UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE MICROBIAL CONVERSION OF SYNTHESIS GAS TO ETHANOL: CHARACTERIZATION AND OPTIMIZATION

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

In partial fulfillment of the requirements for the

Degree of

Doctor of Philosophy

By

Shih-Chuan (Jack) Liou

Norman, Oklahoma

2005

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

THE MICROBIAL CONVERSION OF SYNTHESIS GAS TO ETHANOL:

CHARACTERIZATION AND OPTIMIZATION

A Dissertation APPROVED FOR
THE DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

_____

Dr. Ralph S. Tanner

_____

Dr. Michael J. McInerney

_____

Dr. David P. Nagle

_____

Dr. Mark A. Nanny

_____

Dr. John S. Downard

ACKNOWLEDGEMENTS

The work presented in this dissertation was supported by research grants from Aventine Renewable Energy, Inc., a U.S. Department of Agriculture CSREES Special Research Grant, the Oklahoma Agricultural Experimental Station and the University of Oklahoma Foundation.

I would like to express my sincere gratitude to Dr. Ralph S. Tanner for having been an extraordinary mentor and friend. I have been extremely fortunate to have the benefit of his guidance and experience in the many spheres of microbiology. The advice and support that he has provided me has allowed me to develop professionally and grow maturely as a scientist. Regardless of which field of microbiology one day receives me; I will always apply what I have learned from him, transcend the mere requirements and be the best in my field. Dr. Tanner, thank you so much for these wonderful six years.

I would also like to thank the members of my committee, Dr. Jimmy Ballard, Dr. John Downard, Dr. Michael McInerney, Dr. David Nagle, and Dr. Mark Nanny for finding time in their busy schedules to meet with me. I especially thank Dr. Michael McInerney and Dr. Mark Nanny for their advice and life lessons throughout my time at OU. Thank you for the letters of recommendation.

I thank Dr. Lisa Geig and Neil Wofford for showing me how to use and trouble shoot the fatty acid GC. I thank Chris Struchtemeyer for all his help with molecular techniques. Thank you for all of your patience.

I thank Dr. Matthew Caldwell and Dr. Tamara Marsh for the countless hours of editing, advice and their openness to answer questions and discuss topics whether they were related to my research or to life experiences.

I thank all past and present members of the Tanner lab for their tolerance and helpful discussions. I especially thank Toby Allen and Jason McGuire who have contributed to my research goals.

I am grateful to my best friend, John E. Sacks for the confidence and patience you have shown me throughout the years. I could not have asked for a more loyal friend and confidant.

Special thanks to my family, my brother and sister, Justin S. Liou and Iris S. Liou, for all of their support and encouragement, and my parents, Yao-Meng and Chin-Yeh Liou for keeping me focused on my studies. This work would not have been possible without their support.

Most importantly, I thank my extraordinary friend, Lillian Rojo, M.D who taught me the true meaning of determination and hard work. She has inspired me professionally, and I was so fortunate to have the benefit of her love and friendship throughout the past seven years. Thank you so much for everything.

Finally, I thank my wife Lara for her love and constant words of encouragement. Words could never express how much her love and support has helped me……..

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

As a result of the oil crisis of the 1970's, national research began to explore alternatives to petroleum-based energy sources. Initiatives sponsored by the Department of Energy (DOE) have encouraged the transition from petroleum-based to bio-based energy. DOE projections estimate that products and/or processes based on biological raw materials will replace 10% of the demand on fossil fuels by year 2020. One aspect of this policy has targeted research involving biomass-based energy products not only to limit greenhouse gas emissions but for economic development in rural areas. Traditionally, agricultural crops have served as sources of raw materials (i.e., for the bioconversion to ethanol) such as corn starch and beet sugar (the most prominent bio-based energy product). However, lignocellulosic biomass can also be converted to ethanol by an indirect fermentation process, in which biomass is first gasified (similar to coal gasification, producing carbon monoxide (CO)), followed by fermentation of the carbon monoxide to ethanol. Whereas this process offers a more cost efficient way to bioproduce ethanol, many challenges exist in optimizing synthesis gas fermentation. Microbial strain development through increasing cell densities as well as overcoming the effects of inhibitory synthesis gas constituents are required before large scale fermentation can go online. Microbial enhancements and process improvements have thus far been limited by the number of viable candidates with much of the current work focused on acetogens or butyric acid bacteria as potential microbial catalysts for synthesis gas fermentation. Hence,

our research objectives concentrated on the enrichment, isolation and characterization of anaerobic microorganisms capable of the conversion of carbon monoxide to ethanol.

By screening 310 enrichments from 37 different sources of inocula (e.g., sediments, lagoons, compost, silage, and animal feces), seven potential microbial candidates (strain P7, P11, P14, P20, P21, MU1 and SK1) were isolated for the conversion of synthesis gas to ethanol. The research presented herein focuses on *Clostridium carboxidivorans* strain P7 and is centered on three main topics: phylogeny, microbial physiology, and development for industrial scale purposes.

