast extract, and 1% peptone in 500 ml growth flasks. Cultures were shaken at 200 rpm at 28°C until reaching approximately 0.6 OD_{620}.

Escherichia coli strain V517 was received from A. A. Yousten (Department of Biology, Virginia Tech, Blacksburg). *Escherichia coli* strain V517 was grown in double strength nutrient broth until reaching approximately 0.9 OD_{620} and maintained until use by storing in 66% glycerol at -4°C. Strain V517 used for plasmid isolations was grown, in 50 ml of double strength nutrient broth in 500 ml growth flasks with shaking at 37°C until reaching approximately 0.6 OD_{620}.

Reagents. Sucrose, sodium dodecyl sulfate, glacial acetic acid, xylene cyanol FF, glycerol, phenol, and chloroform were purchased from Fisher Scientific, Fair Lawn, NJ. Ethylenediamine tetra acetic acid, sodium hydroxide, and ethidium bromide were purchased from Sigma Chemical Co., St. Louis, MO. Yeast extract, peptone, and nutrient broth were purchased from Difco Laboratories, Detroit, MI. Ultra pure Tris[hydroxymethyl]aminomethane (Tris) was purchased from United States Biochemical, Cleveland, OH. Concentrated hydrochloric acid was purchased from EM Science Division of EM Industries, Gibbstown, NJ. Lithium chloride was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. Bromophenol blue was purchased from Canalco, Rockville, MD. Glycogen was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. Ethanol was purchased from Aaper Alcohol and Chemical Co., Shelbyville, KY.

Buffers. The buffers referred to in the plasmid isolation procedures were prepared as follows. The E buffer contained 0.04 M Tris, 0.002 M ethylenediamine tetra acetic acid (EDTA), and 15% (w/v) sucrose. It was prepared in sterile water and was adjusted to pH 7.9 with glacial acetic acid. Lysis buffer contained 0.3% (w/v) sodium dodecyl sulfate (SDS), and 5% (v/v) 3 M sodium hydroxide added to 5 ml of a solution of 0.05 M Tris and 15% (w/v) sucrose. The TE buffer contained 10 mM Tris and 1 mM EDTA and was adjusted to pH 8.0 with concentrated hydrochloric acid. Lithium chloride buffer contained 0.4 M lithium chloride in TE buffer. The 50 X TAE buffer contained 242 g Tris, 100 ml of 0.5 M EDTA (adjusted to pH 8.0) and 57.1 ml glacial acetic acid brought to a final volume of one liter with distilled water. Tracking dye contained a 4.2 mg of bromophenol blue and cyanol xylene FF added to 2 ml of a solution containing 50% (w/v) of glycerol and 50% (w/v) 2 X TAE buffer.

Alkaline lysis of cells (11). Five milliliters of each cell culture were centrifuged in an IEC model HN-S centrifuge (Damon/IEC Division) for 10 min at full speed, and the resulting pellet was resuspended in 200 μl of E buffer. Lysis buffer (400 μl) was added to the pellet resuspended in E buffer and gently mixed by wrist action. The cell suspensions were then heated in a 60^{o}C waterbath for 30 min to lyse the cells and denature their DNA. These cell lysates were then cooled to room temperature. The cell lysates were incubated in a 37^{o}C waterbath for 20 min after the addition of 100 μl of 2 M Tris-HCl buffer (pH 7.0) so that plasmid DNA could reanneal. Chromosomal DNA fragments should remain denatured under these conditions.

Phenol-chloroform extraction (11). A 1:1 ratio of phenol and chloroform (1.2 ml total volume) was added to each neutralized cell lysate to remove protein and lipids contained in the cell-lysate mixtures. These mixtures were then mixed gently by wrist action and centrifuged at 13,300 x g (Beckman Model J2-21 centrifuge) for 20 min.

The resulting aqueous top layer (~ 600 µl) was then carefully removed, so as not to disturb the phenol-water interface, and placed in a clean, sterile 1.5 ml microcentrifuge tube. This aqueous phase was then extracted once again with chloroform (600 µl) to remove any residual phenol and then centrifuged in a Fisher Scientific model 235C microcentrifuge at full speed for 8 min. The resulting aqueous top layer was carefully removed in two separate 300 µl volumes, and each was placed into separate clean, sterile 1.5 ml microcentrifuge tubes.

Lithium chloride - ethanol precipitation (11). The following procedure was used to concentrate the plasmid DNA contained in the aqueous phase resulting from the phenol-chloroform and chloroform extractions. Glycogen (1µl of a 20 mg/ml solution) was added to each microcentrifuge tube containing the aqueous phase to serve as a carrier to precipitate DNA from small volumes. One-tenth volume of lithium chloride buffer and 2.5 times the volume (after lithium chloride buffer addition) cold 95% ethanol was added to each tube and vortexed. All tubes were then placed on ice at 4^{o}C for at least 30 min, and then centrifuged in an Eppendorf model 5415 microcentrifuge at 4^{o}C and full speed for 20 min to precipitate the (plasmid) DNA. The resulting pellets were dried at room temperature overnight or air dried in a 43-45^{o}C oven for 15-30 min. The dried pellets were gently suspended in 30 µl of TE buffer and placed in a 43-45^{o}C oven for 15-20 min to help dissolve the pellets. Plasmid preparations were stored at -80^{o}C until use.

Agarose gel electrophoresis and ethidium bromide staining. Samples of plasmid preparations (5 µl) were mixed with 5 µl of TE buffer and 2 µl of tracking dye and loaded into the wells of a 0.7% agarose gel (prepared with 1 x TAE buffer). The samples were electrophoresed in 1 x TAE buffer placed within a Hoefer HE33 submarine agarose gel unit at 60 volts for about 2.5 h until the bromophenol blue dye

front of the tracking dye was about 1.5 cm from the bottom of the gel. Gels were stained for 15 min with 50-100 µl of a 0.5 mg/ml solution of ethidium bromide added to 35 ml of distilled water. Gels were rinsed once with 35 ml of distilled water for 15 min.

Vi alization and photography of the gels. Gels were visualized with a PhotoDyne UV transilluminator and then photographed through an orange filter using a Polaroid camera containing Polaroid 667 black and white film.

lasmid size estimations. All *Gluconobacter* plasmid sizes were estimated by comparison to known molecular weight standards that were run on the same gel. Molecular weight standards used were: (i) plasmids extracted from *E. coli* strain V517 (in kb) 2.1, 2.7, 3.1, 5.1, 5.6, 7.3 & 54 kb and (ii) a super coiled kb ladder (Bethesda Research Laboratories) containing 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, and 16 kb plasmids. The distance these molecular weight standards traveled in the gel was measured from the gel photograph. A standard curve was then constructed on two-cycle semilogarithmic graph paper by plotting each standard's known molecular weight against its distance traveled in the agarose gel. A representative standard curve is shown in Appendix 2A. The *Gluconobacter* plasmid sizes between 2 and 16 kb were then estimated using this standard curve. Plasmid sizes greater than 54 kb were not estimated using these standards and are reported only as large plasmids. Reported plasmid sizes between 16-54 kb result from extrapolations based on the standard curves generated using the molecular weight standards.

RESULTS

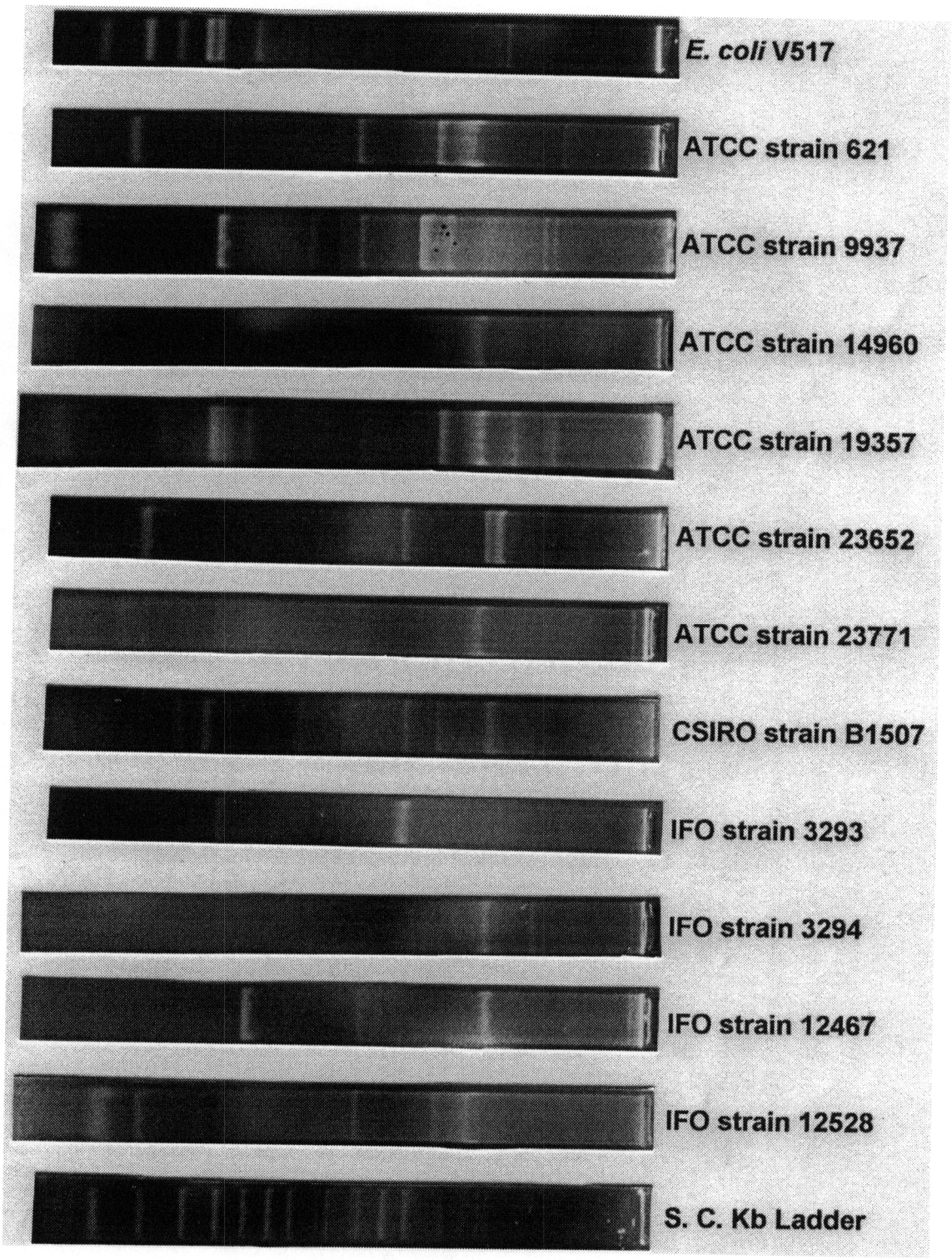
Twenty-two strains representing the three species of *Gluconobacter* were tested for the presence of plasmids. Figures 1 - 3 show representative ethidium bromide-stained agarose gels with one lane from each of the 18 strains that have at least 1 plasmid. The estimated sizes of plasmids from each of the three species are shown in Tables 1 - 3. The data used to make these estimations are shown in Appendices 4B, 4C and 4D.

Plasmid size estimations. The size estimations for plasmids larger than 16 kb are probably not as accurate as those for plasmids between 1 and 16 kb. Even though *E. coli* strain V517 contains a plasmid whose well-established size is 54 kb, this plasmid did not fall within the linear portion of the standard curve plotted using plasmids from 1 to 16 kb (Appendix 2A). Plasmids larger than 100 kb were observed in four strains of *Gluconobacter* (Table 1 and 3). These plasmids were too large to obtain even rough estimations of their size. Therefore, these plasmids are only noted as large plasmids in Tables 1 and 3.

***Gluconobacter oxydans* plasmids.** Plasmids were detected in all 12 strains of *G. oxydans* tested except IFO strain 3244 (Table 1). Some strains contained as few as two plasmids (IFO strains 3293, 3294, and 12467), and two strains (ATCC 19357 and CSIRO B1507) contained nine and six plasmids. Strain ATCC 19357 contained as many as 9 plasmids, including two large plasmids. Strains ATCC 621, ATCC 9937, and IFO 12528 also contained large plasmids. The plasmids isolated from these strains of *G.oxydans* varied in size from 2.2 kb to > 54 kb, although most plasmids were between 2.2 and 25 kb.

***Gluconobacter frateurii* plasmids.** Of the seven strains of *G. frateurii* tested, plasmids were detected in only four strains (Table 2). Plasmids were not detected in *G.*

Figure 1. Representative lanes from photographs of ethidium bromide stained 0.7% agarose gels containing electrophoresed plasmids isolated from strains of *Gluconobacter oxydans*. Molecular weight standards used were from *E. coli* strain V517: (in kb) 2.1, 2.7, 3.1, 5.1, 5.6, & 7.3 and a supercoiled kb ladder (Bethesda Research Laboratories) (2, 3, 4, 5, 6, 7, 8, 10, 12, 14, and 16 kb). Representative lanes were taken from different ethidium bromide stained agarose gels containing the *G. oxydans* plasmids and the molecular weight standards, and therefore, may not align properly in this figure.


E. coli V517
ATCC strain 621
ATCC strain 9937
ATCC strain 14960
ATCC strain 19357
ATCC strain 23652
ATCC strain 23771
CSIRO strain B1507
IFO strain 3293
IFO strain 3294
IFO strain 12467
IFO strain 12528
S. C. Kb Ladder

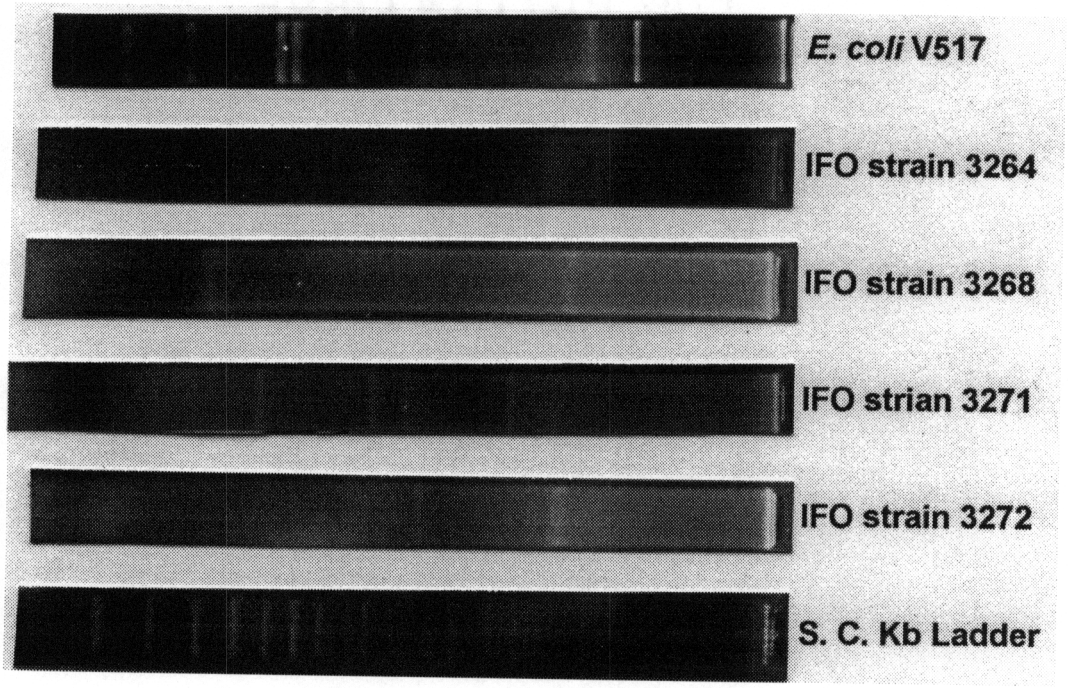

E. coli V517
IFO strain 3264
IFO strain 3268
IFO strian 3271
IFO strain 3272
S. C. Kb Ladder

Figure 2. Representative lanes from photographs of ethidium bromide stained 0.7% agarose gels containing electrophoresed plasmids isolated from strains of *Gluconobacter frateurii.* Molecular weight standards used were from *E. coli* strain V517: (in kb) 2.1, 2.7, 3.1, 5.1, 5.6, & 7.3 and a supercoiled kb ladder (Bethesda Research Laboratories) (2, 3, 4, 5, 6, 7, 8, 10, 12, 14, and 16 kb). Representative lanes were taken from different ethidium bromide stained agarose gels containing the *G. frateurii* plasmids and the molecular weight standards, and therefore, may not align properly in this figure.

Figure 3. Representative lanes from photographs of ethidium bromide stained 0.7% agarose gels containing electrophoresed plasmids isolated from strains of *Gluconobacter asaii*. Molecular weight standards used were from *E. coli* strain V517: (in kb) 2.1, 2.7, 3.1, 5.1, 5.6, & 7.3 and a supercoiled kb ladder (Bethesda Research Laboratories) (2, 3, 4, 5, 6, 7, 8, 10, 12, 14, and 16 kb). Representative lanes were taken from different ethidium bromide stained agarose gels containing the *G. asaii* plasmids and the molecular weight standards, and therefore, may not align properly in this figure.

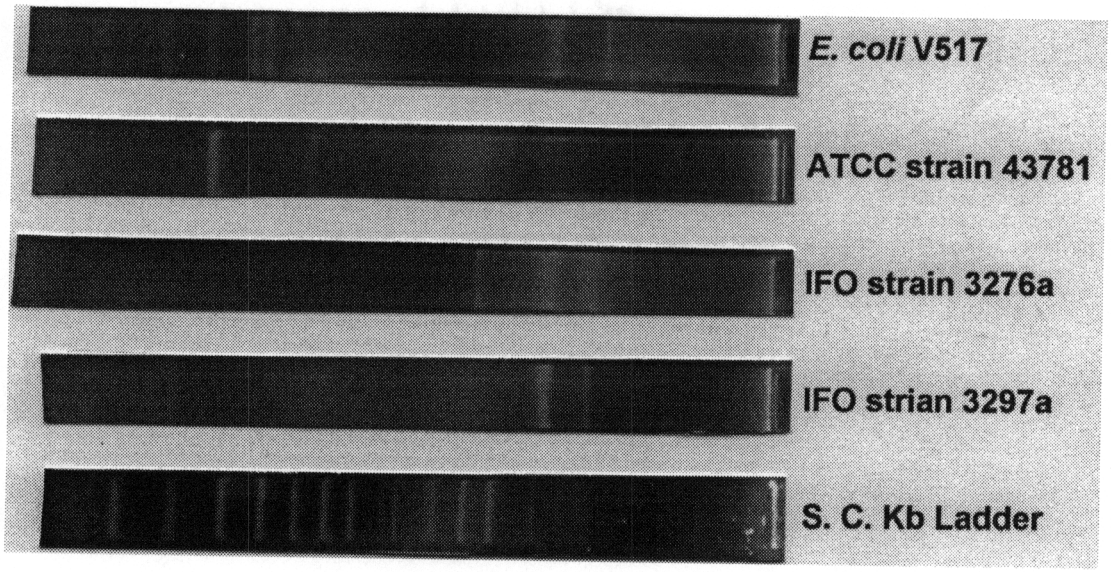

E. coli V517
ATCC strain 43781
IFO strain 3276a
IFO strian 3297a
S. C. Kb Ladder

Table 1. Size estimations of plasmids isolated from strains of *G. oxydans*

Strain [a]	Size estimations (kb) [b]
ATCC 621	2.9 14.8 24.6 one large plasmid
ATCC 9937	5.9 12.7 17.6 27.6 two large plasmids
ATCC 14960	13.4 26.6 45 [c, d]
ATCC 19357	3.1 4.2 5.1 7.8 [c] 16 21.3 28.3 two large plasmids
ATCC 23652	2.8 14.5 [c] 24 [c]
ATCC 23771	2.9 [c] 14. 5 [c] 26.5
CSIRO B1507	3.5 4.2 6.1 7.7 14.5 [c] 18.5 [c]
IFO 3244	none detected
IFO 3293	9.5 20 [c]
IFO 3294	36 [c, d] 54 [c, d]
IFO 12467	6.1 12.3
IFO 12528	2.2 2.9 14.9 26.7 one large plasmid

[a] Abbreviations are: ATCC, American Type Culture Collection, Rockville, MD; CSIRO, Commonwealth Scientific and Industrial Research Organization, New South Wales, Australia; IFO, Institute of Fermentation, Osaka, Japan.

Table 1. (continued)

[b] Plasmid sizes were estimated by comparison to known molecular weight standards that were run on the same gel. Molecular weight standards used were from *E. coli* strain V517: (in kb) 2.1, 2.7, 3.1, 5.1, 5.6, & 7.3 and a super coiled kb ladder (Bethesda Research Laboratories) (2, 3, 4, 5, 6, 7, 8, 10, 12, 14, and 16 kb). Very large plasmids (>100 kb) were observed in some strains and these are noted in the table. However, molecular weight standards much larger than those used must be run on gels containing these plasmids to approximate their large size.

[c] This plasmid has not been resolved in all gels containing plasmid preparations from this strain, therefore, the size estimation is based on fewer replicates than were used to calculate the plasmid sizes from this strain (Appendix B).

[d] This plasmid is a relatively large plasmid whose size is not accurately determined using the molecular weight standards described in footnote b. Higher molecular weight standards must be run on gels to estimate the size of this large plasmid. In addition, the 36 kb plasmid may be the same plasmid as the 54 kb plasmid since it was resolved on a gel where the 54 kb plasmid was not observed.

Table 2. Size estimations of plasmids isolated from strains of *G. frateurii* [a]

Strain	Size estimations (kb)
IFO 3254	none detected
IFO 3264	33 [b]
IFO 3268	3.8
IFO 3270	none detected
IFO 3271	5.0 9.9 [b] 13.5 [b] 22 [b]
IFO 3272	2.1 [b] 12.8 24 [b]
IFO 3286	none detected

[a] Strain culture collection abbreviations and plasmid size estimations are as described in footnotes for Table 1.

[b] This plasmid has not been resolved in all gels containing plasmid preparations from this strain, therefore, the size estimation is based on fewer replicates than were used to calculate the plasmid sizes from this strain (Appendix C).

Table 3. Size estimations of plasmids isolated from strains of *G. asaii* [a]

Strain	Size estimations (kb)
ATCC 43781	3.9 6.8 37 [b]
IFO 3276a	19.4 34 one large plasmid
IFO 3297a	2.0 3.0 3.3 35

[a] Strain culture collection abbreviations and plasmid size estimations are as described in footnotes for Table 1. A very large plasmid (>100 kb) was observed in one of these strains and is noted in the table. However, molecular weight standards much larger than those used must be run on gels containing this plasmid to approximate its large size.

[b] This plasmid has not been resolved in all gels containing plasmid preparations from this strain, therefore, the size estimation is based on fewer replicates than were used to calculate the plasmid sizes from this strain (Appendix D).

frateurii strains IFO 3254, 3270 and 3286. There was a considerably higher proportion of *G. frateurii* strains that lacked plasmids compared with strains from *G.oxydans* (Tables 1 and 2). In addition, strains of *G. frateurii* that had plasmids, did not contain as many plasmids as found in strains of *G. oxydans*. For example, only one plasmid was found in *G. frateurii* IFO strains 3264 and 3268.

As many as four plasmids were found in *G. frateurii* IFO strain 3271 (Table 2); however, three of these plasmids were not consistently resolved in all gels containing plasmids from this strain. The reasons for this are not known. Perhaps these three plasmids are present only in low copy numbers and therefore, plasmid extracts may not contain a great enough concentration to be consistently observed with ethidium bromide. Similarly, two of the three plasmids isolated from *G. frateurii* IFO strain 3272 were not consistently resolved (Table 2). Unlike strains of *G. oxydans*, *G. frateurii* strains appeared to lack large plasmids. The range in plasmid size from *G. frateurii* strains (2.1 kb to 33 kb) was not as large as those found in *G. oxydans* strains (2.2 to > 54 kb).

***Gluconobacter asaii* plasmids.** Unfortunately, only three strains of *G. asaii* are known, and all three were tested for the presence of plasmids (Table 3). All three strains of *G. asaii* contained at least two plasmids. Four plasmids were detected in *G. asaii* IFO strain 3297a. Similar to three strains of *G. oxydans*, *G. asaii* IFO strain 3276a contained a large plasmid that was resolved in only some agarose gels. The plasmids isolated from the three strains of *G. asaii* ranged in size from 2 to 37 kb (excluding the large plasmid from IFO strain 3276a).

DISCUSSION

Plasmids in strains of *Gluconobacter oxydans.* I found that most strains of *Gluconobacter oxydans* contained at least two plasmids (Table 1). One exception was *G. oxydans* IFO strain 3244 which appeared to lack plasmids. Two other investigations also report the lack of plasmids in this strain (2, 11). However, it is possible that plasmids were present but were not detected by the methods used. This strain could contain a low copy number plasmid (or plasmids) not present in high enough concentration to detect using the isolation and detection methods used in these three studies. This strain could also contain one or more large plasmids that were not resolved in these gels.

Fukaya et al. (2) examined five of the same strains of *G. oxydans* that I tested for the presence of plasmids. Although we both agree that all except IFO strain 3244 contain plasmids, several differences exist as to the numbers and sizes of these plasmids. For example, I found that *G. oxydans* ATCC strain 19357 contained at least seven plasmids ranging in size from 3.1 to 28.3 kb plus the possibility of two large plasmids (Table 1). However, Fukaya et al. (2) reported only four plasmids in this strain (2.7 , 11, 12, and > 17 kb). Their 2.7 kb plasmid may possibly be the same as my 3.1 kb plasmid, and their > 17 kb plasmid could be one of at least three plasmids I observed. I was unable to find their 11 and 12 kb plasmids in ATCC strain 19357.

On the other hand, my results using ATCC strain 19357 agree with those of Micales (12) in both numbers of plasmids and their approximate sizes. The only exception is that Micales (12) does not report the two large plasmids or the 7.8 kb plasmid found in my study. Micales (and I) report the following six similar-sized plasmids for ATCC strain 19357: 2.8 (3.1), 3.5 (4.2), 5.9 (5.1), 16 (17.1), 21 (21.3) and 28.3 (29.8).

McKibben (11) did find the two large plasmids that I found in ATCC strain 19357. In addition, McKibben (11) found four other plasmids (4.3, 19.5, 26.8, and 40 kb), but her report of plasmid sizes differs considerably from the sizes I found (Table 1) and those reported by Micales (12).

Reports of the numbers and sizes of plasmids detected from IFO strain 12467 widely differ. Fukaya et al. (2) reported three plasmids in this strain (3.3, 4.1, and 7.4 kb), whereas I observed only two plasmids (6.1 and 12.3 kb). Micales (12) found five plasmids in this strain (4.0, 9.4, 23.4, 76.3, and 117.7 kb) and McKibben (11) found three plasmids (6.4, 26.8, and 260 kb).

My results using *G. oxydans* IFO strain 3294 agree with Fukaya et al. (2). We both found two plasmids, but, we differ in the estimated size of these plasmids. They report 7.8 and > 17 kb plasmids, whereas I found 36 and 54 kb plasmids. However, my plasmid size estimations over 16 kb are rough estimations due to the sizes of the molecular weight standards that I used. McKibben (12) does not report plasmids in this strain. Micales (11) did not examine this strain for plasmids.

Fukaya et al. (2) reported only an 8.6 kb plasmid from *G. oxydans* IFO strain 3293. I also found a similar size plasmid (9.5 kb) and an additional 20 kb plasmid. McKibben (11) did not examine this strain for plasmids. Micales (12) did not examine this strain for plasmids.

McKibben (11) tested four of the remaining seven strains of *G. oxydans* that I screened for the presence of plasmids: ATCC strains 621, 9937, 14960 and IFO strain 12528. For ATCC strain 621, McKibben (11) found a 2.7, 16.2, and a 250 kb plasmid. Our results from ATCC strain 621 agree, except that I detected an additional plasmid approximately 26.7 kb in size (Table 1). Although I observed the large plasmid

McKibben (11) observed, I did not estimate its size. Micales (12) and Fukaya et al., (2) did not examine this strain for the presence of plasmids.

For IFO strain 12528, I isolated four plasmids (2.2, 2.9, 14.9 and 26.7 kb). McKibben (11), however, observed only two plasmids (2.7 and 16.2 kb). Micales (12) and Fukaya et al., (2) did not examine this strain for the presence of plasmids.

For ATCC strain 9937, McKibben (11) found five plasmids (6.4, 13.9, 20, 130, and 210 kb). My results agreed with hers, except that I detected a sixth plasmid ~ 27.6 kb. Micales (12) and Fukaya et al., (2) did not examine this strain for the presence of plasmids.

Our results differ considerably for ATCC strain 14960. I found three plasmids in this strain, (13.4, 26.6, and 45 kb) and McKibben (11) found only a 16 kb plasmid. Micales (12) and Fukaya et al., (2) did not examine this strain for the presence of plasmids.

Plasmids in strains of *Gluconobacter frateurii*. My results from IFO strain 3264 (Table 2) agree with those of McKibben (11), in that we both detected one plasmid. I estimated this plasmid to be 33 kb, and McKibben (11) estimated this plasmid to be 42 kb. These differences in size may be due to fact that my estimations were rough for this plasmid since it is considerably larger than the molecular weight standards I used in these studies. Neither Micales (12) nor Fukaya et al. (2) tested this strain for the presence of plasmids.

Both Fukaya et al. (2) and I found that *G. frateurii* IFO strain 3254 lacks plasmids. McKibben (11) reported a 65 kb plasmid from this strain. Micales (12) did not examine this strain for the presence of plasmids.

I detected four plasmids in IFO strain 3271 (5.0, 9.9, 13.5, and 22 kb) however, I did not consistently resolve the three larger plasmids (9.9, 13.5, and 22 kb) in all of my

agarose gels. Fukaya et al. (2) detected five plasmids (3.5, 6.3, 7.5, 14, and >17 kb) and McKibben (11) detected three plasmids (5.3, 13.5, and 100 kb). The 5.0 kb plasmid detected in my investigation and the 5.3 kb plasmid found by McKibben (12) may be the same as the 6.3 kb plasmid reported by Fukaya et al. (2). Micales (12) did not examine this strain for the presence of plasmids.

Plasmids in strains of *Gluconobacter asaii*. Unfortunately, there are only three known species of *G. asaii* based on DNA-DNA homology studies (13). I detected plasmids in all three strains (Table 3). However, McKibben (11) did not detect plasmids in IFO strain 3297a. I observed four plasmids in IFO strain 3297a ranging in size from 2.0 to 35 kb. McKibben (11) and I agree that ATCC strain 43781 contains three plasmids ranging in size from 3.9 to 42 kb. Our plasmid size estimations of these three plasmids were similar.

For IFO strain 3276a, I found a 19.4 and a 34 kb plasmid. However, McKibben (11) reported only one plasmid (20 kb). Niether Micales (12) nor Fukaya et al. (2) tested any *G. asaii* strains for the presence of plasmids.

Similarities of plasmids within and between species of *Gluconobacter*. Strains of *G. oxydans* contained plasmids very similar in size (Table 1). For example, *G. oxydans* strains ATCC 621, ATCC 23652, ATCC 23771, and IFO 12528 all contained a plasmid that was approximately 2.9 kb. In addition, strains ATCC 621, ATCC 23652, ATCC 23771, CSIRO B1507, and IFO 12528 each contained a 15 kb plasmid. Strains ATCC 621, ATCC 9937, ATCC 14960, ATCC 19357, ATCC 23652, ATCC 23771, and IFO 12528 all contained a plasmid that was between 24 and 28 kb. Strains ATCC 9937, ATCC 19357, CSIRO B1507, and IFO 12467 all contained a plasmid that is between 5 and 6 kb. This was noted within strains of one species and

between species of *Gluconobacter*. These results suggest that these plasmids may have been transferred between *Gluconobacter* strains.

Three strains of *G.oxydans* (ATCC strains 621 and 23652 and IFO strain 12528) contained very similar plasmid profiles. All three strains contained two plasmids that were approximately 2.9 and 15 kb in size. Two of these three strains (ATCC strain 621 and IFO strain 12528) contained a plasmid that was approximately 25 kb. These similarities are consistent with the suggestion that plasmids have been transferred between strains of the same species of *Gluconobacter*.

Strains of *G. frateurii* also contained plasmids of similar size. For example, IFO strains 3271 and 3272 each contained plasmids that are approximately 13 and 23 kb in size.

There also appeared to be similar sized plasmids in some strains of *G. oxydans* and *G. frateurii*. For example, the 22 kb plasmid found in *G. frateurii* IFO strain 3271 and the 24 kb plasmid found in IFO strain 3272 were similar in size to the 24.6 kb plasmid from *G. oxydans* ATCC strain 621, the 21.3 kb plasmid from *G. oxydans* ATCC strain 19357, the 24 kb plasmid from *G. oxydans* ATCC 23552, and the 20 kb plasmid from *G. oxydans* strain IFO 3293 (Tables 1 and 2). The 13.5 kb plasmid from *G. frateurii* IFO strain 3271 and the 12.8 kb plasmid from *G. frateurii* IFO strain 3272 were similar in size to the 12.7 kb plasmid from *G. oxydans* ATCC strain 9937, the 13.4 kb plasmid from *G. oxydans* ATCC strain 14960, and the 12.3 kb plasmid from *G. oxydans* IFO strain 12467 (Tables 1 and 2).

Strains of *G. asaii* also contained plasmids of similar size. For example, all three strains contained a plasmid of approximately 35 kb. Some plasmids isolated from strains of *G. asaii* were also similar in size to plasmids isolated from the other two species of *Gluconobacter* (Tables 1 - 3). For example, the 3.9 kb plasmid from *G. asaii*

ATCC strain 43781 and the 3.0 and 3.3 kb plasmids from *G. asaii* IFO strain 3297a were similar in size to the 3.8 kb plasmid from *G. frateurii* IFO strain 3268, the 2.9 kb plasmid from *G. oxydans* ATCC strain 621, the 3.1 and 4.2 kb plasmids from *G. oxydans* ATCC strain 19357, the 2.8 kb plasmid from *G. oxydans* ATCC strain 23652, the 2.9 kb plasmid from *G. oxydans* ATCC strain 23771, the 3.5 and 4.2 kb plasmids from *G. oxydans* CSIRO strain B1507, and the 2.9 kb plasmid from *G. oxydans* IFO strain 12528.

Some plasmids appeared more often than others within strains of *Gluconobacter*. For example, a plasmid approximately 2-3 kb in size was observed in six strains of *G.oxydans*, and in one strain each of *G. frateurii* and *G. asaii*.

Inconsistently appearing plasmids. The discrepancies between my study and the three previous investigations on plasmid number and size (2, 11, 12) may be due to differences in plasmid isolation techniques, visualization methods or estimation of the size of plasmids. These discrepancies further emphasize the need for electron microscopy as the most accurate method for determining plasmid size (see pages 6-7, Ref. 6). In addition, electron microscopy could reveal plasmids that were undetected using other methods.

My results (Tables 1 - 3), especially with strains of *G. oxydans*, revealed several plasmids that were not consistently resolved in all gels. Reasons for this are unclear, although the presence or absence of a particular plasmid appeared to be related to different plasmid extractions rather than differences between separations of a single extraction. For example, a plasmid that appeared in a gel from one plasmid extraction also appeared in replicate gels containing samples from the same plasmid extraction, whereas some plasmids were seen in one plasmid extraction but not in other extractions from the same strain. It may be that these so-called "inconsistent plasmids"

are present in low copy number and were not always isolated in high enough concentration to appear in all gels. Another possibly related explanation is that some plasmid bands occasionally appeared very light on ethidium bromide-stained agarose gel photographs. For this reason, some bands of plasmid DNA may have been overlooked, because they were too faint or not visible when stained with ethidium bromide. These variations in brightness of plasmid bands may have been due to slight differences in staining the gel with ethidium bromide or in the concentration of plasmid DNA contained in the gel.

I found that four strains of *G. oxydans* (ATCC strain 621, ATCC strain 9937, ATCC strain 19357, and IFO strain 12528) and one strain of *G. asaii* (IFO strain 3276a) contained plasmids that were much larger than the molecular weight standards used in my study (Tables 1 and 3). These plasmids were not always detected. They may have been too large to consistant

ly enter the agarose gels, or they may have been damaged in the extraction procedure because of their large size. Even though I used gentle lysis procedures that were suggested for isolation of large plasmids (17), they may have been damaged during the extraction procedure. These plasmids could also be present in low copy number because of restrictions in the efficiency of DNA replication, and this would further reduce the possibility of their detection.

Plasmid significance in the gluconobacters. The *Gluconobacter* strains used in this study appear genetically complex because, in addition to chromosomal DNA, most strains contain plasmids varying greatly in size. These extrachromosomal elements may contain genetic information relating to some of the important functions of the acetic acid bacteria (14, 15, 20). If genes involved in these important processes are contained on plasmids, then complete characterization of these plasmids may help us

better understand the physiology of the gluconobacters. In addition, these plasmids might be used to develop additional shuttle vectors for use in gene manipulation techniques in the gluconobacters.

My investigation of plasmids within the gluconobacters serves as an important step in the complete characterization of these plasmids and is the first to associate plasmid occurrence among the three species of *Gluconobacter*. The plasmids isolated in this investigation are presently cryptic, although studies are being performed to begin to associate these plasmids to phenotypic characteristics within the gluconobacters.

REFERENCES

1. **Barbour, A. G.** 1990. Antigenic variation of a relapsing fever *Borrelia* species, p. 155-171. *In* L. N. Ornston, A. Balows, and E. P. Greenberg (eds.), Annual review of microbiology, Volume 44, Annual Reviews Inc., Palo Alto, California.
2. **De Ley, J. and Swings, J.** 1984. Genus II. *Gluconobacter* Asai 1935, 689, emend. mut. char. Asai, Iizuka and Komagata 1964, 100, p. 275-278. *In* N.R. Krieg (ed.), Bergey's manual of systematic bacteriology, Volume 1, Williams and Wilkins, Baltimore.
3. **Fukaya, M. , T. Iwata, E. Entani, H. Masai, T. Uozumi, and T. Beppu.** 1985. Distribution and characterization of plasmids in acetic acid bacteria. Agric. Biol. Chem. (Tokyo) **49:** 1349-1355.
4. **Fukaya, M. , K. Tayama, T. Tamaki, H. Tagami, H. Okumura, and T. Beppu.** 1989. Cloning of the membrane-bound aldehyde dehydrogenase gene of *Acetobacter polyoxogenes*. and improvement of acetic acid production by use of the cloned gene. Appl. Environ. Microbiol. **55(1):** 171-176.
5. **Fukaya, M. , H. Okumura, H. Masai, T. Uozumi, and T. Beppu.** 1985. Development of a host-vector system for *Gluconobacter suboxydans*. Agric. Biol. Chem. (Tokyo) **49:** 2407-2411.
6. **Fukaya, M. , H. Okumura, H. Masai, T. Uozumi, and T. Beppu.** 1985. Construction of new shuttle vectors for *Acetobacter*. Agric. Biol. Chem. (Tokyo) **49:** 2083-2090.
7. **Hardy, K.** 1981. Bacterial plasmids, Thomas Nelson and Sons, Ltd., Ontario, Canada.

8. **Jones, D..** 1983. Impact of plasmids and transposons on microbial systematics, p. 119-124. *In* D. Schlessinger (ed.), Microbiology - 1983, American Soc. of Microbiology, Washington, DC

9. **Kinashi, H. , M. Shimaji, and A. Sakai.** 1987. Giant linear plasmids in *Streptomyces*. which code for antibiotic biosynthesis genes. Nature (London) **328:** 454-456.

10. **Kinashi, H. , and M. Shimaji-Murayama.** 1991. Physical characterization of SCP1, a giant linear plasmid from *Streptomyces coelicolor*. J. Bacteriol. **173:** 1523-1529.

11. **Mason, L. M., and G. W. Claus.** 1989. Phenotypic characteristics correlated with DNA-sequence similarities for three species of *Gluconobacter: G. oxydans* (Henneberg, 1897) DeLey 1961, *G. frateruii* sp. nov., and *G. asai* sp. nov.. Int. J. Syst. Bacteriol. **39:** 174-184.

12. **McKibben, L A.** 1992. Characterization of plasmids in *Gluconobacter*. M.S. thesis. Virginia Tech, Blacksburg.

13. **Micales, B K.** 1983. DNA/DNA homology studies on the genus *Gluconobacter*. M.S. thesis. Virginia Tech, Blacksburg.

14. **Micales, B. K., J. L. Johnson, and G. W. Claus.** 1985. Deoxyribonucleic acid homologies among organisms in the genus *Gluconobacter*. Int. J. Syst. Bacteriol. **35:** 79-85.

15. **Okumura, H. , T. Uozumi, and T. Beppu.** 1985. Construction of plasmid vectors and a genetic transformation system for *Acetobacter aceti*. Agric. Biol. Chem. (Tokyo) **49:** 1011-1017.

16. **Qazi, G. N., V. Verma, R. Parshad, and C. L. Chopra.** 1989. Plasmid-mediated direct-glucose oxidation in *Gluconobacter oxydans*. J. Biotechnology **10:** 85-88.

17. **Rainbow, C. .** 1966. Nutrition and Metabolism of Acetic Acid Bacteria. Wallerstein Lab. Commun. **29:** 5-15.

18. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: A laboratory manual (Book 1), 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.

19. **Stanisich, V.A.** 1984. Identification and analysis of plasmids at the genetic level, p. 5-32. *In* P.M. Bennett, and J. Grinsted (ed.), Methods in microbiology, Academic Press, London.

20. **Valla, S. , D. H. Coucheron, and J. Kjosbakken.** 1983. *Acetobacter xylinum.* contains several plasmids: evidence for their involvement in cellulose formation. Arch. Microbiol. **134:** 9-11.

21. **Valla, S. , D. H. Coucheron, and J. Kjosbakken.** 1987. The plasmids of *Acetobacter xylinum* and their interaction with the host chromosome. Mol. Gen. Genet. **208:** 76-83.

APPENDIX 2A

Relationship between distance migrated and the size of DNA molecular weight markers

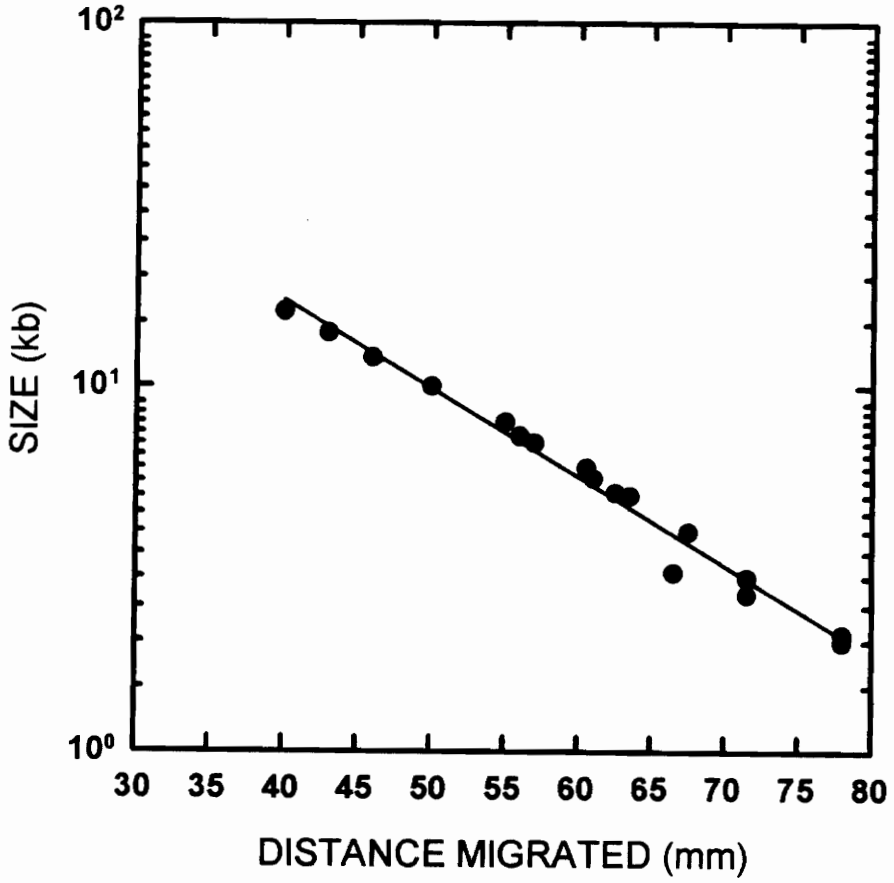


Figure A1. A representative standard curve showing the relationship between distance migrated in a 0.7% agarose gel and the size of various DNA molecular weight standards. Molecular weight standards used were from *E. coli* strain V517: (in kb) 2.1, 2.7, 3.1, 5.1, 5.6, and 7.3 and a supercoiled kb ladder (Bethesda Research Laboratories) (2, 3, 4, 5, 6, 7, 8, 10, 12, 14, and 16 kb). The standard curve was prepared as a result of linear regression analysis using Sigma Plot version 1.02.

APPENDIX 2B

Data used to calculate average plasmid size estimations for strains of *Gluconobacter oxydans* shown in Table 1

Plasmid size estimations in *G. oxydans* ATCC strain 621[a]

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1	2.65, -	15, -	27, -
Cell batch 2:			
Gel 1	2.6, 2.6	14, 14	-, -
Gel 2	2.6	14.5	26
Gel 3	2.6, 2.6	15, 15	26, 26
Cell batch 3:			
Gel 1	2.9, 2.9	13, 14.5	23, 23
Gel 2	2.6, 2.6	15, -	-, -
Gel 3	4.2, 4.2	15.5, 15.5	21, 21
Cell batch 4:			
Gel 1	3.4, 3.4	15, 15	26, 26
Gel 2	2.6, 2.6	15.5, 15.5	23, 23
Cell batch 5:			
Gel 1	2.6, 2.6	15, 15	27, 27
Average:	**2.9**	**14.8**	**24.6**

(continued)

[a] This strain may contain a large plasmid that sometimes is not resolved in these gels. Higher molecular weight standards than those used must be run on these gels to estimate the size of this large plasmid.

Plasmid size estimations in *G. oxydans* ATCC strain 9937[a]

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:				
Gel 1	5.6, 5.6	14, 14	17.5, 17.5	-, 30
Gel 2	6.1, 6.1	11.5, -	17, 17	26, 26
Gel 3	5.7, 5.7	-, -	19.5, 19.5	29, 29
Cell batch 2:				
Gel 1	6.1	12.5	16.5	29
Cell batch 3:				
Gel 1	6.1, 6.1	12, 12	17, 17	26, 26
Average:	**5.9**	**12.7**	**17.6**	**27.6**

[a] This strain may contain large plasmids that are sometimes not resolved in these gels. Higher molecular weight standards than those used must be run on these gels to estimate the size of these larger plasmids.

Plasmid size estimations in *G. oxydans* ATCC 14960

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:			
Gel 1	13.5, 13.5	29, 29	-, -
Gel 2	13.5, 13.5	28, 28	45, 45
Gel 3	-, -	26, 26	-, -
Cell batch 2:			
Gel 1	13	20	-
Average:	**13.4**	**26.6**	**45**

Plasmid size estimations in *G. oxydans* ATCC strain 19357[a]

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1: Gel 1	3.2, 3.2	4.3, 4.3	5.1, 5.1	-, -	15.5, 15.5	20, 20	27, 27
Gel 2	3.2, 3.2	4.3, 4.3	5.0, 5.0	-, -	15.5, -	22, -	-, -
Gel 3	2.8, 2.8	-, -	-, -	-, -	15.5, 15	-, -	-, -
Cell batch 2: Gel 1	-, -	4.1, 4.1	5.1, 5.1	7.8, 7.8	16, 16	22, 22	33, 33
Gel 2	3.1, 3.1	4.1, 4.1	5.0, 5.0	7.8, 7.8	16.5, 17	-, -	26, 26
Cell batch 3: Gel 1	3.6	4.4	5.1	-	17	22	-
Average	**3.1**	**4.2**	**5.1**	**7.8**	**16**	**21.3**	**28.3**

[a] This strain contains two large plasmids that are consistently resolved in these gels. However, higher molecular weight standards than those used must be run on these gels to estimate the size of these large plasmids.

Plasmid size estimations in *G. oxydans* ATCC strain 23652

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1: Gel 1	2.8, 2.8	14.5, 14.5	24, 24
Cell batch 2: Gel 1	2.7	-	-
Average:	**2.8**	**14.5**	**24**

Plasmid size estimations in *G. oxydans* ATCC strain 23771

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:			
Gel 1	2.9, 2.9	14.5, 14.5	28, 28
Gel 2	-, -	-, -	25, 25
Average:	**2.9**	**14.5**	**26.5**

Plasmid size estimations in *G. oxydans* CSIRO strain B1507

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1: Gel 1	3.5	4.3	6.2	7.6	14.5	18.5
Cell batch 2: Gel 2	3.5, 3.5	4.2, 4.2	6.1, 6.1	7.7, 7.7	-, -	-, -
Average	**3.5**	**4.2**	**6.1**	**7.7**	**14.5**	**18.5**

Plasmid size estimations in *G. oxydans* IFO strain 3293

	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:		
Gel 1	9.2, 9.2	20.5, 20.5
Gel 2	9.9, 9.9	19.5, 19.5
Cell batch 2:		
Gel 1	9.6	-
Gel 2	9.3	-
Average:	**9.5**	**20**

Plasmid size estimations in *G. oxydans* strain IFO 3294

	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:		
Gel 1	36, 36	-, -
Gel 2	-, -	48, -
Cell batch 2:		
Gel 1	-	59
Average:	**36**	**54**[a]

[a] This plasmid is a relatively large plasmid whose size is not accurately determined using these molecular weight standards. Higher molecular weight standards than those used must be run on these gels to estimate the size of this large plasmid. In addition, the 36 kb plasmid may in fact be the same plasmid as the 53 kb plasmid since it was resolved on a gel where the 53 kb plasmid was not.

Plasmid size estimations in *G. oxydans* IFO strain 12467

	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:		
Gel 1	6.0, 6.0	12, 12
Gel 2	6.1, 6.1	12.5, 12.5
Cell batch 2:		
Gel 1	6.1	-
Average:	**6.1**	**12.3**

Plasmid size estimations in *G. oxydans* IFO strain 12528[a]

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:				
Gel 1	1.95, 1.95	2.8, 2.8	15, 15	27, 27
Gel 2	2.0	2.8	14.5	24
Gel 3	2.4, 2.4	3.9, -	15.5, 15.5	28, 28
Gel 4	-, -	2.7, 2.7	14.5, -	26, 26
Cell batch 2:				
Gel 1	-	2.6	-	29
Cell batch 3:				
Gel 1	2.2	2.85	14.5	-
Gel 2	-	2.8	14.5	25.5
Average:	**2.2**	**2.9**	**14.9**	**26.7**

[a] This strain may contain a large plasmid that sometimes is not resolved in these gels. Higher molecular weight standards than those used must be run on these gels to estimate the size of this large plasmid.

APPENDIX 2C

Data used to calculate average plasmid size estimations for strains of *Gluconobacter frateurii* shown in Table 2

Plasmid size estimations in *G. frateurii* IFO strain 3264

	Plasmid size (kb)
Cell batch 1: Gel 1	33, 33
Cell batch 2: Gel 1	-
Average:	**33**

Plasmid size estimations in *G. frateurii* IFO strain 3268

	Plasmid size (kb)
Cell batch 1:	
Gel 1	3.8, 3.8
Gel 2	3.7, 3.7
Cell batch 2:	
Gel 1	-
Gel 2	-, -
Average:	**3.8**

Plasmid size estimations in *G. frateurii* IFO strain 3271

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:				
Gel 1	3.3, 3.3	-, -	-, -	-, -
Cell batch 2:				
Gel 1	5.2, 5.2	-, -	-, -	-, -
Cell batch 3:				
Gel 1	4.9, 4.9	10, 10	14, 14	22, 22
Gel 2	5.2, 5.2	9.8, 9.8	12.5, -	22, -
Cell batch 4:				
Gel 1	5.4, 5.4	-, -	-. -	-, -
Gel 2	5.1, 5.1	-, -	-, -	-, -
Cell batch 5:				
Gel 1	5.1	-	-	-
Cell batch 6:				
Gel 1	5.2, 5.2	-, -	-, -	-, -
Cell batch 7:				
Gel 1	5.8, 5.8	-. -	-, -	-, -
Average:	**5.0**	**9.9**	**13.5**	**22**

Plasmid size estimations in *G. frateurii* IFO strain 3272

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:			
Gel 1	-, -	13, 13	-, -
Gel 2	2.05, 2.05	12.5, 12.5	24, -
Cell batch 2:			
Gel 1	-	13	-
Average:	**2.1**	**12.8**	**24**

APPENDIX 2D

Data used to calculated average plasmid size estimations for strains of *Gluconobacter asaii* shown in Table 3

Plasmid size estimations in *G. asaii* ATCC strain 43781

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:			
Gel 1	4.0, 4.0	-, -	37, 37
Gel 2	3.8	7.0	-
Gel 3	3.9, 3.9	7.0, 7.0	-, -
Cell batch 2:			
Gel 1	3.8	6.8	-
Gel 2	3.9, 3.9	6.7, 6.7	-, -
Cell batch 3:			
Gel 1	3.7	6.7	-
Average:	**3.9**	**6.8**	**37**

Plasmid size estimations in *G. asaii* IFO strain 3276a[a]

	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:		
Gel 1	20, 20	35, 35
Gel 2	18.5, 18.5	38, -
Cell batch 2:		
Gel 1	20	27
Average:	**19.4**	**33.8**

[a] This strain may contain a large plasmid that sometimes is not resolved in these gels. Higher molecular weight standards than those used must be run on these gels to estimate the size of this large plasmid.

Plasmid size estimations in *G. asaii* IFO 3297a

	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)	Plasmid size (kb)
Cell batch 1:				
Gel 1	2.1, 2.1	3.2, 3.2	3.4, 3.4	35, 35
Gel 2	2.0	3.0	-	33
Gel 3	1.9, 1.9	2.8, 2.8	3.1, 3.1	36, -
Cell batch 2:				
Gel 1	2.1	3.1	-	36
Average:	**2.0**	**3.0**	**3.3**	**35**

Chapter 3

Attempts to Correlate Phenotypic Characteristics with the Presence of Plasmids from Strains of *Gluconobacter*

SUMMARY

The genus *Gluconobacter* consists of Gram-negative acetic acid bacteria that are isolated from acidic or sugary environments. The gluconobacters do not completely oxidize their substrates to carbon dioxide because they lack a complete tricarboxylic acid cycle. They instead carry out limited oxidations of their substrates, and they are reported to oxidize more than 100 different organic compounds. In addition, the gluconobacters are reportedly resistant to many different antimicrobial agents. Many strains of *Gluconobacter* also contain plasmids. It has been suggested that these plasmids may be responsible for several phenotypic characteristics of the gluconobacters. I wanted to determine whether relationships existed between the presence of plasmids in the gluconobacters and antibiotic resistance or oxidative capability. Strains of *Gluconobacter* were tested for their susceptibility to many different antimicrobial agents using the disc diffusion assay. The minimum inhibitory concentration of antimicrobial agents was determined for two strains using a microdilution method. An attempt to correlate the susceptibility to antimicrobial agents and/or the ability to oxidize various chemical compounds with the presence of plasmids was determined. Most strains that were tested showed similar patterns regarding their susceptibility to antimicrobial agents. This suggests that susceptibility to antimicrobial agents varies little with different strains of *Gluconobacter*. I found no correlation of antimicrobial agent resistance and the presence of plasmids with the strains tested. I

did however, find a possible relationship between plasmids and the ability to oxidize butanol. However, further study of this relationship was precluded by the lack of a genetic marker on any of the plasmids.

INTRODUCTION

Antimicrobial agents are synthetic or natural, chemical compounds that kill or inhibit the growth of microorganisms (2) They are a broad group of chemical compounds that may be divided into antiseptics, disinfectants, and chemotherapeutic agents. Among the chemotherapeutic agents are antibiotics which are natural products of microorganisms that kill or inhibit the growth of other procaryotic or eucaryotic microorganisms in very low concentrations (see page 354, Ref. 2 and pages 2-3, Ref. 10). Antibiotics are grouped into different classes based upon their mechanism of action. For example, beta-lactam antibiotics, such as penicillin, inhibit bacterial cell wall synthesis and aminoglycosides, such as streptomycin, inhibit protein synthesis (see pages 4-5 and 6-8, Ref. 11).

Some microorganisms are resistant to various antibiotics, and these mechanisms of antibiotic resistance vary among microorganisms and antibiotics. A microorganism may be resistant to an antibiotic, because the microorganism lacks a structure that is the target of the antibiotic. Other mechanisms of resistance to antibiotics include lack of permeability of the antibiotic, microbial alteration of the antibiotic, modification of the target site of the antibiotic, alteration of the metabolic pathway affected by the antibiotic, and efflux of the antibiotic from the microbial cell (see page 355, Ref. 2 and page 16, Ref. 3).

One way of measuring antimicrobial activity is to determine the minimum inhibitory concentration (MIC). This method determines the smallest amount of an

antimicrobial agent necessary to inhibit microbial growth (see page 349, Ref. 2 and page 12, Ref. 10). The MIC of an antimicrobial agent can be determined using test tubes or microtiter plates.

Another way of determining antimicrobial activity is the agar diffusion method where small filter paper discs are impregnated with an antimicrobial agent and placed on the surface of an agar plate seeded with a microorganism. The antimicrobial agent diffuses out into the surrounding agar, and a zone of growth-inhibition appears surrounding the disc. This zone occurs where the concentration of the antimicrobial agent exceeds the MIC for that microorganism (see pages 12-13, Ref. 10). Use of this method provides general information, as opposed to quantitative MIC values, relating to the action of an antimicrobial agent on a particular organism (see pages 349-350, Ref. 2).

Plasmids may be associated with resistance to antimicrobial activity (26). For example, the presence of four plasmids in one strain of *Salmonella typhimurium* accounts for resistance to tetracycline, erythromycin, clindamycin, sulfisoxazole, sulfadiazine, triple sulfa, cefoperazone, streptomycin, mezlocillin, piperacillin, carbenicillin, penicillin, ampicillin, and kanamycin (24). Chloramphenicol resistance in other bacteria is often conferred by a chloramphenicol acetyltransferase gene located on so-called R (drug resistance) plasmids (8, 20, 25).

Plasmids may also be associated with certain metabolic activities, such as utilization of simple carbohydrates (such as lactose and raffinose), metabolism of unusual and/or complex carbon sources (such as *n*-alkanes and nicotine), and nitrogen fixation (26).

The genus *Gluconobacter* consists of Gram-negative acetic acid bacteria that are isolated from flowers, honey bees, fruits, cider, vinegar, wine and beer (4, 13, 23).

The gluconobacters are reportedly resistant to many different antimicrobial agents, such as chloramphenicol, erythromycin, nalidixic acid, nitrofurantoin, and penicillin (4). However, there is no information listing which strains of *Gluconobacter* were tested to obtain these results and whether different species of *Gluconobacter* exhibit different susceptibilities to these antimicrobial agents.

The gluconobacters have a strictly respiratory metabolism and use only oxygen as a terminal electron acceptor. They do not completely oxidize their carbon and energy sources to carbon dioxide (1), because they lack a complete tricarboxylic acid cycle (9). Instead, the gluconobacters only partially oxidize these substrates and efficiently excrete their oxidation products into the surrounding medium converting more than 90% of their substrate to product (6, 21). Strains of *Gluconobacter* reportedly oxidize more than 100 substrates (5).

Strains of *Gluconobacter* and *Acetobacter*, a closely related genus, harbor plasmids (7, 15, 16, 27). Some of these plasmids may be involved in important industrial processes involving these bacteria. For example, Okumura et al. (19) suggests that some are responsible for loss of ethanol oxidation ability and resistance to acetic acid in strains of *Acetobacter aceti*. Also, Valla et al. (27) show the possible involvement of plasmids in cellulose biosynthesis in *Acetobacter xylinum*. Qazi et al. (22) show some data that suggests that plasmids may be involved in glucose oxidation and resistance to ampicillin and chloramphenicol in *G. oxydans* ATCC strain 9937.

There are three purposes to my investigation shown in Chapter 3. First, to determine whether specific strains from each *Gluconobacter* species are resistant or susceptible to various antimicrobial agents. I know of no other published information on this subject. Second, to determine whether the resistance or susceptibility to an antimicrobial agent correlates with the presence or absence of a plasmid (see Chapter

2). Third, to compare the ability or inability to oxidize certain substrates (see Chapter 1) with the presence or absence of certain plasmids. The overall goal of this study was to find a phenotypic marker for one or more *Gluconobacter* plasmids.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study were the same as those used in a previous DNA/DNA homology study (17) and tests of related phenotypic characteristics (14). *Gluconobacter oxydans* ATCC strains 621, 9937, and 19357 were received from the American Type Culture Collection. *Gluconobacter oxydans* IFO strain 3244 and IFO strains 3254, 3264, and 3271 were received from Noberto Palleroni (Hoffman-LaRoche, Nutley, NJ). *Gluconobacter asaii* IFO strain 3276 was received from the Institute of Fermentation, and *G. asaii* IFO strain 3297 was a gift from Noberto Palleroni. *Gluconobacter asaii* ATCC strain 43781 was originally received as RS strain 203 from K. G. Rohrback (Department of Plant Pathology, University of Hawaii at Manoa, Honolulu) and later deposited at the American Type Culture Collection. All strains were checked for purity, grown in a complex medium, suspended in a medium containing (w/v) 15% glycerol, 1% yeast extract, and 1% peptone then heat-sealed in glass vials and continuously maintained in liquid nitrogen Dewars. Working stock cultures were prepared by growing the strains in broth containing (w/v) 5% glycerol, 1% yeast extract, and 1% peptone until reaching approximately 0.9 OD_{620} and maintained until use by storing in 66% glycerol at -4^{o}C. Cultures used in agar diffusion antibiotic susceptibility testing were first grown in 50 ml of broth containing (w/v) 5% glycerol, 1% yeast extract, and 1% peptone in 500 ml growth flasks until reaching approximately 0.9 OD_{620} and then heavily streaked onto ATCC mannitol agar slants which contained (w/v) 2.5% mannitol, 0.5% yeast extract,

0.3% peptone, and 2% agar and stored temporarily at -4°C. Cultures used in the minimum inhibitory concentration determinations using the microdilution method were grown in 50 ml of broth containing (w/v) 5% glycerol, 1% yeast extract, and 1% peptone in 500 ml growth flasks until reaching approximately 0.9 OD_{620} .

Reagents. Glycerol was purchased from Fisher Scientific, Fair Lawn, NJ. Mannitol and succinic acid were purchased from Sigma Chemical Co., St. Louis, MO. Yeast extract, peptone, and BiTek agar were purchased from Difco Laboratories, Detroit, MI. Cartridges of BBL® Sensi-Discs® containing the following antimicrobial agents were purchased from Becton Dickinson Co., Cockeysville, MD: ampicillin (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), lincomycin (2 μg), nitrofurantoin (300 μg), nitrofurazone (100 μg), novobiocin (30 μg), penicillin (10 μg), streptomycin (10 μg), sulfamethoxazole trimethoprim (23.75 μg, 1.25 μg), tetracycline (30 μg), and trimethoprim (5 μg).

Antibiotic susceptibility tests using the agar diffusion method. Cells were removed from ATCC mannitol agar slants and suspended in 2 ml of sterile distilled water to achieve a very turbid cell suspension. A 0.3 ml sample of each turbid cell suspension was spread-inoculated onto the surface of an ATCC mannitol agar plate. Small filter paper discs, impregnated with various antimicrobial agents (BBL® Sensi-Disc® System), were either dispensed onto the surface of the inoculated ATCC mannitol plates with a BBL® Sensi-Disc® 8-Place Dispenser (Becton Dickinson Co.), and using sterile forceps, Additional BBL® Sensi-Discs® impregnated with different antimicrobial agents were placed on the surface of the inoculated ATCC mannitol plates. All plates were incubated at 28°C for 48 h, except those containing *G. asaii* IFO strain 3297a, which were incubated for 72 h. The plates were then observed for

the presence of zones of inhibition surrounding the BBL® Sensi-Discs®. Strains that demonstrated a zone of inhibition of any size were considered susceptible to that antimicrobial agent. All strains were tested at least twice.

Minimum inhibitory concentrations of antimicrobial agents using the microdilution method. Cultures were harvested by centrifugation in a Beckman Model J2-21 centrifuge at 7500 x g for 10 min. After discarding the supernatant fluid, the cell pellets were resuspended in sterile 0.2 M succinate buffer (pH 6.2) until the turbidity matched that of a # 0.5 McFarland standard. Two milliliters of this turbid cell suspension was used to inoculate 200 ml of broth containing (w/v) 5% glycerol, 1% yeast extract, and 1% peptone. The inoculated broth was then placed into a sterile plastic container, and 200 µl of the inoculated broth was dispensed into each well of a Sceptor® System microtiter panel (Becton Dickinson Co.) using a multitip pipettor. The panels were incubated at 28°C for 48 h. The minimum inhibitory concentrations of the antimicrobial agents contained within the wells of the Sceptor® panels were visually determined by observing the panels for growth (turbidity). In order to test cultures with a wide variety of antimicrobial agents, the following Sceptor® Panel systems were used: Sceptor® System Enteric MIC Panel, Sceptor® System Streptococcus MIC Panel, Sceptor® System Staphylococcus MIC Panel, Sceptor® System Psedomonas MIC Panel, Sceptor® System Gram Positive Breakpoint ID Panel, and the Sceptor® System Gram Negative Breakpoint ID Panel. *G. oxydans* ATCC strain 621 and IFO strain 3244 were tested in duplicate using each of these six panels.

Substrate oxidations. Six strains of *Gluconobacter* were tested for their ability to oxidize 13 substrates using the tetranitroblue tetrazolium (TNBT) reduction assay described in Chapter 1. The criteria used to determine the substrate oxidation by each strain was as follows: (+) indicated oxidation greater than 0.9 mmole of substrate

oxidized per mg cell protein x 10^{-5}; (–) indicated no oxidation; (±) indicated oxidation less than 0.9 mmole of substrate oxidized per mg cell protein x 10^{-5}.

RESULTS AND DISCUSSION

Susceptibility to antimicrobial agents. Nine strains of *Gluconobacter* were tested by the agar diffusion method for their susceptibility to different antimicrobial agents (Tables 1 - 3).

All four strains of *G. oxydans* tested were susceptible to kanamycin, novobiocin, and streptomycin (Table 1). Three of four strains of *G. oxydans* (ATCC strains 621, 9937 and 19357) were susceptible to tetracycline. Only ATCC strain 9937 was susceptible to ampicillin. All four of these *G. oxydans* strains were resistant to the other 11 antibiotics tested (cephalothin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, lincomycin, nitrofurantoin, nitrofurazone, penicillin, sulfamethoxazole-trimethoprim, and trimethoprim).

Table 1. Antimicrobial-agent susceptibility in *G. oxydans* strains as determined by the agar diffusion method

Antimicrobial Agent [c]	Presence (+) or absence of (–) inhibition zone for each strain [a] (Number of plasmids present in each strain)[b]			
	ATCC 621 (4)	ATCC 9937 (5)	ATCC 19357 (9)	IFO 3244 (0)[d]
Ampicillin	–	+	–	–
Cephalothin	–	–	–	–
Chloramphenicol	–	–	–	–
Ciprofloxacin	–	–	–	–
Erythromycin	–	–	–	–
Gentamicin	–	–	–	–
Kanamycin	+	+	+	+
Lincomycin	–	–	–	–
Nitrofurantoin	–	–	–	–
Nitrofurazone	–	–	–	–
Novobiocin	±	+	+	+
Penicillin	–	–	–	–
Streptomycin	+	+	+	+
Sulfamethoxazole - Trimethoprim	–	–	–	–
Tetracycline	±	+	+	–

Table 1. (continued)

Trimethoprim	–	–	–	–

[a] Results shown reflect antibiotic suseptibility testing on at least two replicate plates for each strain. A plus (+) indicates that a zone of inhibition was observed surrounding the antimicrobial-agent impregnated filter paper discs. A minus (–) indicates that no zone of inhibition was observed. A plus/minus (±) indicates that a zone of inhibition was observed but not on all replicate plates.

[b] Plasmid isolations were performed as described in Chapter 2 of this dissertation.

[c] Concentrations of antimicrobial agents contained in the BBL® Sensi-Discs® are listed in the Materials and Methods. Antimicrobial agents shown in shaded box were tested in other *Gluconobacter* species.

[d] No plasmids were detected in this strain.

Table 2. Antimicrobial-agent susceptibility in *G. frateurii* as determined by the agar diffusion method

Antibiotimicrobial Agent [c]	Presence (+) or absence (–) of inhibition zone for each strain[a] (Number of plasmids present in each strain)[b]		
	IFO 3254 (0)[d]	IFO 3264 (1)	IFO 3271 (4)
Ampicillin	+	–	–
Chloramphenicol	–	–	–
Erythromycin	–	–	–
Kanamycin	+	+	+
Nitrofurantoin	–	–	–
Novobiocin	+	+	+
Penicillin	–	–	–
Streptomycin	+	+	+
Tetracycline	+	+	+
Trimethoprim	–	–	–

[a] Results shown reflect antibiotic susceptibility testing on at least two replicate plates for each strain. A plus (+) indicates that a zone of inhibition was observed surrounding the antimicrobial-agent impregnated filter paper discs. A minus (–) indicates that no zone of inhibition was observed. A plus/minus (±) indicates that a zone of inhibition was observed but not on all replicate plates.

[b] Plasmid isolations were performed as described in Chapter 2 of this dissertation.

Table 2. (continued)

[c] Concentrations of antimicrobial agents contained in the BBL® Sensi-Discs® are listed in the Materials and Methods. Antimicrobial agents shown in shaded box were tested in other *Gluconobacter* species.

[d] No plasmids were detected in this strain.

Table 3. Antimicrobial-agent susceptibility in *G. asaii* strains as determined by the agar diffusion method

Antibimicrobial Agent [c]	Presence (+) or absence (–) of inhibition zone for each strain[a] (Number of plasmids present in each strain)[b]		
	CSIRO RS203b (3)	IFO 3276a (3)	IFO 3297a (4)
Ampicillin	–	±	±
Chloramphenicol	–	+	–
Erythromycin	–	–	–
Kanamycin	+	+	±
Nitrofurantoin	–	–	–
Novobiocin	±	±	±
Penicillin	–	–	–
Streptomycin	±	+	+
Tetracycline	±	+	–
Trimethoprim	–	–	–

[a] Results shown reflect antibiotic susceptibility testing on at least two replicate plates for each strain. A plus (+) indicates that a zone of inhibition was observed surrounding the antimicrobial-agent impregnated filter paper discs. A minus (–) indicates that no zone of inhibition was observed. A plus/minus (±) indicates that a zone of inhibition was observed but not on all replicate plates.

[b] Plasmid isolations were performed as described in Chapter 2 of this dissertation.

Table 3. (continued)

[c] Concentrations of antimicrobial agents contained in the BBL® Sensi-Discs® are listed in the Materials and Methods. Antimicrobial agents shown in shaded box were tested in other *Gluconobacter* species.

All three strains of *G. frateurii* tested were susceptible to kanamycin, novobiocin, streptomycin, and tetracycline (Table 2). One strain, (IFO strain 3254) was susceptible to ampicillin. All three of these strains were resistant to chloramphenicol, erythromycin, nitrofurantoin, penicillin, and trimethoprim.

All three strains of *G. asaii* tested were susceptible to kanamycin, novobiocin, and streptomycin (Table 3). In addition: IFO strains 3276a and 3297a were susceptible to ampicillin; ATCC strain 43781 and IFO strain 3276a were susceptible to tetracycline; and IFO strain 3276a was susceptible to chloramphenicol. All three of these strains were resistant to erythromycin, nitrofurantoin, penicillin, and trimethoprim.

All nine strains (representing the three species of *Gluconobacter)* were susceptible to kanamycin, novobiocin, and streptomycin. All *Gluconobacter* strains were resistant to erythromycin, nitrofurantoin, penicillin, and trimethoprim. Of the 10 antibiotics commonly tested on all three *Gluconobacter* species, only three yielded different susceptibility results (ampicillin, chloramphenicol, and tetracycline).

My use of the disc diffusion method showed that most strains are identical in their antibiotic susceptibility (Tables 1 - 3). For this reason, antimicrobial agent susceptibility cannot be used to distinguish strains of *Gluconobacter.* I found only three exceptions to this generalization. First, four of the nine strains were susceptible to ampicillin, but at least one strain of each species was susceptible to this antibiotic. Thus, ampicillin susceptibility must not be limited to one species of *Gluconobacter.* Second, all but two strains were susceptible to tetracycline. These two strains belong to two species, therefore, tetracycline susceptibility must not be limited to one *Gluconobacter* species. Third, all strains except *G. asaii* IFO strain 3276a were resistant to chloramphenicol, thus, chloramphenicol resistance is not species specific.

Table 4 shows the results of antimicrobial-agent susceptibility testing using Sceptor®Panel analysis for *G. oxydans* ATCC strain 621 and IFO strain 3244. Both strains were able to grow in the presence of most of these antimicrobial agents at the lowest concentrations. Both strains were susceptible (no growth at any concentration) to amikacin and ceftrizoxime. Only ATCC strain 621 was susceptible to ceftazidime and pipercillin. These microdilution panels can be used to determine the minimum inhibitory concentration (MIC) for each of these antimicrobial agents. These values are reported in Appendix 3A, Table A1.

Different patterns of antibiotic susceptibility are often used to distinguish different strains from one another in various genera of bacteria, especially pathogens (24, 25). However, the use of antimicrobial susceptibility to distinguish strains of *Gluconobacter* is very limited.

The gluconobacters showed resistance to many different antimicrobial agents (Tables 1 - 4). Based on the sugary and acidic environments where the gluconobacters are isolated, it follows that these acetic acid bacteria would be resistant to many antimicrobial agents. For example, resistance to these compounds would give the gluconobacters a distinct advantage over other bacteria and especially fungi growing in these environments. Resistance to many of these antimicrobial compounds may have appeared early in the evolution of the gluconobacters and, therefore, has become a property related to the genus as a whole.

Comparison between resistance to antimicrobial agents and plasmid presence. The number of plasmids found in each *Gluconobacter* strain (Chapter 2) is shown in parentheses below the strain number in data columns of Tables 1 - 4. When this information was compared to their susceptibility to various antimicrobial agents, the

Table 4. Antimicrobial-agent susceptibility in *G. oxydans* ATCC strain 621 and IFO strain 3244 using Sceptor® panel analysis

Antimicrobial agent[b] (lowest concentration in μg/ml)	Presence (+) or absence (–) of growth at lowest concentration [a]	
	ATCC 621 (4 plasmids)[c]	IFO 3244 (0)[d]
Amikacin (2)	–	–
Amoxicillin ().12)	+	+
Ampicillin (0.025)	+	+
Aztreonam (2)	+	+
Cefazolin (1)	+	+
Cefoperazone (8)	+	+
Cefotaxime (1)	+	+
Cefotetan (1)	+	+
Ceftaxime (8)	+	+
Ceftazidime (4)	+	+
Ceftriaxone (2)	+	+
Ceftrizoxime (8)	–	–
Cefuroxime (4)	+	+
Cephalothin (1)	+	+
Chloramphenicol (8)	+	+
Ciprofloxacin (0.25)	+	+
Clavulonic acid (0.06)	+	+
Clindamycin (0.12)	+	+
Erythromycin (0.12)	+	+
Gentamicin (0.25)	+	+
Imipemem (0.5)	+	+
Mezlocillin (4)	+	+
Nitrofurantoin (32)	+	+
Norfloxacin (2)	+	+
Oxacillin (0.12)	+	+
Penicillin (0.12)	+	+
Pipercillin (4)	–	+
Rifampin (1)	+	+
Sulbactam (0.13)	+	+
Sulfamethoxazole (9.5)	+	+
Tetracycline (4)	+	+
Ticarcillin (2)	+	+
Tobramycin (0.25)	+	+

Table 4. (continued)

Trimethoprim (0.5)	+	+
Vancomycin (0.5)	+	+

[a] Strains that grew in the microtiter-plate well containing the lowest concentration of each antimicrobial agent were interpreted as resistant. Strains that did not grow in the well with the lowest concentration of the antimicrobial agent were interpreted as susceptible. Each antimicrobial agent was tested at least twice for each strain.

[b] Antimicrobial agents shown in shaded box were tested in other *Gluconobacter* strains using the disc diffusion method (Tables 1 - 3).

[c] Plasmid isolations were performed as described in Chapter 2 of this dissertation.

[d] No plasmids were detected in this strain.

gluconobacters appeared to exhibit the same susceptibility to antimicrobial agents whether or not plasmids were detected in these strains. For example, *G. oxydans* IFO strain 3244 was resistant to tetracycline, but it does not contain plasmids. However, all the strains that were susceptible to tetracycline, except *G. frateurii* IFO strain 3254, contained at least one plasmid. All strains of *Gluconobacter* were resistant to erythromycin, nitrofurantoin, penicillin, and trimethoprim regardless of whether they contained plasmids. Similarly, all strains were susceptible to kanamycin, novobiocin, and streptomycin even though they contained plasmids.

Antimicrobial resistance in the gluconobacters is not associated with the presence of antibiotic resistance plasmids often found in bacteria (12, 18, 24, 26). My results suggest that antibiotic resistance mechanisms within the gluconobacters is probably associated with chromosomal DNA and not plasmid DNA. These results further emphasize the possibility that mechanisms for antibiotic resistance may have appeared early in the evolution of the genus *Gluconobacter* and therefore, is associated with the more stable chromosomal DNA as opposed to plasmid DNA. In addition, some mechanisms of antibiotic resistance in bacteria are not directly associated with the presence of a particular gene encoding an enzyme involved in resistance. For example, if a bacterial cell lacks a structure that is the target of an antibiotic, the absence of this structure may not have simply evolved for the sake of resistance to an antimicrobial compound. However, resistance to an antimicrobial compound does give the cell an advantage. Also, an alteration in one of a bacterial cell's metabolic pathways may result in the inhibition of the antimicrobial agent. This change in a metabolic pathway probably did not evolve as a mechanism for antibiotic resistance, but for some metabolic advantage. My findings suggest that although the gluconobacters are resistant to a number of antimicrobial compounds, their mechanism

(or mechanisms) of antibiotic resistance cannot be easily associated with the presence of plasmids within these bacteria.

Comparison between oxidative capability and plasmid presence. Most strains of *Gluconobacter* were able to oxidize most of the 13 substrates tested, at least to a small extent, regardless of whether they contained plasmids (Table 5). However, I observed one possible correlation between oxidation capability and plasmid presence. *Gluconobacter oxydans* ATCC strain 621 oxidized butanol and this strain contains four plasmids, whereas *G. oxydans* IFO strain 3244 did not oxidize butanol, and it appears to lack plasmids. This finding suggests that butanol oxidation may be plasmid related. To better characterize butanol oxidation in these two strains of *G. oxydans*, I determined that I must further investigate the possible involvement of plasmids.

In 1989, Qazi et al. (22) report that the loss of membrane-bound NADP-independent glucose dehydrogenase activity and the loss of ampicillin and chloramphenicol resistance is associated with the curing of plasmids in *G. oxydans* ATCC strain 9937. However, these authors did not insert the plasmids back into the cured strain and show a recovery of glucose dehydrogenase activity, ampicillin or chloramphenicol resistance. Such a recovery in glucose dehydrogenase activity, ampicillin or chloramphenicol resistance would more strongly support the idea of these phenotypic characteristics being plasmid-encoded.

Table 5. Substrate oxidation and the presence of plasmids in selected strains of *Gluconobacter*

Strain	Number of plasmids	Substrate oxidized [a]												
		Bd	Gl	Mt	Mn	Ri	Bu	Cy	Fr	Na	Ra	Et	Gu	So
G. oxydans														
ATCC 621	4	+	+	+	+	±	+	+	+	±	±	+	+	+
IFO 3244	0	+	+	+	+	+	–	+	+	+	±	+	+	+
G. frateurii														
IFO 3271	4	+	+	+	+	+	+	+	+	+	±	+	+	+
IFO 3254	0	+	+	+	+	+	+	±	+	+	+	+	+	+
G. asaii														
ATCC 43781	3	+	+	+	+	+	+	+	+	+	±	+	+	+
IFO 3297a	4	–	±	–	–	–	–	±	±	–	±	+	±	±

Table 5. (continued)

[a] Substrate oxidation was determined with the use of a tetrazolium reduction assay (Chapter 2 of this dissertation. A plus (+) = oxidation; a minus (-) = no oxidation a plus-minus (±) = oxidation less than 1 mmole of substrate oxidized per mg cell protein x 10^{-5}. Substrate abbreviations are: Bd = butanediol;Gl = glycerol; Mt = maltose; Mn = mannose; Ri = ribitol; Bu = butanol; Cy = cyclopentanol; Fr = fructose; Na = Na acetate; Ra = raffinose; Et = ethanol; Gu = glucose; So = sorbitol.

To further study the possibility of butanol oxidation being plasmid related in strains of *Gluconobacter*, I first wanted to determine if the presence of the plasmids from *G. oxydans* ATCC strain 621 affected this strain's ability to oxidize butanol. One way to accomplish this would be to cure this strain of its plasmids and determine if recovery in butanol oxidation occurred. Due to the difficulty in curing strains of bacteria of their plasmids (N. Palleroni, personal communication with G. W. Claus), I decided that it would be better to transform *G. oxydans* IFO strain 3244 with each of the plasmids from *G. oxydans* ATCC strain 621 and observe whether butanol oxidation occurred in the transformed strain.

A genetic marker for each of these plasmids was necessary to know whether IFO strain 3244 was successfully transformed by these plasmids. Since I determined that antibiotic resistance was not related to the presence of plasmids within the gluconobacters, antibiotic resistance could not be used as a genetic marker for these two strains.

I also thought of using butanol oxidation as a possible genetic marker. However, I did not know which of these plasmids, if any, contained the genes necessary for butanol oxidation. Therefore, butanol oxidation as a genetic marker would only be useful in the transformation of one plasmid.

Gluconobacter oxydans ATCC strain 621 and IFO strain 3244 were both able to grow on agar medium containing butanol as a sole source of carbon and energy (Appendix 3A). I cannot easily explain these results except to suggest that over the time of two days incubation, IFO strain 3244 was able to slightly oxidize butanol. However, this strain oxidized butanol at a rate much slower than that which I was able to detect after the 60 min incubation period of the TNBT reduction assay (Chapter 1).

I feel that I have exhausted most possibilities of a natural genetic marker existing on *Gluconobacter* plasmids. Heavy metal resistance in many bacteria is associated with the presence of plasmids (Stanisich, 1984), and I have not investigated the possibility that heavy metal resistance may be plasmid encoded in the gluconobacters.

To learn more about the phenotypic characteristics *Gluconobacter* plasmids may encode, some sort of genetic marker needs to be either identified or inserted into these plasmids. For example, a reporter gene such as luciferase or GUS (Beta-glucuronidase) could possibly be inserted into one or more of these plasmids. On the other hand, such an insertion is time consuming and might also disrupt a gene of interest such as the gene(s) for butanol oxidation.

Although the plasmids within the gluconobacters presently remain cryptic, we now have a better understanding of which phenotypic characteristics are not likely associated with these plasmids. Antibiotic resistance and probably many limited oxidations are not associated with plasmids in these strains of *Gluconobacter*.

ACKNOWLEDGEMENTS

I would like to thank Mark Mikales for his assistance in determining antimicrobial agent susceptibility using the agar diffusion method and Sceptor® Panel analysis.

REFERENCES

1. **Asai, T.** 1968. Acetic acid bacteria: Classification and biochemical activities, University of Tokyo Press, Tokyo.
2. **Brock, T.D., M.T. Madigan, J.M. Martinko, and J. Parker.** 1994. Biology of microorganisms, 7th ed. Prentice Hall, Englewood Cliffs, NJ.
3. **Connamacher, R.H.** 1976. Inducible bacterial resistance, p. 8-66. *In* F.E. Hahn (ed.), Acquired resistance of microorganisms to chemotherapeutic drugs, S. Karger AG, Basel, Switzerland.
4. **De Ley, J., and Swings, J.** 1984. Genus II. *Gluconobacter* Asai 1935, 689, emend. mut. char. Asai, Iizuka and Komagata 1964, 100, p. 275-278. *In* N.R. Krieg and J. G. Holt (eds.), Bergey's manual of systematic bacteriology, Volume 1, Williams and Wilkins, Baltimore.
5. **Edwards, D E.** 1990. Diversity of limited oxidations accomplished by *Gluconobacter oxydans*. M.S. thesis. Virginia Tech, Blacksburg.
6. **Feshami, B. A., and G. W. Claus.** 1991. A nonchromatographic assay for sorbitol in chemically-complex growth media containing sorbose. J. Indust. Microbiol. **7:** 57-61.
7. **Fukaya, M. , T. Iwata, E. Entani, H. Masai, T. Uozumi, and T. Beppu.** 1985. Distribution and characterization of plasmids in acetic acid bacteria. Agric. Biol. Chem. (Tokyo) **49:** 1349-1355.
8. **Gaffney, D. , and T. J. Foster.** 1978. Cholramphenicol acetyltransferase determined by R plasmids from gram-negative bacteria. J. Gen. Microbiol. **109:** 351-358.
9. **Greenfield, S. , and G. W. Claus.** 1969. Isocitrate dehydrogenase and glutamate synthesis in *Acetobacter suboxydans*. J. Bacteriol. **100:** 1264-1270.

10. **Hammond, S.M., and P.A. Lambert.** 1978. Antibiotics and antimicrobial action, Edward Arnold (Publishers) Limited, London.

11. **Ivler, D.** 1974. Antimicrobial drugs: Mechanisms and factors influencing their action, p. 3-11. *In* B.M. Kagan (ed.), Antimicrobial therapy, 2nd ed. W. B. Saunders Company, Philadelphia, PA.

12. **Jones, D.** 1983. Impact of plasmids and transposons on microbial systematics, p. 119-124. *In* D. Schlessinger (ed.), Microbiology - 1983, American Soc. of Microbiology, Washington, D. C..

13. **Lambert, B. , K. Kersters, F. Gossele, J. Swings, and J. De Ley.** 1981. Gluconobacters from honey bees. Antonie van Leeuwenhoek J. Microbiol. Serol. **47:** 147-157.

14. **Mason, L. M., and G. W. Claus.** 1989. Phenotypic characteristics correlated with DNA-sequence similarities for three species of *Gluconobacter: G. oxydans* (Henneberg, 1897) DeLey 1961, *G. frateruii* sp. nov., and *G. asai* sp. nov.. Int. J. Syst. Bacteriol. **39:** 174-184.

15. **McKibben, L A.** 1992. Characterization of plasmids in *Gluconobacter.* M.S. thesis. Virginia Tech, Blacksburg.

16. **Micales, B K.** 1983. DNA/DNA homology studies on the genus *Gluconobacter.* M.S. thesis. Virginia Tech, Blacksburg.

17. **Micales, B. K., J. L. Johnson, and G. W. Claus.** 1985. Deoxyribonucleic acid homologies among organisms in the genus *Gluconobacter.* Int. J. Syst. Bacteriol. **35:** 79-85.

18. **Mickel, S. , V. Arena, Jr., and W. Bauer.** 1977. Physical properties and gel electrophoresis behavior of R12-derived plasmid DNAs. Nucleic Acids Research. **4:** 1465-1482.

19. **Okumura, H. , T. Uozumi, and T. Beppu.** 1985. Construction of plasmid vectors and a genetic transformation system for *Acetobacter aceti*. Agric. Biol. Chem. (Tokyo) **49:** 1011-1017.

20. **Proctor, N. , and R. Rownd.** 1982. Rosanilins: indicator dyes for chloramphenicol-resistant enterobacteria containing chloramphenicol acetyltransferase. J. Bacteriol. **150:** 1375-1382.

21. **Pronk, J. T., P. R. Levering, W. Olijve, and J. P. Dijken.** 1989. Role of NADP-dependent and quinoprotein glucose dehydrogenase in gluconic acid production by *Gluconobacter oxydans*. Enzyme Microb. Technol. **11:** 160-164.

22. **Qazi, G. N., V. Verma, R. Parshad, and C. L. Chopra.** 1989. Plasmid-mediated direct-glucose oxidation in *Gluconobacter oxydans*. J. Biotechnology **10:** 85-88.

23. **Rainbow, C.** 1966. Nutrition and metabolism of acetic acid bacteria. Wallerstein Lab. Commun. **29:** 5-15.

24. **Schuman, J. D., E. A. Zottola, and S. K. Harlander.** 1989. Preliminary characterization of a food-borne multiple-antibiotic-resistant *Salmonella typhimurium* Strain. Appl. Environ. Microbiol. 2344-2348.

25. **Shaw, W. V.** 1976. Chloramphenicol acetyltransferase in *Streptomyces*. J. Gen. Microbiol. **94:** 159-166.

26. **Stanisich, V.A.** 1984. Identification and analysis of plasmids at the genetic level, p. 5-32. *In* P.M. Bennett, and J. Grinsted (ed.), Methods in microbiology, Academic Press, London.

27. **Valla, S. , D. H. Coucheron, and J. Kjosbakken.** 1983. *Acetobacter xylinum*. contains several plasmids: evidence for their involvement in cellulose formation. Arch. Microbiol. **134:** 9-11.

APPENDIX 3A

Minimum inhibitory concentrations of antimicrobial agents using Sceptor® panel analysis for *G. oxydans* ATCC strain 621 and IFO strain 3244

Table A1. Minimum inhibitory concentrations of various antimicrobial agents and the presence of plasmids using Septor panel analysis for *G. oxydans* ATCC strain 621 and IFO strain 3244

Antimicrobial agent[b]	Minimum inhibitory concentration for each strain [a] (μg/ml)	
	ATCC 621 (4 plasmids)[c]	IFO 3244 (0)[d]
Amikacin	< 2	< 4
Amoxicillin	4	2
Ampicillin	4	4
Aztreonam	> 32	> 32
Cefazolin	> 32	> 32
Cefoperazone	> 32	> 32
Cefotaxime	16	16
Cefotetan	> 64	> 64
Ceftaxime	16	32
Ceftazidime	4	8
Ceftriaxone	8	4
Ceftrizoxime	< 8	< 8
Cefuroxime	> 16	> 16
Cephalothin	> 32	> 32
Chloramphenicol	> 8	> 16
Ciprofloxacin	8	> 2
Clavulonic acid	2	1
Clindamycin	> 8	> 8
Erythromycin	> 8	> 8
Gentamicin	< 2	8
Imipemem	1	1
Mezlocillin	32	16
Nitrofurantoin	> 128	> 128
Norfloxacin	> 8	> 8
Oxacillin	> 8	> 8
Penicillin	> 8	> 8
Pipercillin	< 16	16
Rifampin	1	> 4
Sulbactam	2	2
Sulfamethoxazole	> 152	> 152
Tetracycline	2	> 8
Ticarcillin	16	16
Tobramycin	4	8

Table A1. (continued)

Trimethoprim	> 16	> 16
Vancomycin	> 16	> 16

[a] Minimum inhibitory concentrations were determined from at least two replicates. The MIC shown accounts for differences between the two replicates and the greatest and/or the least concentration exhibited by the strain for each antimicrobial agent is shown.

[b] Antimicrobial agents shown in shaded box were tested in other *Gluconobacter* strains using the disc diffusion method.

[c] Plasmid isolations were performed as described in Chapter 2 of this dissertation.

[d] No plasmids were detected in this strain.

APPENDIX 3B

Growth on butanol as a sole oxidizable carbon source

Gluconobacter oxydans ATCC strain 621 contains four plasmids (Chapter 2). In experiments using the TNBT reduction assay, I found that this strain oxidizes butanol, whereas *G. oxydans* IFO strain 3244, a strain which I could not detect plasmids, was unable to oxidize butanol. I wanted to investigate whether the genes necessary for butanol oxidation were encoded on a plasmid in ATCC strain 621. To accomplish this, I wished to separately transform IFO strain 3244 with each of the plasmids from ATCC strain 621 and then determine if IFO strain 3244 gained the ability to oxidize butanol. However, this was not possible because none of these plasmids contain a known genetic marker. For this reason, I thought that growth on solid medium containing only butanol as an oxidizable carbon source might serve as a genetic marker for one plasmid that might encode the gene (or genes) necessary for butanol oxidation.

To test for growth on butanol I first prepared a subculture of *G. oxydans* ATCC strain 621 and IFO strain 3244 in 50 ml of broth medium containing (w/v) 5% glycerol, 1% yeast extract, and 1% peptone to approximate 0.90 OD_{620} . Each culture was centrifuged, and the resulting pellets were washed twice in 0.2 M succinate buffer (pH 6.2) to remove any carbon source remaining from the growth media. The pellets were resuspended in 30 ml of 0.2 M succinate buffer (pH 6.2). A basal medium containing (w/v) 1% yeast extract, 1% peptone, and 2% agar was used in the experiment to determine growth on butanol. The cell suspensions for both cultures were streaked onto each of four plates containing only the basal medium. Four plates containing the basal medium plus mannitol where streaked in the same manner. These plates were used to test culture viability. A plate of basal medium and basal medium plus mannitol were each placed into four separate glass gallon jars. One jar contained a beaker of

distilled water. The second jar contained a beaker of 1% butanol, and the third jar contained a beaker of 5% butanol. The fourth jar contained a beaker of 100% butanol. All four jars were sealed and incubated at 28°C. After 72 h of incubation, the plates were examined for growth.

Growth was observed on all plates, although, growth was heaviest on plates containing mannitol. Growth was observed on basal media placed in the jar with a beaker containing 1% butanol. Growth was also observed on basal medium placed in the jar with the beaker containing 5% butanol. Only slight growth appeared on basal media that was placed in the jar containing a beaker of 100% butanol. Very little growth occurred on basal medium in the jar containing water. These results showed that ATCC strain 621 and IFO strain 3244 utilized very little of either the yeast extract or peptone as a carbon source. In addition, both strains utilized butanol as a sole source of carbon when it was supplied to them in the atmosphere of the glass jars.

These results seem to contradict previous results, because IFO strain 3244 grew on butanol but did not show butanol oxidation in the TNBT reduction assay (Chapter 1). One possible explanation is that *G. oxydans* IFO strain 3244 could exhibit oxidation of butanol during the one hour incubation period used for the TNBT reduction assay, but it may have oxidized butanol enough to grow slowly on butanol over the period of 72 h.

Chapter 4

Hybridization Studies with Plasmids from the Three Species of *Gluconobacter*

SUMMARY

The genus *Gluconobacter* consists of Gram-negative rod-shaped bacteria that are characterized as acetic acid bacteria, because they generate acidic products from the oxidation of substrates, in particular, acetic acid from ethanol. At the present time, some of these rapid limited oxidations have great value to chemical and pharmaceutical industries, however, little is known about the genetics of the gluconobacters. At the present time, most plasmids isolated from strains of acetic acid bacteria are cryptic in that they encode no known phenotypic characteristics. Some investigators suggest that genes carried on plasmids of acetic acid bacteria are involved in some of the enzymatic processes that make them industrially useful. I used hybridization analysis of plasmid DNA to determine the relatedness of genetic material from strains representing the three species of *Gluconobacter*. Plasmids were first isolated from low-melting agarose gels and then non-radioactively labeled using the Genius 1 kit from Boehringer-Mannheim Biochemicals. The plasmid probes were hybridized against plasmids from other strains using the Southern blot technique. The hybridization results indicate that more homology exists between the plasmids within the same species than between plasmids from other species of *Gluconobacter*. Plasmid probes from strains of *G. oxydans* and *G. asaii* did not hybridize with any plasmids from *G. frateurii* strains. However, plasmid probes from *G. frateurii* did hybridize with a few plasmids from *G. oxydans* strains but not with plasmids from *G. asaii* strains. All of these results suggests the possibility of natural transfer of plasmids between different

strains of the same species, and at least limited natural transfer of plasmids between the three species of *Gluconobacter*.

INTRODUCTION

Many types of bacteria belonging to vastly diverse metabolic groups contain plasmids (20). Plasmids are usually circular molecules of double-stranded DNA that replicate autonomously, are stably inherited, posses the ability to transfer to other bacterial cells, and are independent of the host cell chromosome (see page 1, Ref. 7), (9). However, not all plasmids share these basic characteristics. For example, some plasmids have the ability to become integrated into the host cell chromosome and replicate along with it (9), and some plasmids in strains of *Streptomyces* are linear (11.; 10).

Plasmid characterization in bacteria is important for several reasons. First, plasmids have become important research tools for genetic analysis of many different organisms. For example, plasmids are often used as vectors for the transfer and characterization of genetic elements (see page 2, Ref. 7). Second, bacterial plasmids are responsible for many phenotypic characteristics in bacteria, such as antibiotic resistance, heavy metal resistance, metabolism of diverse compounds, nitrogen fixation, toxin production, gas vacuole formation, and conjugal properties (see page 1, Ref. 7, 9, 20). Third, plasmids may affect the classification of bacteria since bacterial plasmids encode many different phenotypic characteristics (9).

Genetic transfer of information between bacteria through transformation, transduction, and conjugation is believed responsible for much of bacterial diversity over time (9). Bacterial plasmids, therefore, contribute to bacterial variation. It is common for strains isolated from similar environments and/or belonging to closely

related taxonomic groups to contain a plasmid (or plasmids) common to those strains (9).

Acetic acid bacteria are Gram-negative, rod-shaped bacteria that generate acidic products from the oxidation of substrates, in particular, acetic acid from ethanol (2, 18). There are two genera of acetic acid bacteria: *Acetobacter* and *Gluconobacter* (2). Most plasmids isolated from strains of acetic acid bacteria are cryptic in that the types of genes carried on these plasmids are not known. However, several investigators suggest that the genetic information carried on plasmids of some acetic acid bacteria is associated with ethanol and glucose oxidation, acetic acid resistance, and cellulose biosynthesis (15, 17, 21).

Acetic acid bacteria are important due to their involvement in vinegar production and sorbitol oxidation. Since plasmids may be involved in some of these industrial processes, further characterization of plasmids from strains of *Acetobacter* and *Gluconobacter* has become increasingly more important. Hybrid shuttle vectors comprised of plasmid DNA isolated from acetic acid bacteria and plasmid DNA from strains of *Escherichia coli* are being used to study plasmid involvement in these processes (4, 5, 15). Genetic modifications using these hybrid shuttle vectors may also lead to strain improvements which may increase the efficiencies of industrial processes involving acetic acid bacteria (6).

One approach to further plasmid characterization is to study the similarities between plasmids. Restriction enzyme digests would be useful for two reasons. First, restriction maps could be compared to determine similarities between the number and types of restriction sites in these plasmids. Second, restriction maps could identify possible sights for use in hybrid-shuttle-vector development.

Another approach to determine relatedness between plasmids is hybridization analysis. Deoxyribonucleic acid hybridization or reassociation experiments are used as methods of determining the relatedness between different genetic material (8, 12.). One advantage of using hybridization studies is that this method shows more specificity than restriction mapping. In other words, this technique identifies larger portions of plasmid DNA sequences that are similar than restriction mapping does (see page 199, Ref. 1).

The purpose of this study was to perform hybridization analyses with plasmids isolated from *Gluconobacter* strains and to determine if similar genetic material exists between plasmids within one strain, or within strains of the same species, or between strains from different species. Perhaps this information might also provide indirect evidence that *Gluconobacter* plasmids were transferred between strains sometime in the past.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study were the same as those used in a previous DNA/DNA homology study (14) and tests of related phenotypic characteristics (13). *Gluconobacter oxydans* ATCC strains 621, 9937, 14960, 19357, and 23771 were received from the American Type Culture Collection, and *G. oxydans* IFO strains 3244, 3293, 3294, & 12528 and ATCC strain 23652 were received from Noberto Palleroni (Hoffman-LaRoche, Nutley, NJ). *Gluconobacter oxydans* IFO strain 12467 was received from the Institute of Fermentation (Osaka, Japan) and *G. oxydans* CSIRO strain B1507 was received from Division of Food Resources, Commonwealth Scientific and Industrial Research Organization (Ryde, Australia). *Gluconobacter frateurii* IFO strains 3254, 3264, 3268,

3270, 3271, 3272, and 3286 were also received from Noberto Palleroni. *Gluconobacter asaii* IFO strain 3276 was received from the Institute of Fermentation and *Gluconobacter asaii* IFO strain 3297 was a gift from Noberto Palleroni. *Gluconobacter asaii* ATCC strain 43781 was originally received from K. G. Rohrback (Department of Plant Pathology, University of Hawaii at Manoa, Honolulu) and later deposited at the American Type Culture Collection. All strains were checked for purity, grown in a complex medium, suspended in a medium containing (w/v) 15% glycerol, 1% yeast extract, and 1% peptone, then heat-sealed in glass vials and continuously maintained in liquid nitrogen Dewars. Working-stock cultures were prepared by growing the strains in broth containing (w/v) 5% glycerol, 1% yeast extract, and 1% peptone until reaching approximately 0.9 OD_{620} and maintained until use by storing in 66% glycerol at -4°C. Cells used in the plasmid isolations and subsequent hybridization experiments were grown in 50 ml of broth containing (w/v) 5% glycerol, 1% yeast extract, and 1% peptone in 500 ml growth flasks. Incubated cultures were shaken at 200 rpm at 28°C until reaching approximately 0.6 OD_{620}.

Escherichia coli strain V517 was received from A. A. Yousten (Department of Biology, Virginia Tech, Blacksburg). *Escherichia coli* strain V517 was grown in double strength nutrient broth until reaching approximately 0.9 OD_{620}. This culture was stored in 66% glycerol at -4°C. Cells used in plasmid isolations and subsequent hybridization experiments were grown with shaking in 50 ml of double strength nutrient broth, in 500 ml growth flasks, at 37°C until reaching approximately 0.6 OD_{620}.

Reagents. Sucrose, sodium dodecyl sulfate, glacial acetic acid, xylene cyanol FF, glycerol, phenol, sodium chloride, magnesium chloride, sodium citrate, and chloroform were purchased from Fisher Scientific, Fair Lawn, NJ. Ethylenediamine tetraacetic acid, sodium hydroxide, ethidium bromide, and N-lauroyl sarcosine (sodium

salt) were purchased from Sigma Chemical Co., St. Louis, MO. Yeast extract, peptone, and nutrient broth were purchased from Difco Laboratories, Detroit, MI. Ultrapure Tris[hydroxymethyl]aminomethane (Tris) was purchased from United States Biochemical, Cleveland, OH. Concentrated hydrochloric acid was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. Ethanol was purchased from Aaper Alcohol and Chemical Co., Shelbyville, KY.

Buffers and solutions. The E buffer contained 0.04 M Tris, 0.002 M ethylenediamine tetraacetic acid (EDTA), and 15% (w/v) sucrose prepared in sterile water and adjusted to pH 7.9 with glacial acetic acid. Lysis buffer contained 0.3% (w/v) sodium dodecyl sulfate (SDS), and 5% (v/v) 3 M sodium hydroxide added to 5 ml of a solution of 0.05 M Tris and 15% (v/v) sucrose. The TE buffer contained 10 mM Tris and 1 mM EDTA, and this was adjusted to pH 8.0 with concentrated hydrochloric acid. Lithium-chloride buffer contained 0.4 M lithium chloride in TE buffer. Fifty x TAE buffer contained 242 g Tris, 100 ml 0.5 M EDTA (adjusted to pH 8.0), and 57.1 ml of glacial acetic acid brought to a final volume of one liter with distilled water. Tracking dye contained about 4.2 mg each of bromophenol blue and xylene cyanol FF added to 2 ml of a solution containing 50% (w/v) of glycerol and 50% (w/v) of 2 X TAE buffer. Genius Buffer #1 contained 0.1 M Tris and 0.15 M sodium chloride. Genius Buffer #2 contained 2% (w/v) of blocking reagent (supplied in the Genius 1 kit) in Genius Buffer #1. Genius Buffer #3 contained 0.1 M Tris, 0.1 M sodium chloride, and 0.05 M magnesium chloride, and this was adjusted to pH 9.5 and filtered through a membrane having a pore size of 0.45 μm. Anti-DIG alkaline phosphatase buffer contained a 1:5000 dilution of antibody conjugate (supplied in the Genius 1 kit) diluted in Genius Buffer #2. Color-substrate solution contained 45 μl of NBT solution and 35 μl of X-phosphate (both supplied in the Genius 1 kit) in 10 ml of Genius Buffer #3. Twenty x

SSC wash solution contained 3 M sodium chloride and 0.3 M sodium citrate at a pH of 7.0. Two x SSC, SDS and 0.1 x SSC, SDS each contained 0.1% (w/v) of sodium dodecyl sulfate. Hybridization solution contained in (w/v): 1% of blocking reagent, 0.1% of N-lauroyl sarcosine (sodium salt), and 0.02% of sodium dodecyl sulfate in 5 x SSC wash solution.

Plasmid preparations. Plasmids preparations were made from bacterial cell cultures using alkaline cell lysis, phenol-chlorofom extraction, and lithium chloride-ethanol precipitation as described in Chapter 2.

Plasmid extraction from low-melting agarose gels. Samples of plasmid preparations for probe isolation (15 μl) were mixed with 2 μl of tracking dye and loaded into wells of a 0.7% low-melting agarose gel (agarose dissolved in 1 X TAE buffer). The samples were electrophoresed, and the gels stained as previously described in Chapter 2. The desired plasmid band was visualized using a PhotoDyne UV transilluminator, excised from the gel with an ethanol-sterilized razor blade, and placed into a separate, sterile, 1.5 ml microcentrifuge tube. The excised agarose "plug" containing the plasmid band was melted at 70°C for 15 min. The volume of the melted agarose was estimated, and about twice that volume of TE buffer was added, so that the final agarose concentration was much less than 0.5%. The tube of melted agarose and TE buffer was again heated at 70°C for 5 min. A volume of phenol:chloroform (1:1) (equal to that contained in the tube) was added to the hot solution, vortexed for 1 min, and this tube was left at room temperature for 5 min placed at -80°C for 30 min. The mixtures was centrifuged in an Eppendorf (model 5415) microcentrifuge at full speed for 15 min at 4°C. The resulting aqueous phase was carefully removed, placed in a sterile 1.5 ml microcentrifuge tube, and stored at -20°C until use in the lithium chloride-ethanol precipitation described below. A volume of TE buffer (equal to the

remaining phenol phase) was added to the tube, and the phenol-chloroform extraction was repeated. The new aqueous phase was placed in a separate, sterile, 1.5 ml microcentrifuge tube and stored at -20°C until used in the lithium chloride-ethanol precipitation described below.

Lithium chloride-ethanol precipitation of extracted plasmid DNA. One microliter of glycogen (20 mg/ml), one-half the volume of lithium chloride buffer (to the volume contained in the tube), and 2.5 X volume of cold 95% ethanol (to the volume contained in the tube after lithium chloride buffer addition) was added to the aqueous phase that resulted from the 2nd phenol-chloroform extraction. This solution contained the extracted plasmid DNA. This tube was vortexed briefly, held at -20°C overnight (or -80°C for 30 min), and centrifuged in an Eppendorf (model 5415) microcentrifuge at full speed and at 4°C for 20 min to precipitate and pellet the plasmid DNA. The resulting pellets were dried as previously described in Chapter 2, suspended in 15 µl of TE buffer and stored at -80°C until use.

Probe labeling. Plasmids to be non-radiactively labeld were randomly selected from strains representing each of the three species of *Gluconobacter*. At least two plasmid probes were prepared from each of the three species. Plasmid DNA extracted and purified as described in the above two sections, was first denatured by boiling for 10 min then placed on ice for about 15 min. The plasmids were non-radioactively labeled with digoxigenin-11-UTP using a random primed reaction, as described in the instructions included with the Nonradioactive Genius 1 DNA Labeling and Detection Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Reaction mixtures for labeling contained 10 µl of extracted and purified DNA, 2 µl of hexanucleotide mixture, 1 µl of Klenow enzyme (DNA polymerase), and 5 µl of sterile, distilled water. This reaction mixture was incubated for at least 20 h in a 37°C waterbath, and 2 µl of a 0.2 M

solution of EDTA (pH 8) was added to stop the reaction. The labeled plasmid probe was precipitated with 2.5 µl of lithium chloride buffer and 75 µl of cold 70% ethanol by placing this mixture at -80°C for 30 min, and centrifuging in an Eppendorf (model 5415) microcentrifuge at full speed for 20 min at 4°C. The resulting pellet was air dried for 2-3 h and resuspended in 50 µl of TE buffer. This non-radioactively labeled plasmid probe was stored at -20°C until use.

Estimation of the concentration of non-radioactively labeled plasmid DNA. The following dilutions of digoxigenin (DIG) - labeled control DNA were prepared in DNA dilution buffer: 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl, and 0.1 pg/µl. The DIG-labeled control DNA and the DNA dilution buffer were supplied in the Genius 1 kit. The following dilutions of the DIG-labeled plasmid DNA were also prepared: 1:5, 1:50, 1:500, 1:5,000, and 1:50,000. One microliter of each DNA dilution (both labeled control and labeled plasmid DNA) was spotted onto a piece of positively charged nylon membrane (Boehringer Mannheim Biochemicals, Indianapolis, IN). The DNA was fixed to the nylon membrane with UV light using a Fisher Scientific (model FB-UVXL-1000) UV crosslinker and selecting the optimal crosslinking setting. The nylon membrane was placed in a sealable plastic bag along with 20 ml of Genius Buffer #2. The bag was sealed and incubated at room temperature for 5 min. The Genius Buffer #2 was removed, and 20 ml of anti-DIG alkaline phosphatase buffer (antibody conjugate) was added to the bag. The bag was sealed once again and incubated at room temperature for 5 min. The nylon membrane was removed from the bag and washed twice with ~ 20 ml of Genius Buffer #1 for 5 min at room temperature. The nylon membrane was placed in a new, sealable plastic bag along with 20 ml of Genius Buffer #3. The bag was sealed and incubated for 2 min at room temperature. The Genius Buffer #3 was replaced with 10 ml of color substrate solution, the bag was sealed and incubated at

room temperature overnight in the dark (without shaking). The developed nylon membrane was stored in 10 ml of TE buffer within the sealed plastic bag. The DIG-labeled plasmid DNA concentration on the nylon membrane was estimated by visual comparison to the DIG-labeled control DNA. A representative nylon membrane used to estimate plasmid DNA concentration is shown in Appendix 4A.

Southern transfer. To test for hybridization with the DIG-labeled plasmid probe, 10 µl samples of plasmid preparations were mixed with 2 µl of tracking dye and loaded into the wells of a 0.7% agarose gel prepared in 1 X TAE buffer. Samples were electrophoresed in 1 X TAE buffer in a PhotoDyne (model 1214) gel unit set at 90 volts until the bromophenol blue tracking dye front was about 4 cm from the bottom of the gel (about 3 h). Each gel was stained for 15 min with 175-350 µl of a solution containing 0.5 mg/ml of ethidium bromide added to 120 ml of distilled water. The gel was rinsed with 120 ml of distilled water for 15 min and depurinated with 0.3 N hydrochloric acid for about 15 min (until the bromophenol blue dye front of the tracking dye turned to a yellowish color). The gel was soaked in 0.4 N sodium hydroxide for about 15 min (until the bromophenol blue dye front of the tracking dye turned back to blue).

A Southern transfer apparatus (19) was assembled in the following manner. The casting tray of the PhotoDyne gel unit was inverted and placed in a glass dish containing 0.4 N sodium hydroxide. The gel was placed face-up on top of two sheets of Whatmann 3MM paper. The Whatmann 3MM paper was placed on the inverted gel casting tray so that the ends of these sheets served as wicks in 0.4 N sodium hydroxide. A piece of plastic wrap was placed around the edges of the gel to prevent evaporation of the sodium hydroxide. A sheet of positively charged nylon membrane was cut the same size as the gel, wetted in 0.4 N sodium hydroxide, and placed on top

of the gel. Two additional sheets of Whatmann 3MM paper were wetted with 0.4 N sodium hydroxide and placed on top of the nylon membrane. A stack of paper towels (about 4 inches thick) was placed on top of the Whatmann 3MM paper. A piece of plexi-glass was placed on top of the paper towel stack, and this was weighted down by a water-filled glass bottle. This alkaline transfer of plasmid DNA from the agarose gel to the nylon membrane was done overnight. The Southern transfer apparatus was disassembled, and the transferred DNA was fixed to the nylon membrane with UV light as before (see Estimation of the concentration of non-radioactively labeled plasmid DNA).

Hybridization. The nylon membrane with the affixed transferred plasmid DNA was placed in a sealable plastic bag containing 20 ml of hybridization buffer (preheated to 65^{o}C). The bag was sealed, and placed at 65^{o}C for 2 h. This procedure blocks the nylon membrane to prevent non-specific binding of the DIG-labeled plasmid DNA. The DIG-labeled plasmid DNA (2.5 ng/ml) was denatured as before and added to 10 ml of hybridization buffer. The hybridization buffer was discarded, the hybridization buffer containing the DIG-labeled plasmid DNA was added, the bag was sealed, and incubated at 65^{o}C overnight. The nylon membrane was removed from the bag and washed twice with 2 x SSC, SDS solution at room temperature for 5 min and twice again with 0.1 x SSC, SDS solution (prewarmed to 65^{o}C) at 65^{o}C for 15 min.

Detection of DIG-labeled plasmid DNA. The nylon membrane was rinsed once with Genius Buffer #1 then incubated in ~ 100 ml of Genius Buffer #2 at room temperature for 30 min. The nylon membrane was placed in a sealable plastic bag containing 20 ml of anti-DIG alkaline phosphatase buffer (antibody conjugate) and incubated at room temperature for 30 min. After incubation, the nylon membrane was washed twice with ~ 20 ml of Genius Buffer #1 at room temperature for 15 min and

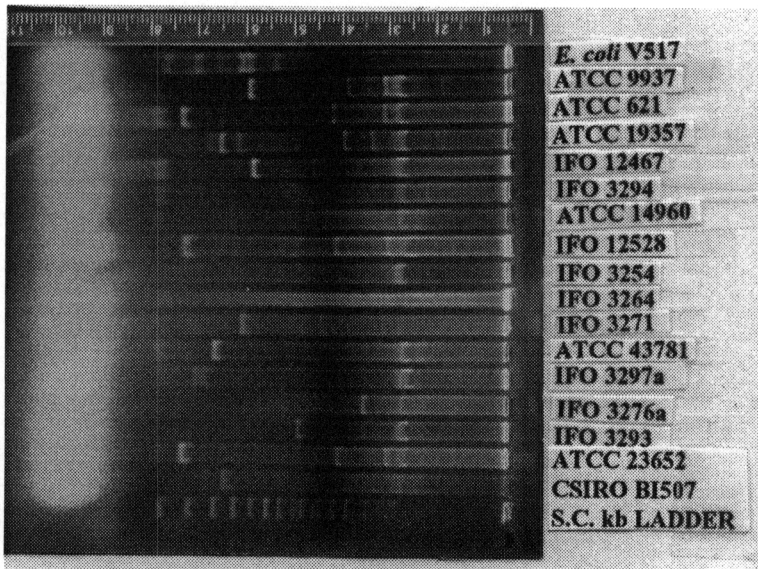
placed in a new, sealable, plastic bag containing 20 ml of Genius Buffer #3 for about 2 min. The Genius Buffer #3 was removed and replaced with 10 ml of color substrate solution. The nylon membrane was allowed to remain in this solution overnight in the dark at room temperature without shaking. The color substrate solution was replaced with 10 ml of TE buffer. The developed nylon membrane was stored indefinitely at room temperature.

RESULTS

The hybridization results within single strains and between the three species of *Gluconobacter* are shown in Figures 1 - 7 and summarized in Tables 1 - 7. The gels pictured on the right of these figures are not shown actual size and, therefore, the plasmid bands do not line up with those shown in the Southern blot on the left of these figures. Generally, plasmids from the same species appeared to hybridize with one another more than between species. Plasmid probes from strains of *G. oxydans* and *G. asaii* did not hybridize with any plasmids from strains of *G. frateurii*. However, plasmid probes from *G. frateurii* strains did hybridize with a few plasmids from strains of *G. oxydans* but not with plasmids from strains of *G. asaii*.

Hybridizations using plasmid probes from strains of *Gluconobacter oxydans*. Agarose gels containing the plasmids used in hybridization experiments and

Figure 1. Ethidium-bromide stained agarose gel of electrophoresed plasmids (left) and the Southern-blot analysis of this gel using the 5.9 kb plasmid from *G. oxydans* ATCC strain 9937 as the probe (right). The lanes shown in the Southern blots are reversed from those shown in the agarose gel. Estimates of plasmid size and which plasmids hybridize with this probe are summarized in Table 1.


E. coli V517
ATCC 9937
ATCC 621
ATCC 19357
IFO 12467
IFO 3294
ATCC 14960
IFO 12528
IFO 3254
IFO 3264
IFO 3271
ATCC 43781
IFO 3297a
IFO 3276a
IFO 3293
ATCC 23652
CSIRO BI507
S.C. kb LADDER

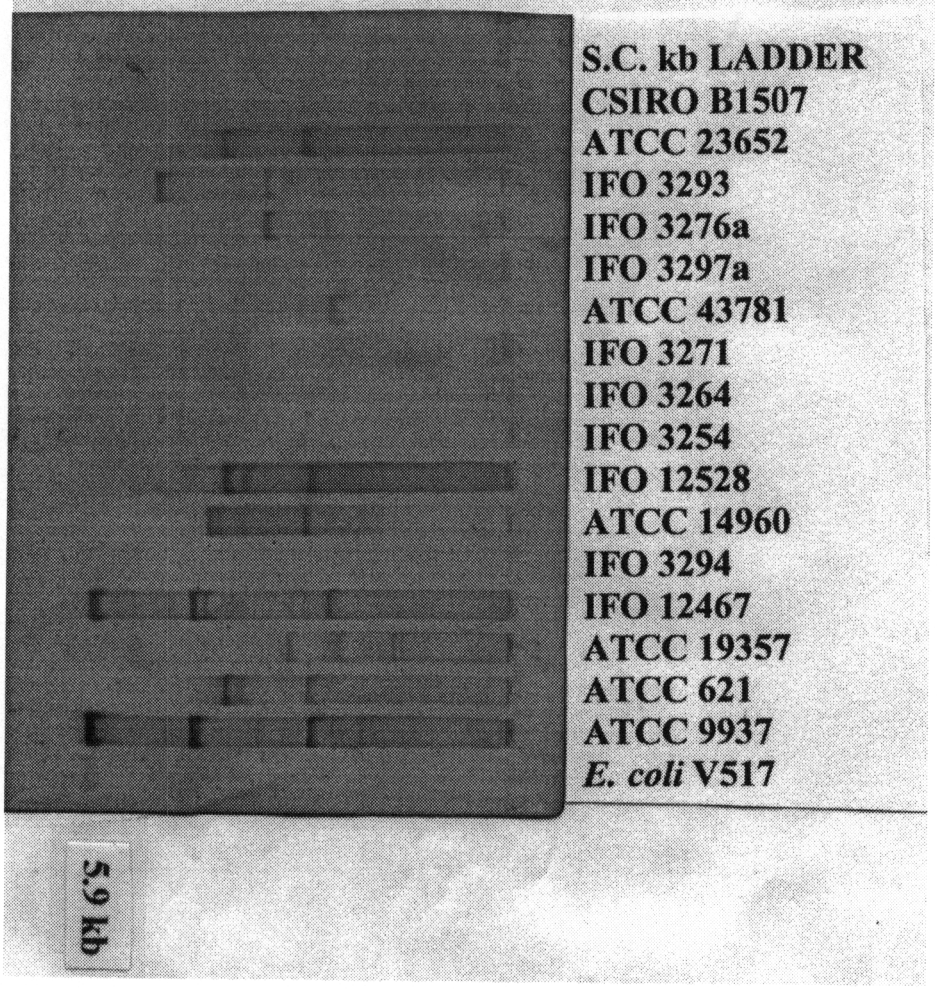

S.C. kb LADDER
CSIRO B1507
ATCC 23652
IFO 3293
IFO 3276a
IFO 3297a
ATCC 43781
IFO 3271
IFO 3264
IFO 3254
IFO 12528
ATCC 14960
IFO 3294
IFO 12467
ATCC 19357
ATCC 621
ATCC 9937
E. coli V517
5.9 kb

Figure 2. Ethidium-bromide stained agarose gel of electrophoresed plasmids (left) and the Southern-blot analysis of this gel using the 2.9 kb plasmid from *G. oxydans* ATCC strain 621 as the probe (right). The lanes shown in the Southern blots are reversed from those shown in the agarose gel. Estimates of plasmid size and which plasmids hybridize with this probe are summarized in Table 2.

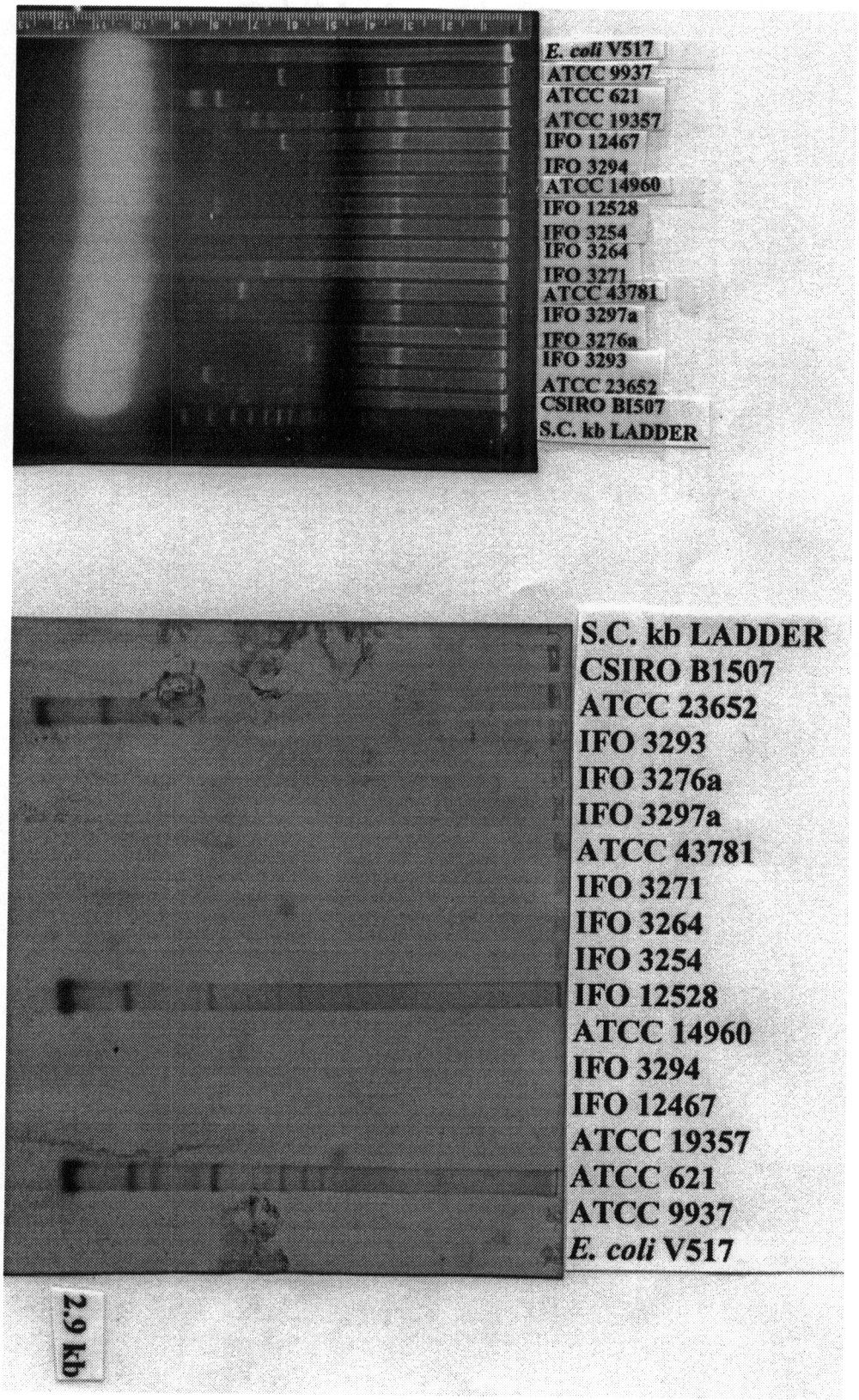

E. coli V517
ATCC 9937
ATCC 621
ATCC 19357
IFO 12467
IFO 3294
ATCC 14960
IFO 12528
IFO 3254
IFO 3264
IFO 3271
ATCC 43781
IFO 3297a
IFO 3276a
IFO 3293
ATCC 23652
CSIRO BI507
S.C. kb LADDER
S.C. kb LADDER
CSIRO B1507
ATCC 23652
IFO 3293
IFO 3276a
IFO 3297a
ATCC 43781
IFO 3271
IFO 3264
IFO 3254
IFO 12528
ATCC 14960
IFO 3294
IFO 12467
ATCC 19357
ATCC 621
ATCC 9937
E. coli V517
2.9 kb

Figure 3. Ethidium-bromide stained agarose gel of electrophoresed plasmids (left) and the Southern-blot analysis of this gel using the 16 kb plasmid from *G. oxydans* ATCC strain 19357 as the probe (right). The lanes shown in the Southern blots are reversed from those shown in the agarose gel. Estimates of plasmid size and which plasmids hybridize with this probe are summarized in Table 3.

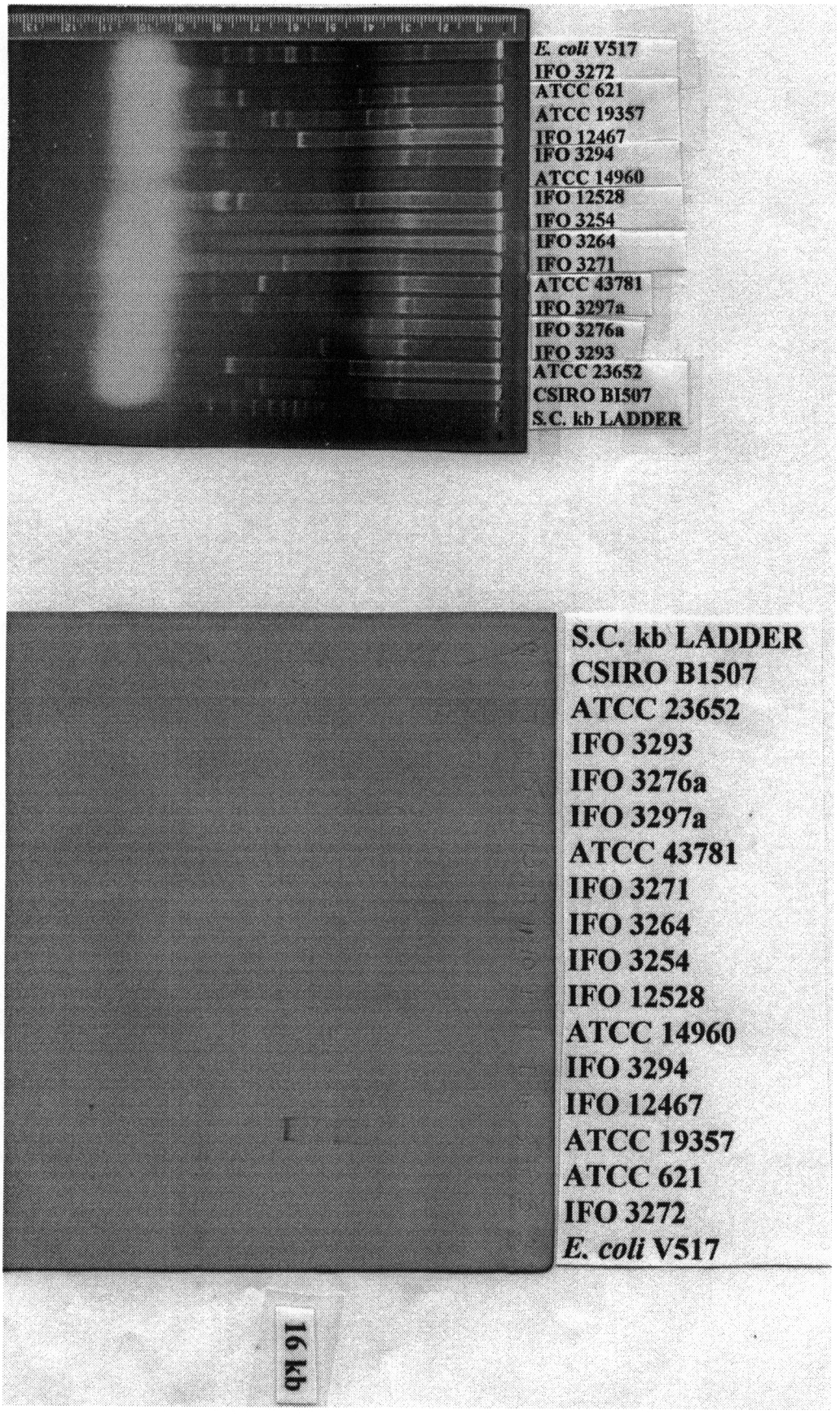

E. coli V517
IFO 3272
ATCC 621
ATCC 19357
IFO 12467
IFO 3294
ATCC 14960
IFO 12528
IFO 3254
IFO 3264
IFO 3271
ATCC 43781
IFO 3297a
IFO 3276a
IFO 3293
ATCC 23652
CSIRO B1507
S.C. kb LADDER
S.C. kb LADDER
CSIRO B1507
ATCC 23652
IFO 3293
IFO 3276a
IFO 3297a
ATCC 43781
IFO 3271
IFO 3264
IFO 3254
IFO 12528
ATCC 14960
IFO 3294
IFO 12467
ATCC 19357
ATCC 621
IFO 3272
E. coli V517
16 kb

Figure 4. Ethidium-bromide stained agarose gel of electrophoresed plasmids (left) and the Southern-blot analysis of this gel using the 5.0 kb plasmid from *G. frateurii* IFO strain 3271 as the probe (right). The lanes shown in the Southern blots are reversed from those shown in the agarose gel. Estimates of plasmid size and which plasmids hybridize with this probe are summarized in Table 4.

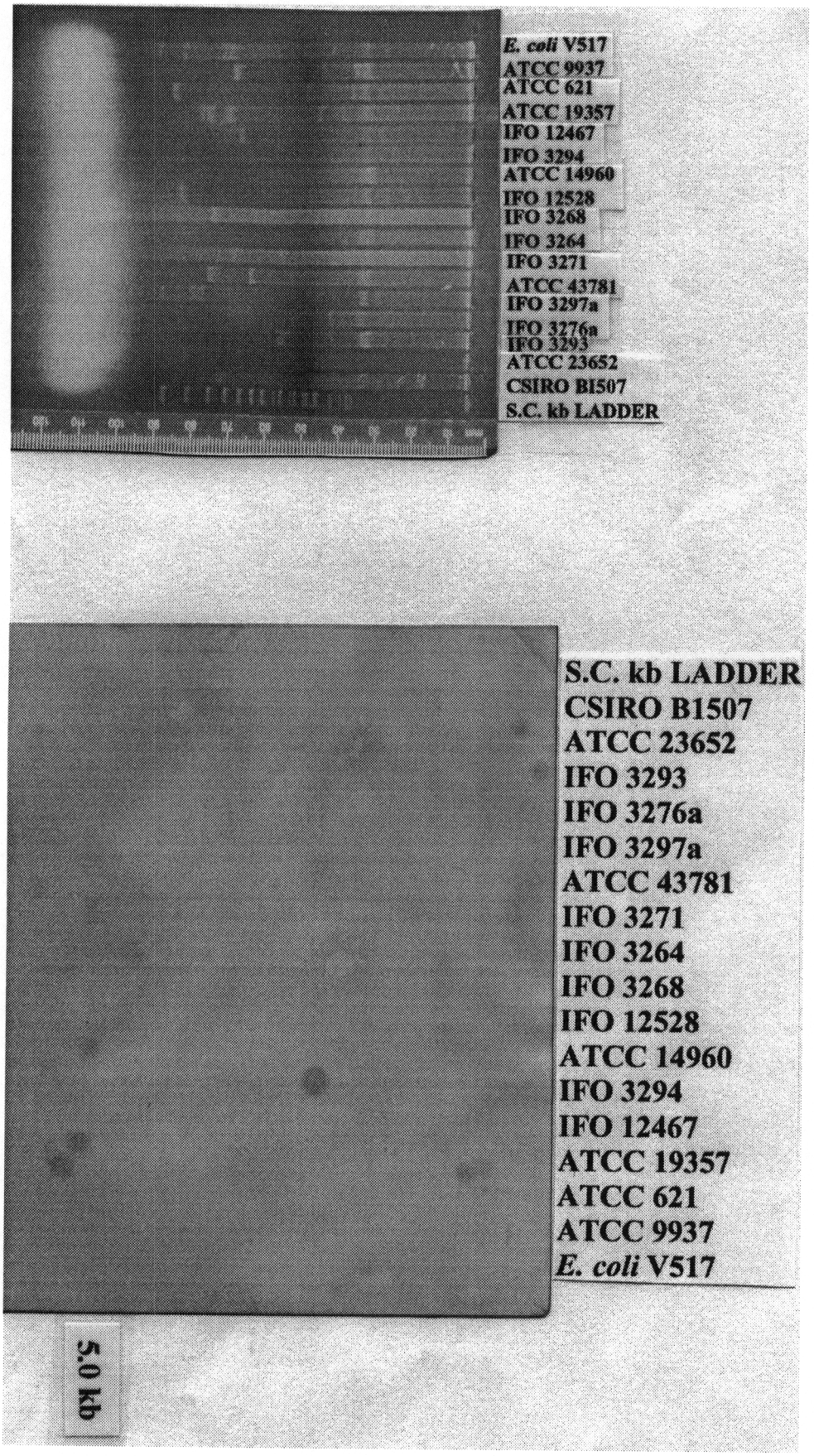

E. coli V517
ATCC 9937
ATCC 621
ATCC 19357
IFO 12467
IFO 3294
ATCC 14960
IFO 12528
IFO 3268
IFO 3264
IFO 3271
ATCC 43781
IFO 3297a
IFO 3276a
IFO 3293
ATCC 23652
CSIRO B1507
S.C. kb LADDER
S.C. kb LADDER
CSIRO B1507
ATCC 23652
IFO 3293
IFO 3276a
IFO 3297a
ATCC 43781
IFO 3271
IFO 3264
IFO 3268
IFO 12528
ATCC 14960
IFO 3294
IFO 12467
ATCC 19357
ATCC 621
ATCC 9937
E. coli V517
5.0 kb

Figure 5. Ethidium-bromide stained agarose gel of electrophoresed plasmids (left) and the Southern-blot analysis of this gel using the 3.8 kb plasmid from *G. frateurii* IFO strain 3268 as the probe (right). The lanes shown in the Southern blots are reversed from those shown in the agarose gel. Estimates of plasmid size and which plasmids hybridize with this probe are summarized in Table 5.

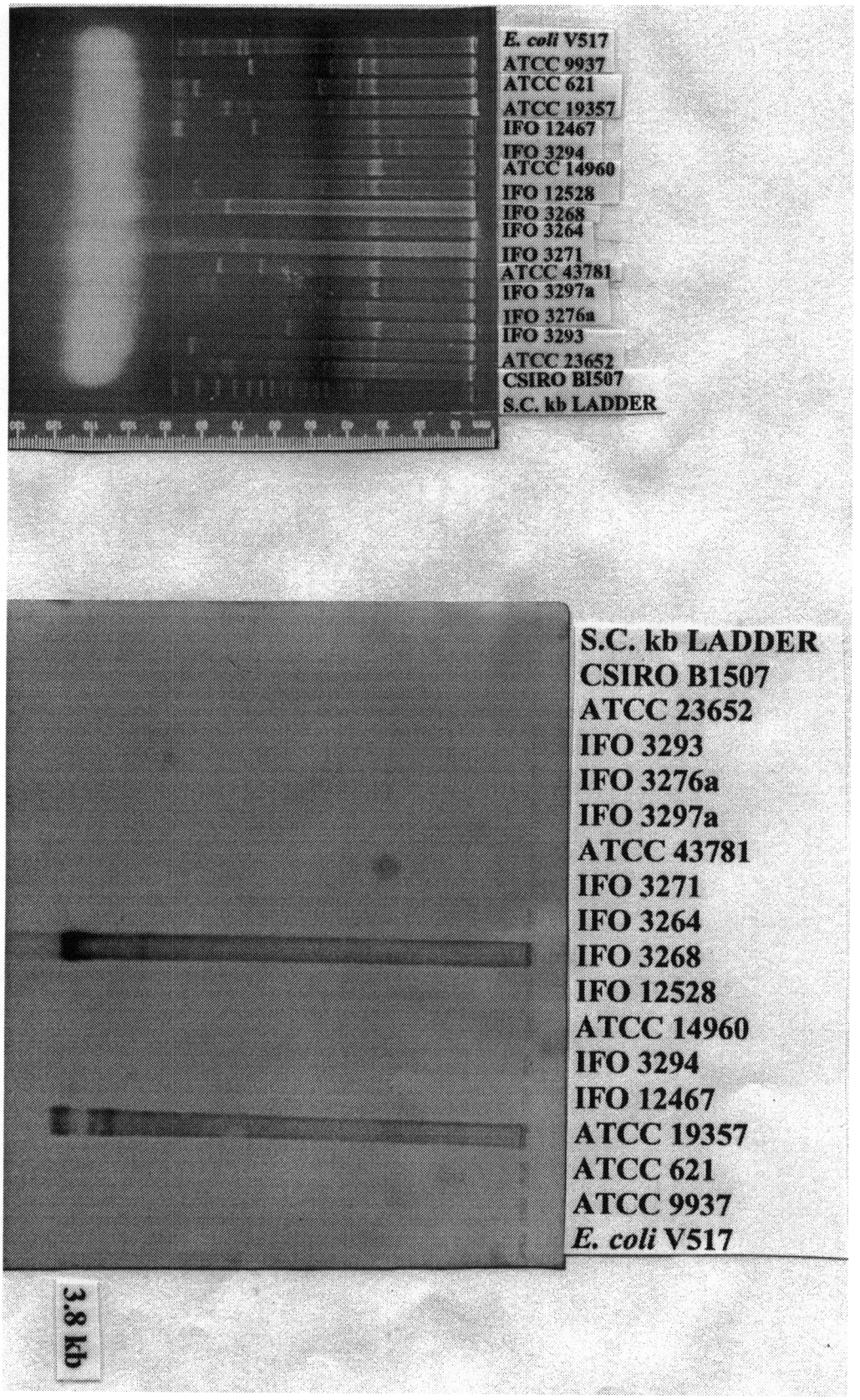

E. coli V517
ATCC 9937
ATCC 621
ATCC 19357
IFO 12467
IFO 3294
ATCC 14960
IFO 12528
IFO 3268
IFO 3264
IFO 3271
ATCC 43781
IFO 3297a
IFO 3276a
IFO 3293
ATCC 23652
CSIRO BI507
S.C. kb LADDER
S.C. kb LADDER
CSIRO B1507
ATCC 23652
IFO 3293
IFO 3276a
IFO 3297a
ATCC 43781
IFO 3271
IFO 3264
IFO 3268
IFO 12528
ATCC 14960
IFO 3294
IFO 12467
ATCC 19357
ATCC 621
ATCC 9937
E. coli V517
3.8 kb

Figure 6. Ethidium-bromide stained agarose gel of electrophoresed plasmids (left) and the Southern-blot analysis of this gel using the 3.9 kb plasmid from *G. asaii* ATCC strain 43781 as the probe (right). The lanes shown in the Southern blots are reversed from those shown in the agarose gel. Estimates of plasmid size and which plasmids hybridize with this probe are summarized in Table 6.

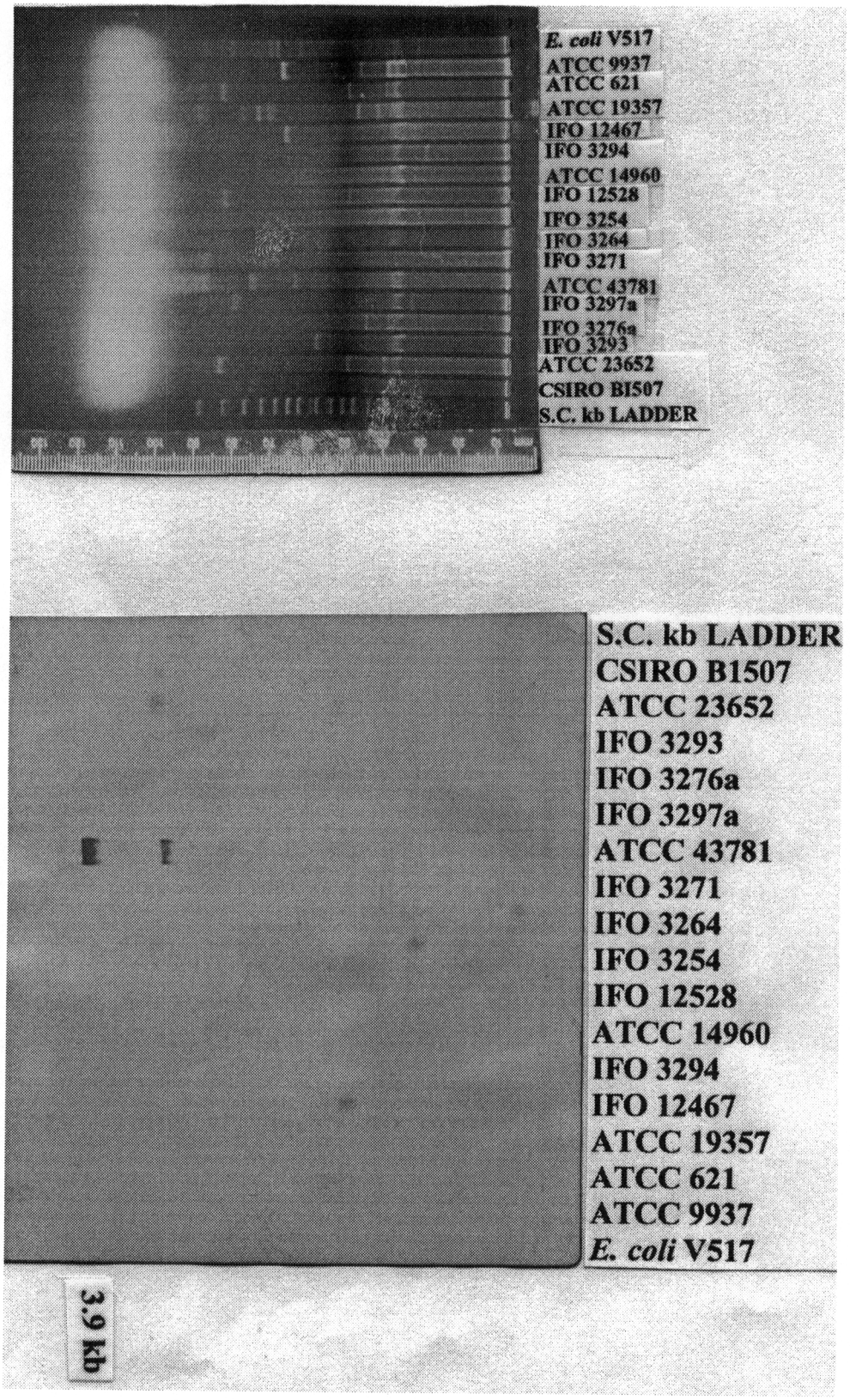

E. coli V517
ATCC 9937
ATCC 621
ATCC 19357
IFO 12467
IFO 3294
ATCC 14960
IFO 12528
IFO 3254
IFO 3264
IFO 3271
ATCC 43781
IFO 3297a
IFO 3276a
IFO 3293
ATCC 23652
CSIRO BI507
S.C. kb LADDER
S.C. kb LADDER
CSIRO B1507
ATCC 23652
IFO 3293
IFO 3276a
IFO 3297a
ATCC 43781
IFO 3271
IFO 3264
IFO 3254
IFO 12528
ATCC 14960
IFO 3294
IFO 12467
ATCC 19357
ATCC 621
ATCC 9937
E. coli V517
3.9 kb

Figure 7. Ethidium-bromide stained agarose gel of electrophoresed plasmids (left) and the Southern-blot analysis of this gel using the 6.8 kb plasmid from *G. asaii* ATCC strain 43781 as the probe (right). The lanes shown in the Southern blots are reversed from those shown in the agarose gel. Estimates of plasmid size and which plasmids hybridize with this probe are summarized in Table 7.

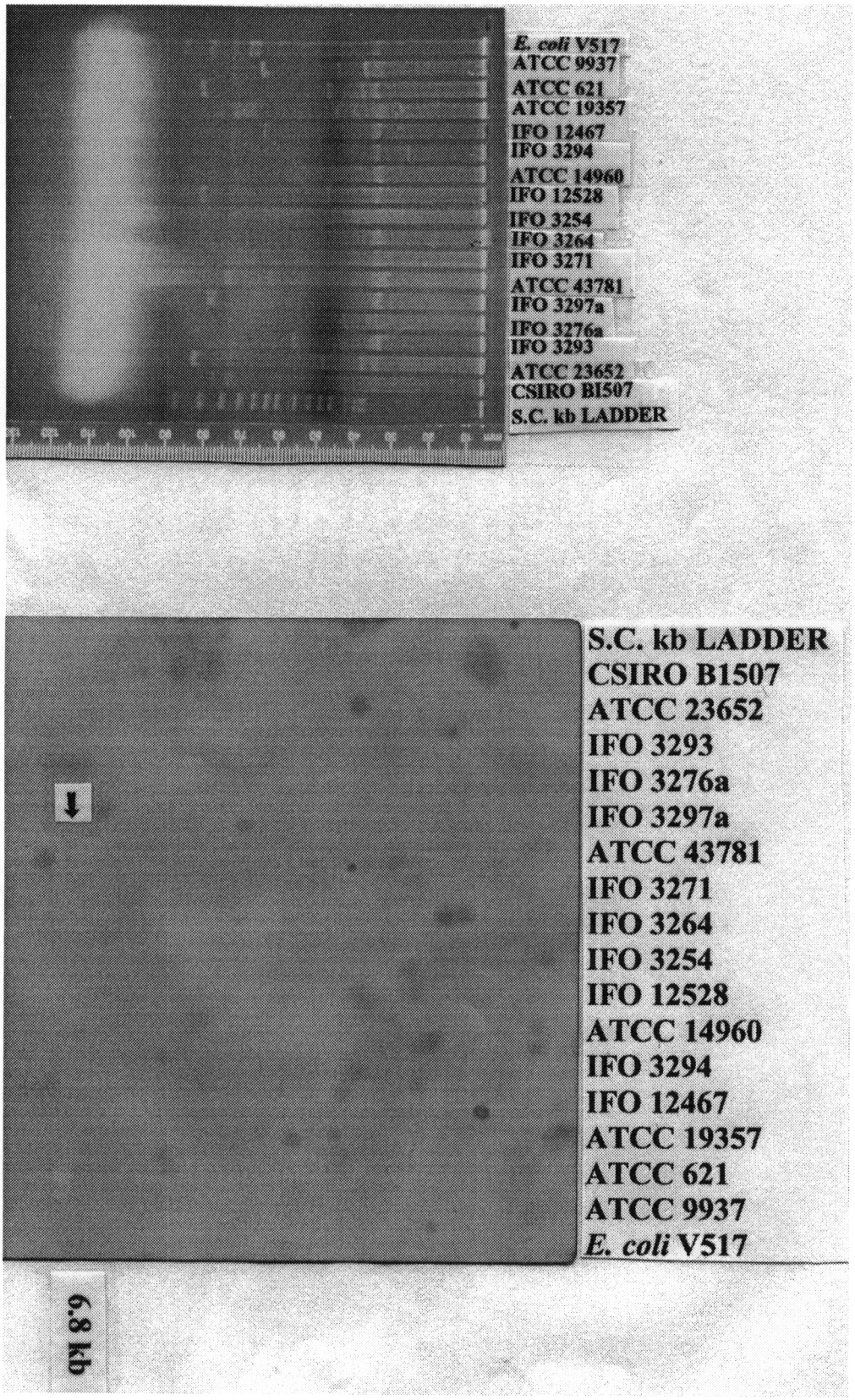

E. coli V517
ATCC 9937
ATCC 621
ATCC 19357
IFO 12467
IFO 3294
ATCC 14960
IFO 12528
IFO 3254
IFO 3264
IFO 3271
ATCC 43781
IFO 3297a
IFO 3276a
IFO 3293
ATCC 23652
CSIRO BI507
S.C. kb LADDER
S.C. kb LADDER
CSIRO B1507
ATCC 23652
IFO 3293
IFO 3276a
IFO 3297a
ATCC 43781
IFO 3271
IFO 3264
IFO 3254
IFO 12528
ATCC 14960
IFO 3294
IFO 12467
ATCC 19357
ATCC 621
ATCC 9937
E. coli V517
6.8 kb

Table 1. Hybridization between the 5.9 kb plasmid from *G. oxydans* ATCC strain 9937 and plasmids from other strains

Strain [a]	Species	Plasmids hybridizing with probe (in kb)	Plasmids NOT hybridizing with probe (in kb)
ATCC 9937	*G. oxydans*	5.9 12.7 17.6	27.6 two large plasmids
ATCC 621	*G. oxydans*	14.8 24.6	2.9 large plasmid
ATCC 14960	*G. oxydans*	13.4 26.6	45
ATCC 19357	*G. oxydans*	21.3 28.3 two large plasmids	3.1 4.2 5.1 7.8 16
ATCC 23652	*G. oxydans*	14.5 24	2.8
CSIRO B1507	*G. oxydans*	none	3.5 4.2 6.1 7.7 14.5 18.5
IFO 3293	*G. oxydans*	9.5 20	none
IFO 3294	*G. oxydans*	none	36 54
IFO 12467	*G. oxydans*	6.1 12.3	none
IFO 12528	*G. oxydans*	14.9 26.7	2.2 2.9 large plasmid
IFO 3254	*G. frateurii*	none	none
IFO 3264	*G. frateurii*	none	33
IFO 3271	*G. frateurii*	none	5.0 9.9 13.5 22
ATCC 43781	*G. asaii*	37	3.9 6.8
IFO 3276a	*G. asaii*	19.4	34 large plasmid
IFO 3297a	*G. asaii*	none	2.0 3.0 3.3 35

Table 1. (continued)

[a] Abbreviations are: ATCC = American Type Culture Collection, Rockville, MD; CSIRO = Commonwealth Scientific and Industrial Research Organization, New South Wales, Australia; IFO = Institute of Fermentation, Osaka, Japan.

Table 2. Hybridization between the 2.9 kb plasmid from *G. oxydans* ATCC strain 621 and plasmids from other strains

Strain [a]	Species	Plasmids hybridizing with probe (in kb)	Plasmids NOT hybridizing with probe (in kb)
ATCC 621	*G. oxydans*	2.9 14.8 24.6	large plasmid
ATCC 9937	*G. oxydans*	5.9 27.6	12.7 17.6 two large plasmids
ATCC 14960	*G. oxydans*	none	13.4 26.6 45
ATCC 19357	*G. oxydans*	none	3.1 4.2 5.1 7.8 16 21.2 28.3 two large plasmids
ATCC 23652	*G. oxydans*	2.8 unidentified plasmid	14.5 24
CSIRO B1507	*G. oxydans*	none	3.5 4.2 6.1 7.7 14.5 18.5
IFO 3293	*G. oxydans*	9.5 20	none
IFO 3294	*G. oxydans*	none	36 54
IFO 12467	*G. oxydans*	none	6.1 12.3
IFO 12528	*G. oxydans*	2.9 14.9 26.7	2.2 large plasmid
IFO 3254	*G. frateurii*	none	none
IFO 3264	*G. frateurii*	none	33
IFO 3271	*G. frateurii*	none	5.0 9.9 13.5 22
ATCC 43781	*G. asaii*	none	3.9 6.8 37
IFO 3276a	*G. asaii*	none	19.4 34 large plasmid

Table 2. (continued)

IFO 3297a	*G. asaii*	none	2.0	3.0	3.3	35

[a] Abbreviations are: ATCC = American Type Culture Collection, Rockville, MD; CSIRO = Commonwealth Scientific and Industrial Research Organization, New South Wales, Australia; IFO = Institute of Fermentation, Osaka, Japan.

Table 3. Hybridization between the 16 kb plasmid from *G. oxydans* ATCC strain 19357 and plasmids from other strains

Strain [a]	Species	Plasmids hybridizing with probe (in kb)	Plasmids NOT hybridizing with probe (in kb)
ATCC 19357	*G. oxydans*	16 21.2 28.3	3.1 4.2 5.1 7.8 two large plasmids
ATCC 621	*G. oxydans*	14.8 24.6	2.9 large plasmid
ATCC 14960	*G. oxydans*	none	13.4 26.6 45
ATCC 23652	*G. oxydans*	14.5 24	2.8
CSIRO B1507	*G. oxydans*	18.5	3.5 4.2 6.1 7.7 14.5
IFO 3293	*G. oxydans*	none	9.5 20
IFO 3294	*G. oxydans*	none	36 54
IFO 12467	*G. oxydans*	none	6.1 12.3
IFO 12528	*G. oxydans*	14.9 26.7	2.2 2.9 large plasmid
IFO 3254	*G. frateurii*	none	none
IFO 3264	*G. frateurii*	none	33
IFO 3271	*G. frateurii*	none	5.0 9.9 13.5 22
IFO 3272	*G. frateurii*	none	2.05 12.8 24
ATCC 43781	*G. asaii*	37	3.9 6.8
IFO 3276a	*G. asaii*	19.4	34 large plasmid
IFO 3297a	*G. asaii*	35	2.0 3.0 3.3

Table 3. (continued)

[a] Abbreviations are: ATCC = American Type Culture Collection, Rockville, MD; CSIRO = Commonwealth Scientific and Industrial Research Organization, New South Wales, Australia, IFO = Institute of Fermentation, Osaka, Japan.

Table 4. Hybridization between the 5.0 kb plasmid from *G. frateurii* IFO strain 3271 and plasmids from other strains

Strain [a]	Species	Plasmids hybridizing with probe (in kb)	Plasmids NOT hybridizing with probe (in kb)
IFO 3271	*G. frateurii*	5.0 9.9	13.5 22
ATCC 621	*G. oxydans*	none	2.9 14.8 24.6 large plasmid
ATCC 9937	*G. oxydans*	27.6	5.9 12.7 17.6 two large plasmids
ATCC 14960	*G. oxydans*	none	13.4 26.6 45
ATCC 19357	*G. oxydans*	4.2	3.1 5.1 7.8 16 21.3 28.3
ATCC 23652	*G. oxdans*	none	2.8 14.5 24
CSIRO B1507	*G. oxydans*	none	3.5 4.2 6.1 7.7 14.5 18.5
IFO 3293	*G. oxydans*	none	9.5 20
IFO 3294	*G. oxydans*	none	36 54
IFO 12467	*G. oxydans*	none	6.1 12.3
IFO 12528	*G. oxydans*	none	2.2 2.9 14.9 26.7 large plasmid
IFO 3264	*G. frateurii*	none	33
IFO 3268	*G. frateurii*	none	3.8
ATCC 43781	*G. asaii*	none	3.9 6.8 37
IFO 3276a	*G. asaii*	none	19.4 34 large plasmid

Table 4. (continued)

IFO 3297a	*G. asaii*	none	2.0	3.0	3.3	35

[a] Abbreviations are: ATCC = American Type Culture Collection, Rockville, MD; CSIRO = Commonwealth Scientific and Industrial Research Organization, New South Wales, Australia; IFO = Institute of Fermentation, Osaka, Japan.

Table 5. Hybridization between the 3.8 kb plasmid from *G. frateurii* IFO strain 3268 and plasmids from other strains

Strain [a]	Species	Plasmids hybridizing with probe (in kb)	Plasmids NOT hybridizing with probe (in kb)
IFO 3268	*G. frateurii*	3.8	---
ATCC 621	*G. oxydans*	2.9	14.8 24.6 large plasmid
ATCC 9937	*G. oxydans*	5.9	12.7 17.6 27.6 two large plasmids
ATCC 14960	*G. oxydans*	none	13.4 26.6 45
ATCC 19357	*G. oxydans*	3.1 4.2 5.1	7.8 16 21.3 28.3
ATCC 23652	*G. oxdans*	2.8	14.5 24
CSIRO B1507	*G. oxydans*	4.2	3.5 6.1 7.7 14.5 18.5
IFO 3293	*G. oxydans*	none	9.5 20
IFO 3294	*G. oxydans*	none	36 54
IFO 12467	*G. oxydans*	none	6.1 12.3
IFO 12528	*G. oxydans*	2.9	2.2 14.9 26.7 large plasmid
IFO 3264	*G. frateurii*	none	33
IFO 3271	*G. frateurii*	none	5.0 9.9 13.5 22
ATCC 43781	*G. asaii*	none	3.9 6.8 37
IFO 3276a	*G. asaii*	none	19.4 34 large plasmid

Table 5. (continued)

IFO 3297a	*G. asaii*	none	2.0	3.0	3.3	35

[a] Abbreviations are: ATCC = American Type Culture Collection, Rockville, MD; CSIRO = Commonwealth Scientific and Industrial Research Organization, New South Wales, Australia; IFO = Institute of Fermentation, Osaka, Japan.

Table 6. Hybridization between the 3.9 kb plasmid from *G. asaii* ATCC strain 43781 and plasmids from other strains

Strain [a]	Species	Plasmids hybridizing with probe (in kb)	Plasmids NOT hybridizing with probe (in kb)
ATCC 43781	*G. asaii*	3.9 6.8	37
ATCC 621	*G. oxydans*	none	2.9 14.8 24.6 large plasmid
ATCC 9937	*G. oxydans*	none	5.9 12.7 17.6 27.6 two large plasmids
ATCC 14960	*G. oxydans*	none	13.4 26.6 45
ATCC 19357	*G. oxydans*	none	3.1 4.2 5.1 7.8 16 21.3 28.3 two large plasmids
ATCC 23652	*G. oxdans*	none	2.8 14.5 24
CSIRO B1507	*G. oxydans*	3.5 6.1	4.2 7.7 14.5 18.5
IFO 3293	*G. oxydans*	none	9.5 20
IFO 3294	*G. oxydans*	none	36 54
IFO 12467	*G. oxydans*	none	6.1 12.3
IFO 12528	*G. oxydans*	none	2.2 2.9 14.9 26.7 large plasmid
IFO 3254	*G. frateurii*	none	none
IFO 3264	*G. frateurii*	none	33
IFO 3271	*G. frateurii*	none	5.0 9.9 13.5 22
IFO 3276a	*G. asaii*	none	19.4 34 large plasmid

Table 6. (continued)

IFO 3297a	*G. asaii*	none	2.0	3.0	3.3	35

[a] Abbreviations are: ATCC = American Type Culture Collection, Rockville, MD; CSIRO = Commonwealth Scientific and Industrial Research Organization, New South Wales, Australia; IFO = Institute of Fermentation, Osaka, Japan.

Table 7. Hybridization between the 6.8 kb plasmid from *G. asaii* ATCC strain 43781 and plasmids from other strains

Strain [a]	Species	Plasmids hybridizing with probe (in kb)	Plasmids NOT hybridizing with probe (in kb)
ATCC 43781	*G. asaii*	6.8 3.9	37
ATCC 621	*G. oxydans*	none	2.9 14.8 24.6 large plasmid
ATCC 9937	*G. oxydans*	none	5.9 12.7 17.6 27.6 two large plasmids
ATCC 14960	*G. oxydans*	none	13.4 26.6 45
ATCC 19357	*G. oxydans*	none	3.1 4.2 5.1 7.8 16 21.3 28.3 two large plasmids
ATCC 23652	*G. oxydans*	none	2.8 14.5 24
CSIRO B1507	*G. oxydans*	none	3.5 4.2 6.1 7.7 14.5 18.5
IFO 3293	*G. oxydans*	none	9.5 20
IFO 3294	*G. oxydans*	none	36 54
IFO 12467	*G. oxydans*	none	6.1 12.3
IFO 12528	*G. oxydans*	none	2.2 2.9 14.9 26.7 large plasmid
IFO 3254	*G. frateurii*	none	none
IFO 3264	*G. frateurii*	none	33
IFO 3271	*G. frateurii*	none	5.0 9.9 13.5 22

Table 7. (continued)

IFO 3276a	*G. asaii*	none	19.4	34	large plasmid		
IFO 3297a	*G. asaii*	none	2.0	3.0	3.3	35	

[a] Abbreviations are: ATCC = American Type Culture Collection, Rockville, MD; CSIRO = Commonwealth Scientific and Industrial Research Organization, New South Wales, Australia; IFO = Institute of Fermentation, Osaka, Japan.

the resulting Southern blots from three plasmid probes of strains of *G. oxydans*, are shown in Figures 1 - 3. Summaries of these hybridizations are presented in Tables 1 - 3.

When the 5.9 kb plasmid from *G. oxydans* ATCC strain 9937 was used as the probe, I found that it hybridized with many plasmids from strains of *G. oxydans*, two plasmids from strains of *G. asaii*, but it did not hybridize with any plasmids from strains of *G. frateurii* (Figure 1 and Table 1). It appeared that there was no relationship between the probe size and the size of plasmids this probe hybridized with. The 5.9 kb plasmid probe from *G. oxydans* ATCC strain 9937 hybridized with plasmids such as the 6.1 kb plasmid from *G. oxydans* IFO strain 12467, and the 26.6, 28.3, and the 26.7 kb plasmids from *G. oxydans* ATCC strains 14960 and 19357 and IFO strain 12528. The 5.9 kb probe also hybridized with the two large plasmids from *G. oxydans* ATCC strain 19357. However, the 5.9 kb probe did not hybridize with other plasmids in this size range. For example, the 5.9 kb probe did not hybridize with the 6.1 kb plasmid from *G. oxydans* CSIRO strain B1507 or the 6.8 kb plasmid from *G. asaii* ATCC strain 43781. Also, the 27.6 kb plasmid from *G. oxydans* ATCC strain 9937 (the strain from which the plasmid probe was isolated) did not hybridize with the 5.9 kb probe.

The smallest plasmid with which the 5.9 kb probe hybridized was the 6.1 kb plasmid from *G. oxydans* IFO strain 12467. The 5.9 kb probe from *G. oxydans* ATCC strain 9937 did not hybridize with plasmids that were smaller than itself (Figure 1 and Table 1). For example, the 5.9 kb probe did not hybridize with: the 2.9 kb plasmid from *G. oxydans* ATCC strain 621; the 3.1, 4.2, and 5.1 kb plasmids from *G. oxydans* ATCC strain 19357; the 2.0, 3.0, and 3.3 kb plasmids from *G. asaii* IFO strain 3297a; and the smaller plasmids isolated from strains of *G. oxydans* and *G. asaii*.

When the 2.9 kb plasmid from *G. oxydans* ATCC strain 621 was used as a probe, it only hybridized with plasmids from some strains of the same species (Figure 2 and Table 2). The 2.9 kb probe hybridized with three of the four plasmids isolated from the same strain from which the plasmid probe was obtained. The 2.9 kb probe hybridized with plasmids of similar size contained in other strains of *Gluconobacter*, such as the 2.8 kb plasmid from ATCC strain 23652 and the 2.9 kb plasmid from IFO strain 12528. Other similar sized plasmids from strains of *G. oxydans* did not hybridize with the 2.9 kb probe such as the 3.5 kb plasmid from CSIRO strain B1507. Similarly, other similar sized plasmids from strains of *G. asaii* did not hybridize with the 2.9 kb probe such as the 3.0 or 3.3 kb plasmids from IFO strain 3297a.

It is interesting to me that the 2.9 kb probe from *G. oxydans* ATCC strain 621 hybridized with the 5.9 kb plasmid from *G. oxydans* ATCC strain 9937 (Figure 2 and Table 2), but the 5.9 kb probe from *G. oxydans* ATCC strain 9937 did not hybridize with the 2.9 kb plasmid from ATCC strain 621 (Figure 1 and Table 1). Even when the experiment was repeated, the 5.9 kb probe showed no hybridization with the 2.9 kb plasmid from ATCC strain 621 (Data not shown).

When the 16 kb plasmid from *G. oxydans* ATCC strain 19357 was used as a probe, it only hybridized with plasmids from other strains of *G. oxydans* and *G. asaii* (Figure 3 and Table 3). The 16 kb probe from *G. oxydans* ATCC strain 19357 did not hybridize with plasmids from *G. frateurii*. This 16 kb probe hybridized with some plasmids that were similar in size or larger. For example, it hybridized with the 14.8 kb plasmid from *G. oxydans* ATCC strain 621, the 14.5 kb plasmid from *G. oxydans* ATCC strain 23652, the 18.5 kb plasmid from *G. oxydans* CSIRO strain B1507, the 14.9 kb plasmid from *G. oxydans* IFO strain 12528, and the 19.4 kb plasmid from *G. asaii* IFO strain 3276a. There were, however, plasmids of similar size to the 16 kb probe that did

not hybridize with it, such as the 13.4 kb plasmid from *G. oxydans* ATCC strain 14960, the 14.5 kb plasmid from *G. oxydans* CSIRO strain B1507, and the 13.5 kb plasmid from *G. frateurii* IFO strain 3271. This 16 kb probe also did not hybridize with any smaller plasmids (between 2.0 and 12.3 kb) from any of the three species of *Gluconobacter.*

Hybridizations using plasmid probes from strains of *Gluconobacter frateurii.* Agarose gels containing the plasmids used in hybridization experiments and the resulting Southern blots from two plasmid probes of strains of *G. frateurii*, are shown in Figures 4 and 5. Summaries of these hybridizations are presented in Tables 4 and 5.

When the 5.0 kb plasmid from *G. frateurii* IFO strain 3271 was used as a probe, I found that this plasmid hybridized only with some plasmids from this same strain and with one plasmid from each of two other strains of *G. oxydans* (Figure 4 and Table 4). The 5.0 kb probe hybridized with: the 9.9 kb plasmid also isolated from *G. frateurii* IFO strain 3271; the 27.6 kb plasmid from *G. oxydans* ATCC strain 9937; and the 4.2 kb plasmid from *G. oxydans* ATCC strain 19357. There were also many plasmids similar in size to those showing positive hybridization results with the 5.0 kb probe to which it did not hybridize. For example, the 5.0 kb probe did not hybridize with the 9.5 kb plasmid from *G. oxydans* IFO strain 3293, the 26.6 kb plasmid from *G. oxydans* ATCC 14960 or the 26.7 kb plasmid from *G. oxydans* IFO strain 12528.

When the 3.8 kb plasmid from *G. frateurii* IFO strain 3268 was used as a probe, it only hybridized with plasmids isolated from other strains of *G. oxydans* (Figure 5 and Table 5). These results were similar to the hybridization results using the 5.0 kb probe from *G. frateurii* IFO strain 3271. However, the 3.8 kb probe from IFO strain 3268 hybridized with different plasmids than those which hybridized with the 5.0 kb plasmid

from IFO strain 3271 with one exception (both the 3.8 kb and the 5.0 kb probes hybridized with the 4.2 kb plasmid from *G. oxydans* ATCC 19357). The 3.8 kb probe from *G. frateurii* IFO strain 3271 hybridized with some plasmids of similar size. However, there were several plasmids within this size range with which the 3.8 kb probe did not hybridize (the 3.5 and 6.1 kb plasmids from *G.oxydans* CSIRO strain B1507, the 6.1 kb plasmid from *G. oxydans* IFO strain 12467, the 2.2 kb plasmid from *G. oxydans* IFO strain 12528, the 5.0 kb plasmid from *G. frateurii* IFO strain 3271, the 3.9 kb plasmid from *G. asaii* ATCC strain 43781, and the 2.0, 3.0, and 3.3 kb plasmids from *G. asaii* IFO strain 3297a).

Hybridizations using plasmid probes from strains of *Gluconobacter asaii*. Agarose gels containing the plasmids used in hybridization experiments and the resulting Southern blots from two plasmid probes of strains of *G. asaii* are shown in Figures 6 and 7. Summaries of these hybridizations are presented in Tables 6 and 7.

When the 3.9 kb plasmid isolated from *G. asaii* ATCC stain 43781 was used as a probe, it hybridized with the 6.8 kb plasmid from the same strain and with the 3.5 and 6.1 kb plasmids from *G. oxydans* CSIRO strain B1507 (Figure 6 and Table 6). With only a few plasmids giving positive hybridization results, it was not possible to determine if there was a relationship between the probe used and the sizes of the plasmids hybridizing with it. However, other plasmids with sizes similar to the 3.9 kb probe did not hybridize with it.

When the 6.8 kb plasmid from *G. asaii* ATCC strain 43781 was used as a probe, it only hybridized with the 3.9 kb plasmid isolated from the same strain (Figure 7 and Table 7). This result compares favorably with the 3.9 kb probe hybridizing with the 6.8 kb plasmid from this strain (Figure 6 and Table 6).

DISCUSSION

These results showed that each plasmid probe hybridized more frequently with plasmids from strains of the same species than with plasmids from other *Gluconobacter* species (Figures 1 - 7 and Tables 1 - 7). My results also showed that plasmids from strains of *G. oxydans* and *G. asaii* did not hybridize with plasmids from strains of *G. frateurii* (Figures 1 - 7 and Tables 1 - 7). A few hybridizations occurred between plasmids from *G. frateurii* and *G. oxydans* but only when *G. frateurii* plasmids were used as the probes (Figures 4 and 5 and Tables 4 and 5).

These results suggest that at some time in the past, *Gluconobacter* plasmids might possibly have transferred between strains of the same species and even between some species. Plasmid transfer may occur by conjugation, transformation, or transduction (9). Transformation refers to the uptake of genetic material from the surrounding environment whereas, conjugation is the exchange of DNA between two bacteria through direct contact. Transduction refers to bacteriophage-mediated DNA transfer (see page 21, Ref. 7). Although all three methods of DNA transfer are believed to contribute greatly to the diversity and evolution of bacteria (9), conjugation is believed to be the most common method of plasmid transfer among bacteria (see page 21, Ref. 7).

Palleroni et al. (16) report the conjugative transfer of wide host range plasmids from strains of *Escherichia coli* to strains of *G. oxydans* and the subsequent conjugative transfer of these plasmids to strains of *Pseudomonas putida*. Their results clearly demonstrate the ability ot the gluconobacters to participate in conjugative DNA transfer. In addition, Fukaya et al. (3) report that strains of *Gluconobacter* can be successfully transformed with plasmid DNA.

My results suggest that *Gluconobacter* plasmid transfers may have resulted in the fusing of two or more plasmids into one large plasmid. For example, the 2.9 kb probe from *G. oxydans* ATCC strain 621 hybridized with the much larger 24.6 kb plasmid isolated from the same strain, with the 27.6 kb plasmid from *G. oxydans* ATCC strain 9937, with the 20 kb plasmid from *G. oxydans* IFO strain 3293, and with the 26.7 kb plasmid from *G. oxydans* IFO strain 12528. These examples of a small plasmid hybridizing with plasmids at least eight times larger suggest either that this small plasmid was derived from one of the larger plasmids, or that the smaller plasmid combined with other plasmids forming those large plasmids.

Other plasmid transfers within the gluconobacters may be the result of the division of a larger plasmid into smaller plasmids. For example, the 16 kb probe from *G. oxydans* ATCC strain 19357 only hybridized with plasmids from strains of *G. oxydans* and *G. asaii* that were similar in size or larger (Figure 3 and Table 3). Larger plasmids such as the 28.3 kb plasmid from *G. oxydans* ATCC strain 19357, the 26.7 kb plasmid from *G.oxydans* IFO strain 12528, the 37 kb plasmid from *G. asaii* ATCC strain 43781, and the 35 kb plasmid from *G. asaii* IFO strain 3297a may have divided into smaller plasmids such as the 16 kb plasmid used as a probe. However, there were other plasmids of similar size to those listed above to which this 16 kb probe did not bind such as the 26.6 kb plasmid from *G. oxydans* ATCC strain 14960, the 33 kb plasmid from *G. frateurii* IFO strain 3264, and the 34 kb plasmid from *G. asaii* IFO strain 3276a.

These hybridization studies also support the suggestion that three strains of *G. oxydans* were very similar to one another based on their plasmid profiles. *G. oxydans* ATCC strains 621 and 23652, and IFO strain 12528 all contained plasmids approximately 3, 15, and 25 kb in size. When the 2.9 kb plasmid from ATCC strain 621

was used as a probe, it hybridized with the 14.8 and 24.6 kb plasmids from the same strain. The 2.9 kb probe from ATCC 621 also hybridized with the 2.8 kb plasmid from ATCC strain 23652 and with the 2.9, 14.9, and 26.7 kb plasmids from IFO strain 12528. This same 2.9 kb probe was used in another experiment that was performed to determine the ability of the labeled plasmid DNA to bind to different concentrations of plasmid DNA loaded into wells of an agarose gel (Appendix 4A). Figure B1 of Appendix 4B shows that the 2.9 kb probe hybridized with all plasmids except the very large plasmids within these three strains of *G. oxydans*. These results suggest that these three *G. oxydans* strains may be closely related to one another, especially regarding the genetic composition of their plasmids.

One discrepancy in these hybridization studies is that the 2.9 kb probe from *G. oxydans* ATCC strain 621 hybridized with the 5.9 kb plasmid form *G. oxydans* ATCC strain 9937, but the 5.9 kb probe from ATCC strain 9937 did not hybridize with the 2.9 kb plasmid from ATCC strain 621. The reason for this is not clear. One possible explanation is that another plasmid exists that is very similar in size to the 5.9 kb plasmid from ATCC strain 9937 but is in a low concentration such that the eithidium bromide does not stain this plasmid band. However, the 2.9 kb plasmid probe from ATCC strain 621 may have actually hybridized to this additional plasmid and not the 5.9 kb plasmid from ATCC strain 9937. This would explain why the 2.9 kb plasmid probe from ATCC strain 621 appeared to hybridize with the 5.9 kb plasmid from ATCC strain 9937 when the reverse situation did not occur.

Occasionally, the developed nylon membranes (resulting from the Southern transfer and subsequent hybridizations) showed bands that were not observed in the ethidium-bromide stained agarose gels. It is conceivable that these "hybridization" bands were due to variations in the extent of supercoiling of the *Gluconobacter*

plasmids. These different states of supercoiling were probably present in low concentration, and therefore, were undetected by the ethidium bromide used to stain plasmid DNA in agarose gels. However, the probes may have detected these low-copy number plasmid bands by hybridization. The 2.9 kb plasmid from *G. oxydans* ATCC strain 621 hybridized with a plasmid that was not observed on the ethidium-stained agarose gel and was not similar in size to any other plasmid isolated from that strain (Figure 2 and Table 2). This observation may also be explained by the higher specificity of the probe for the plasmid DNA than ethidium bromide. This probe may have been used to identify a plasmid that was otherwise undetected in the ethidium-bromide stained agarose gels.

These hybridization studies strongly suggest that plasmids from different *Gluconobacter* strains are related to one another, especially plasmids from strains of the same species. Through evolution, these plasmids may have passed from strain to strain and then recombined with one another and/or the cell's chromosomal DNA resulting in the plasmids now observed in these strains.

These results further emphasize that there are three species within this genus since plasmids appear more related between strains of the same species than between species. This is also the first study to characterize plasmids among the three species of *Gluconobacter*.

These hybridization studies are the beginning to further understanding the importance of these plasmids within the gluconobacters. Although the types of phenotypic characteristics these plasmids may encode remains a mystery, the sequence of some of these plasmids appears to have been conserved throughout the evolution of the gluconobacters.

ACKNOWLEDGMENTS

I would like to acknowledge Laura McKibben for preliminary hybridization studies that showed that the Genius 1 kit can be used to non-radioactively label plasmid DNA.

REFERENCES

1. **Darnell, J., H. Lodish, and D. Baltimore.** 1990. Molecular cell biology, 2nd ed. W. H. Freeman and Company, New York.
2. **De Ley, J., and Swings, J..** 1984. Genus II. *Gluconobacter* Asai 1935, 689, emend. mut. char. Asai, Iizuka and Komagata 1964, 100, p. 275-278. *In* N.R. Krieg and J. G. Holt (eds.), Bergey's manual of systematic bacteriology, Volume 1, Williams and Wilkins, Baltimore.
3. **Fukaya, M. , K. Tayama, H. Okumura, H. Masai, T. Uozumi, and T. Beppu.** 1985. Improved transformation method for *Acetobacter.* with plasmid DNA. Agric. Biol. Chem. (Tokyo) **49:** 2091-2097.
4. **Fukaya, M. , H. Okumura, H. Masai, T. Uozumi, and T. Beppu.** 1985. Construction of new shuttle vectors for *Acetobacter.* Agric. Biol. Chem. (Tokyo) **49:** 2083-2090.
5. **Fukaya, M. , H. Okumura, H. Masai, T. Uozumi, and T. Beppu.** 1985. Development of a host-vector system for *Gluconobacter suboxydans.* Agric. Biol. Chem. (Tokyo) **49:** 2407-2411.
6. **Fukaya, M. , Y. S. Park, and K. Toda.** 1992. Improvement of acetic acid fermentation by molecular breeding and process development. Journal of Applied Bacteriology **73:** 447-454.
7. **Hardy, K.** 1981. Bacterial plasmids, Thomas Nelson and Sons, Ltd., Ontario, Canada.
8. **Johnson, J. L.** 1973. Use of nucleic-acid homologies in the taxonomy of anaerobic bacteria. Int. J. Syst. Bacteriol. **23:** 308-315.

9. **Jones, D.** 1983. Impact of plasmids and transposons on microbial systematics, p. 119-124. *In* D. Schlessinger (ed.), Microbiology - 1983, American Soc. of Microbiology, Washington, D. C..

10. **Kinashi, H. , M. Shimaji, and A. Sakai.** 1987. Giant linear plasmids in *Streptomyces*. which code for antibiotic biosynthesis genes. Nature (London) **328:** 454-456.

11. **Kinashi, H. , and M. Shimaji-Murayama.** 1991. Physical characterization of SCP1, a giant linear plasmid from *Streptomyces coelicolor*. J. Bacteriol. **173:** 1523-1529.

12. **Lachance, M.** 1980. Simple method for determination of Deoxyribonucleic acid relatedness by thermal elution in hydroxyapatite microcolumns. Int. J. Syst. Bacteriol. **30:** 433-436.

13. **Mason, L. M., and G. W. Claus.** 1989. Phenotypic characteristics correlated with DNA-sequence similarities for three species of *Gluconobacter: G. oxydans* (Henneberg, 1897) DeLey 1961, *G. frateruii* sp. nov., and *G. asai* sp. nov.. Int. J. Syst. Bacteriol. **39:** 174-184.

14. **Micales, B. K., J. L. Johnson, and G. W. Claus.** 1985. Deoxyribonucleic acid homologies among organisms in the genus *Gluconobacter*. Int. J. Syst. Bacteriol. **35:** 79-85.

15. **Okumura, H. , T. Uozumi, and T. Beppu.** 1985. Construction of plasmid vectors and a genetic transformation system for *Acetobacter aceti*. Agric. Biol. Chem. (Tokyo) **49:** 1011-1017.

16. **Palleroni, N. J., C. W. Despreaux, K. R. Rittweger, and G. Higgins.** 1993. Transfer of wide host range plasmids to *Gluconobacter oxydans* and isolation of primes by intergeneric mating. sumbitted.

17. **Qazi, G. N., V. Verma, R. Parshad, and C. L. Chopra.** 1989. Plasmid-mediated direct-glucose oxidation in *Gluconobacter oxydans*. J. Biotechnology **10:** 85-88.

18. **Rainbow, C.** 1966. Nutrition and metabolism of acetic acid bacteria. Wallerstein Lab. Commun. **29:** 5-15.

19. **Sambrook, J., E.F. Fritsch & T. Maniatis.** 1989. Molecular cloning: A laboratory manual (Book 1), 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.

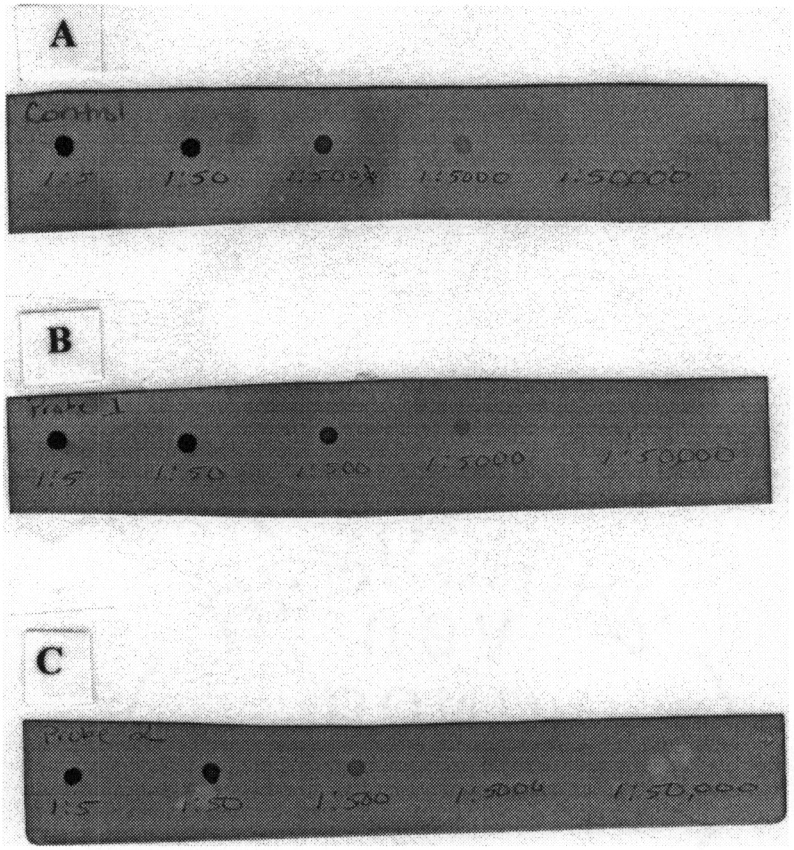
20. **Stanisich, V.A.** 1984. Identification and analysis of plasmids at the genetic level, p. 5-32. *In* P.M. Bennett, and J. Grinsted (ed.), Methods in microbiology, Academic Press, London.

21. **Valla, S. , D. H. Coucheron, and J. Kjosbakken.** 1987. The plasmids of *Acetobacter xylinum* and their interaction with the host chromosome. Mol. Gen. Genet. **208:** 76-83.

APPENDIX 4A

Estimation of the yield of digoxigenin-labeled plasmid DNA

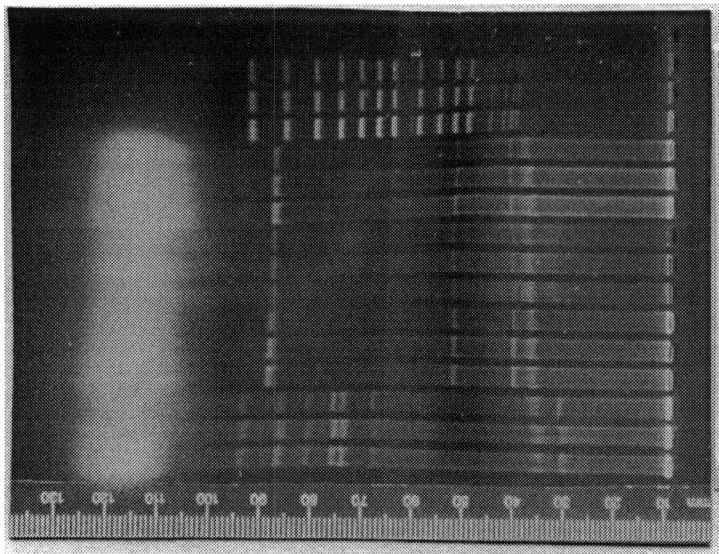
Figure B1. Ethidium-bromide stained agarose gel of electrophoresed plasmids (left) and the Southern-blot analysis of this gel using the 2.9 kb plasmid from *G. oxydans* ATCC strain 621 as the probe (right). This gel contained different volumes of plasmid DNA from each of the three *G. oxydans* strains, the plasmid DNA from *E. coli* strain V517, and the supercoiled kb ladder. The following volumes of each plasmid preparation and the supercoiled kb ladder loaded into the wells of the agarose gel : 5 μl, 10 μl and 15 μl. This hybridization experiment was used to determine if the ability of the 2.9 kb probe to hybridize with the plasmids was affected by different concentrations of plasmid DNA.


A
Control
1:5
1:50
1:500
1:5000
1:50000
B
1:5
1:50
1:500
1:5000
1:50000
C
1:5
1:50
1:500
1:5000
1:50,000

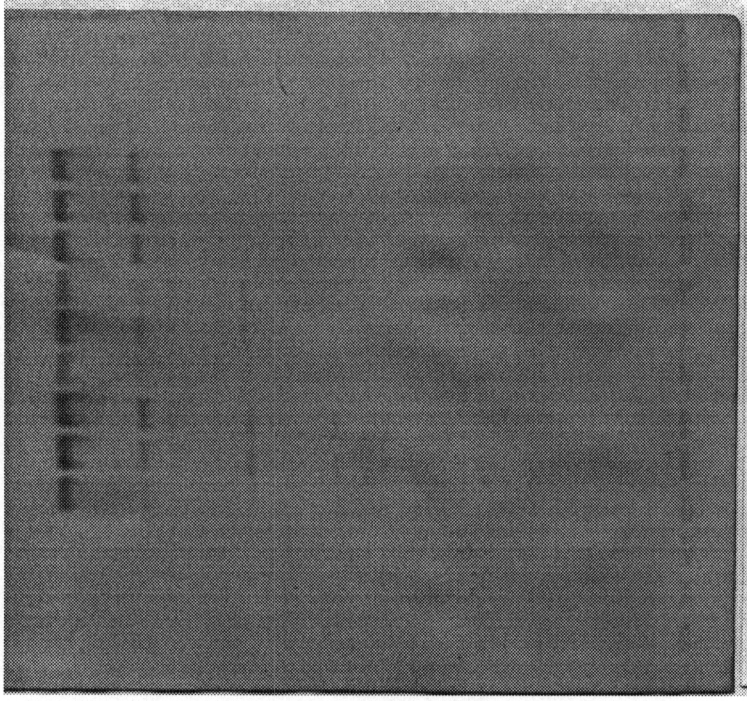
APPENDIX 4B

Effect of DNA concentration on Southern analysis using digoxigenin-labeled plasmid DNA

Figure A1. Representative membrane used to estimate the yield of digoxigenin-labeled plasmid DNA. (A) Dilutions of labeled control DNA spotted in 1 μl quantities on a positively charged nylon membrane. From left to right: 1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl, and 0.1 pg/μl of labeled control DNA. (B) Dilutions of one preparation of digoxigenin-labeled plasmid DNA spotted in 1 μl quantities on the positively charged nylon membrane. Dilutions from left to right: 1:5, 1:50, 1:500, 1:5,000, and 1:50,000. (C) Dilutions of another preparation of digoxigenin-labeled plasmid DNA spotted in 1 μl quantities on the positively charged nylon membrane. Dilutions from left to right: 1:5, 1:50, 1:500, 1:5,000, and 1:50,000.



S.C. kb LADDER (5µl)
S.C. kb LADDER (10 µl)
S.C. kb LADDER (15 µl)
ATCC 621 (5 µl)
ATCC 621 (10 µl)
ATCC 621 (15 µl)
IFO 12528 (5 µl)
IFO 12528 (10 µl)
IFO 12528 (15 µl)
ATCC 23652 (5 µl)
ATCC 23652 (10 µl)
ATCC 23652 (15 µl)
E. coli V517 (5 µl)
E. coli V517 (10 µl)
E. coli V517 (15 µl)



E. coli V517 (15 µl)
E. coli V517 (10 µl)
E. coli V517 (5 µl)
ATCC 23652 (15 µl)
ATCC 23652 (10 µl)
ATCC 23652 (15 µl)
IFO 12528 (15 µl)
IFO 12528 (10 µl)
IFO 12528 (5 µ)
ATCC 621 (15 µl)
ATCC 621 (10 µl)
ATCC 621 (5 µl)
S.C. kb LADDER (15 µl)
S.C. kb LADDER (10 µl)
S.C. kb LADDER (5 µl)

DISSERTATION CONCLUSIONS

Chapter 1. Development and use of a tetrazolium assay for measuring useful biotransformations in *Gluconobacter* strains.

1. A distinct advantage of the TNBT reduction assay is the broad range of optima such as pH, temperature, and substrate concentration.
2. *Gluconobacter* strains from the same species show as much variation in oxidative ability as strains from different species.
3. Other advantages of the TNBT reduction assay include the ability to use whole cells as opposed to membrane fractions or purified enzymes, the potential to be miniaturized and/or used qualitatively, and the ability to perform the assay in a short amount of time.

Chapter 2. Characterization of plasmids from the three species of *Gluconobacter*.

1. Eighteen strains of *Gluconobacter* contain plasmids; four strains do not.
2. Most *Gluconobacter* strains contain from one to nine plasmids and they range in size from 2 to > 54 kb.
3. Five of the 22 *Gluconobacter* strains contain plasmids larger than 100 kb.
4. Many strains of *Gluconobacter* contain plasmids of similar size suggesting that plasmids may have been transferred between strains.

Chapter 3. Attempts to correlate phenotypic characteristics with *Gluconobacter* plasmids.

1. Susceptibility to antimicrobial agents within the gluconobacters is not strain specific or species specific, therefore antimicrobial-agent susceptibility cannot be used as a phenotypic characteristic to distinguish strains of this genus.
2. Strains of *Gluconobacter* are resistant to many different antimicrobial agents tested.
3. Antimicrobial resistance mechanisms within the gluconobacters are most likely associated with chromosomal DNA and not plasmid DNA.
4. Oxidations carried out by membrane-bound dehydrogenases within the gluconobacters do not appear to be plasmid related. The oxidation of butanol may be an exception.
5. To date, *Gluconobacter* plasmids do not encode natural genetic markers such as antimicrobial agent resistance or the ability to utilize a particular carbon source.

Chapter 4. Hybridization among plasmids from the three species of *Gluconobacter*.

1. *Gluconobacter* plasmids hybridize more frequently with plasmids from strains of the same species than with plasmids from other *Gluconobacter* species.
2. *Gluconobacter* plasmids may have transferred between strains of the same species and even between species at some time in the past.

SUGGESTIONS FOR FUTURE INVESTIGATORS

1. Screen more strains of *Gluconobacter* using the TNBT reduction assay to determine how they oxidize the same 13 substrates used in this study. These results can be compared to the existence of plasmids within these strains to determine if any other possible correlations are observed between plasmids and oxidation capability.

2. Determine heavy metal susceptibility for *Gluconobacter* strains whose plasmid profiles are characterized in this study. Heavy metal resistance, if it occurs, may be plasmid encoded, and therefore, might serve as a genetic marker for use in further plasmid analyses.

3. Perform hybridization analysis using plasmid probes from both *Gluconobacter* and *Acetobacter*. If hybridization occurs between genera, this will suggest that plasmid transfer is possible between these closely related genera of acetic acid bacteria.

4. Further characterize *Gluconobacter* plasmids with the use of restriction enzymes. Restriction mapping of these plasmids may lead to greater use of these plasmids as DNA vectors. Restriction mapping should reveal restriction sites that could be used for cloning a genetic marker (reporter gene) into these plasmids to allow for their further study.

5. Develop DNA probes using published sequences of membrane-dehydrogenase genes from strains of *Gluconobacter* (or *Acetobacter*), and see if these probes will hybridize to *Gluconobacter* plasmids and/or chromosomal DNA. Positive hybridizations will indicate whether some of these genes (or portions of them) are encoded on plasmids, chromosomal DNA, or both.

CURRICULUM VITAE

LORI LYNN BROOKMAN
July, 1995

Birthdate and Place:

May 24, 1967 Baltimore, Maryland

Addresses:

Residence: 750 Tall Oaks Drive Apt. 12500A Blacksburg, VA 24060 Ph. (703) 951-7351

Office: 4005 Derring Hall Department of Biology Virginia Tech Blacksburg, VA 24061-0406 Ph. (703) 231-3997

Education:

1989 Bachelor of Science (*cum laude*) Virginia Tech
Major-Biology (Microbiology emphasis) GPA: 3.5/4.0

1995 Ph. D. Expected Virginia Tech
Microbial Physiology GPA: 3.8/4.0

Research Interest:

Physiology of Bacteria

Research Experience:

1988-1989 Undergraduate - "Isolation of gibberellic acid degrading bacteria from soil"

1989-1991 Graduate - "Phytohormone biosynthesis by rhizobacteria in culture and in soil"

1992-present Graduate - "Physiological significance of plasmids within the genus *Gluconobacter*"

Teaching Experience:

1989 **Principles of Biology Laboratory.** Three lab sections each with ~ 30 students. Totally responsible for teaching the laboratory. Subject matter included fetal pig dissection, plant physiology and morphology.

1990-1991 **Soil Microbiology Laboratory.** Two lab sections each with ~ 15 students. Totally responsible for teaching the laboratory. Subject matter included chemical properties of soils, enumeration of soil microbial populations, most probable number technique, and soil biochemical analysis.

1990-1994 **General Microbiology Laboratory.** A total of 13 lab sections (2 sections each for 6 semesters). Totally responsible for teaching the laboratory. Subject matter included microscopy, staining and cell structure, growth and enumeration of bacteria, metabolic products, molecular biology, medical microbiology, environmental microbiology, food microbiology and unknown identification.

1993 **Pathogenic Bacteriology Laboratory.** One lab section with ~ 30 students. Totally responsible for teaching the laboratory. Genera studied included *Streptococcus, Corynebacterium, Mycobacterium, Haemophilus,* various genera belonging to *Enterobacteriaceae,* anaerobic bacteria, *Treponema* and others. Laboratory exercises included isolation and identification of unknown microorganisms, fluorescent antibody test, co-agglutination test, ELISA, use of several commercially available kits, and the use of anaerobic culture techniques.

1993 **Cell Biology.** Attended lectures and assisted faculty member by holding weekly review sessions pertaining to material discussed in lecture. Met with students individually to answer questions pertaining to lecture material.

Laboratory Preparation Experience:

1995 **Culture curator.** Given the responsibility of preparing all cultures and setting-up demonstrations for use in microbiology laboratories. Coordinated weekly meetings with Graduate Teaching Assistants.

Research Grants Received:

1991 "Influence of several environmental parameters on indole acetic acid synthesis in *Azotobacter*". Graduate Research Development Project.

1992 "Development of a rapid screening assay for potentially useful *Gluconobacter* biotransformations". Graduate Research Development Project.

1993 "Use of a tetranitroblue tetrazolium assay in the determination of species related oxidations within the genus *Gluconobacter*". Sigma Xi Grant-In-Aid of Research.

Research Grants Submitted:

1992 "The use of tetranitroblue tetrazolium in the characterization of useful *Gluconobacter* biotransformations". Sigma Xi Grant-In-Aid of Research.

1993 "Determination of antibiotic susceptibility of two strains of *Gluconobacter*". Sigma Xi Grant-In-Aid of Research.

1994 "Location of a membrane-bound glucose dehydrogenase gene in strains of *Gluconobacter*". Sigma Xi Grant-In-Aid of Research.

Unpublished Reports:

1992 "Development of a rapid screening assay for potentially useful *Gluconobacter* biotransformations". Progress report submitted to the Graduate Student Assembly, Virginia Tech.

1993 "Determining the oxidative characteristics of intact cells of *Gluconobacter* species using the tetranitroblue tetrazolium reduction assay". Submitted to Research and Development, Genencor International, Inc.

Publications:

Manuscript in preparation:

1995 Brookman, L. L. and G. W. Claus. "Characterization of plasmids among the three species of *Gluconobacter*. To be submitted to the Journal of Industrial Microbiology

Manuscript submitted:

1995 Brookman, L. L. and G. W. Claus. "Development and use of a tetranitroblue tetrazolium reduction assay in the determination of oxidative capabilities among three species of *Gluconobacter*". Submitted to the Journal of Industrial Microbiology.

Published Abstracts:

1993 Brookman, L. L. and G. W. Claus. "Development and use of a tetrazolium assay for measuring useful biotransformations in *Gluconobacter* strains". Abstracts of the 93rd General Meeting of the American Society for Microbiology. Page 324; Abstract O-34.

1994 Brookman, L. L. and G. W. Claus. "Types and similarities of plasmids from selected strains of *Gluconobacter*". Presented at the 18th Annual Mid-Atlantic Extrachromosomal Elements & Molecular Genetics Meeting held in Virginia Beach on 4-6 November. In press.

1995 Brookman, L. L. and G. W. Claus. "Types and similarities of plasmids from selected strains of *Gluconobacter*". Abstracts of the 95th General Meeting of the American Society for Microbiology. Page 380; Abstract O-68.

Unpublished Research Presentations:

1992 Brookman, L. L. and G. W. Claus. "Development and use of a tetrazolium assay for measuring useful biotransformations in *Gluconobacter* species". Paper read at the Annual Meeting, Virginia Branch-American Society for Microbiology held in Williamsburg on 13-14 November.

1993 Brookman, L. L. and G. W. Claus. "A comparison between plasmid profiles and the limited oxidations of *Gluconobacter* species". Paper read at the Annual Meeting, Virginia Branch-American Society for Microbiology held in Lexington on 12-13 November.

1994 Brookman, L. L. and G. W. Claus. "Types and similarities of plasmids from selected strains of *Gluconobacter*". Paper read at the Annual Meeting, Virginia Branch-American Society for Microbiology held in Richmond on 2-3 December.

Other Meetings Attended:

1991	Annual Meeting, Virginia Branch-American Society for Microbiology held in Blacksburg on 1-2 November.
1992	92nd General Meeting of the American Society for Microbiology held in New Orleans, LA on 27-30 May.
1994	94th General Meeting of the American Society for Microbiology held in Las Vegas, NV on 23-27 May.

Seminars Given:

1990	"Phytohormone production by rhizobacteria". Given during Graduate Microbiology Seminar, Virginia Tech.
1993	"The genetics of the acetic acid bacteria". Given during Graduate Microbiology Seminar, Virginia Tech.

Positions Held:

1988-1989	Secretary, Virginia Tech Biology Club
1991-1992	Member At-Large, Biology Association of Graduate Students, Virginia Tech
1992-1994	Treasurer, Biology Graduate Student Association, Virginia Tech

Community Service:

1993	Instructed molecular biology short course for Graham High School A.P. Biology class (Bluefield, VA)

Honors and Awards:

Cum laude graduate

Instructional Fees Scholarships

Honorary Societies:

Phi Sigma

Golden Key National Honor Society

Garnet and Gold Women's Honor Society

Gamma Beta Phi National Honor Society

Sigma Xi Scientific Research Society - Associate Member

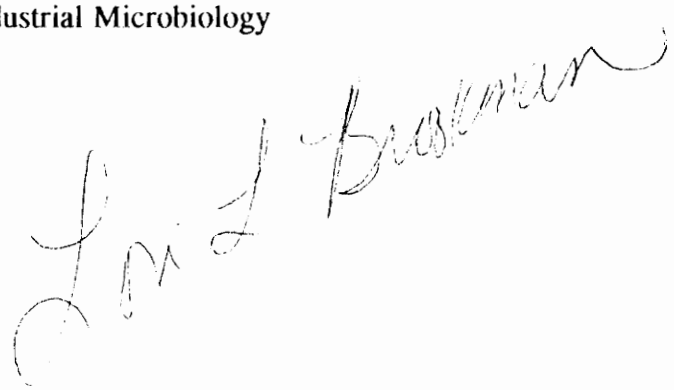
Professional Societies:

American Society for Microbiology

Virginia Branch-American Society for Microbiology

Virginia Academy of Science

Society of Industrial Microbiology



CHARACTERIZATION OF PLASMIDS AMONG THE THREE SPECIES OF *GLUCONOBACTER*

by

Lori L. Brookman

G. William Claus, Chairman

Department of Biology

Although much is known about the physiology of limited oxidations in the gluconobacters, little is known of their genetics, especially the plasmids. The initial purpose of this dissertation was to determine if *Gluconobacter* plasmids correlate with oxidative ability and/or antibiotic resistance. Twenty-two strains representing the three species of *Gluconobacter* were examined for the presence of plasmids. Plasmids were detected in all but four strains, and they ranged in size from 2 to > 54 kb. I determined susceptibility to antimicrobial agents by various strains of *Gluconobacter* using agar diffusion assays. Most strains showed almost identical susceptibility to the antimicrobial agents tested, and no relationship was observed between plasmids and antimicrobial agent resistance. I also developed an assay that used an unusual artificial electron acceptor, tetranitroblue tetrazolium and then tested the ability of six strains to oxidize 13 chemical compounds. Although most strains were able to oxidize the 13 compounds tested, they accomplished this with varying extents of oxidation. These differences were noted even with strains representing the same species. Only one possible correlation between plasmids and oxidative ability was found, and that was between the presence of plasmids and the ability to oxidize butanol. However, I

chose not to study this further, because these plasmids had no known genetic markers for tracking plasmid transformations into *Gluconobacter* strains that lacked plasmids. To further characterize *Gluconobacter* plasmids, I used hybridization analysis to determine plasmid relatedness between strains representing the three species. Isolated plasmids were non-radioactively labeled and used to probe plasmids from other strains of *Gluconobacter*. I found that more similarities existed between plasmids from strains of the same species, although, plasmids from strains of different species also hybridized with one another. I did not observe a relationship between the size of the probes and the plasmids they hybridized with, however, all probes hybridized with plasmids that were at least as large as the probe used. Although these plasmids remain cryptic, I found that portions of the DNA sequence of some plasmids has been conserved over time.