Phylogenetically, *C. carboxidivorans* is very similar to *Clostridium drakei* (99.8%) and *Clostridium scatologenes* (99.7%) in their 16S rRNA gene sequences. All three strains are in cluster I (subcluster Ic) of *Clostridium* group I. The DNA-DNA reassociation value of *C. carboxidivorans* to *C. drakei* and to *C. scatologenes* was 31.8% and 50.2%, respectively. Using a threshold value of 70% for the definition of the same species, these three clostridia each represent a different species. *C. carboxidivorans* is able to convert C_1 substrates to ethanol with relatively high efficiency. A fermentation balance of *C. carboxidivorans* when grown on carbon monoxide yielded ethanol, acetate, butanol, and butyrate as end products. These same end products were also observed when *C. carboxidivorans* was grown on fructose. Growth of *C. carboxidivorans* required the addition of pantothenic acid, para-amino benzoic acid (PABA), and biotin. Growth on gaseous substrates was stimulated with the addition of isoleucine and proline. Chemolithotrophic growth did not occur without an initial addition of

carbon dioxide (CO_2) with CO as the primary substrate. Increased ethanol production by *C. carboxidivorans* strain P7 was achieved by changing physiochemical properties of the growth media (i.e., pH, Fe^{+2}) or by adding exogenous ethanol or the metabolic inhibitors trifluoroacetate or fluoroacetate. In addition, growth of *C. carboxidivorans* was increased from an initial value of 0.7 grams per liter to 10 grams per liter through growth medium optimization. Because of the results presented here, further studies to determine the commercial potential for the production of biofuels from CO by strain P7 seem warranted.

INTRODUCTION

The development of renewable biofuels (primarily ethanol from biomass) is a national priority motivated by both economic and environmental concerns including the reduction of petroleum imports and greenhouse gas emissions and enhancement of the domestic fuel supply (Dale, 2003). Biomass primarily refers to agricultural crops or plant material, but other sources of biomass such as residues from agricultural practices, forestry and organic components of municipal and industrial wastes are generating greater interest (National Energy Policy, 2001). Biomass is a continuous and constantly replenished energy crop and can be directly and indirectly converted into liquid fuels or "biofuels." The most common biofuels are ethanol and biodiesel. Biodiesel is used mainly as a diesel additive made from vegetable oils, animal fats, algae, or even recycled cooking greases (National Energy Policy, 2001). Ethanol is made directly by fermenting any biomass that is rich in carbohydrates, such as corn.

The current practice for producing ethanol involves the saccharification fermentation of simple sugars from cornstarch (Ladisch and Svarczkopf, 1991). Though the technology is well established and the raw material (corn) is readily available, particularly in the Midwestern states of US, the limiting factors for this technology are its dependence on corn as the feedstock and the complexities (pretreatment) associated with the release of polymeric sugars prior to fermentation (Mielenz, 2001). Through this process, the United States currently produces about 5 billion liters of ethanol from starch crops, mainly corn.

However, the projected target for ethanol production is between 38-53 billion liters per year or enough for a 10% ethanol blend of gasoline for use in the US (Mielenz, 2001). Therefore, the demand for more ethanol is great, and alternative means of producing ethanol are necessary in order to meet this objective (Mielenz, 2001; Dale, 2003).

The use of lignocellulosic biomass as a feedstock for a direct fermentation process to produce ethanol is an attractive alternative (Dale, 2003). Lignocellulosic biomass is an inexpensive source of carbohydrate and is abundant (Mielenz, 2001). Lignocellulosic feedstocks (e.g. agricultural residues, herbaceous energy crops, and short rotation hardwoods) are composed of 35-50% cellulose, 20-40% hemicellulose, and 15-30% lignin (Claassen et al., 1999). The cost of producing ethanol from lignocellulosic biomass has been greater than the cost of gasoline; however, continued development of a direct fermentation method has resulted in inexpensive and effective processing technology for the breakdown of lignocellulosic material to fermentable sugars (Lynd, 1996; Claassen et al., 1999; Mielenz, 2001). For example, the simultaneous saccharification and fermentation (SSF) process and simultaneous saccharification cofermentation (SSCF) process were developed to significantly reduce production costs by conducting cellulose hydrolysis simultaneously with fermentation of the glucose (Mielenz, 2001). New methods in molecular biology and genetic engineering have created recombinant strains of *Saccharomyces*, *Escherichia coli* and *Zymomonas mobilis* that utilize xylose and arabinose sugars (constituents of lignocellulose) in a direct fermentation process (Mielenz, 2001;

Ingram et al. 1998; Ingram et al. 1999). However, these strains do not currently use polyphenolic lignin and other 'extractables' that constitute 15-30% of the plant's matrix. Continuous improvement of these strains will be necessary to create an ideal strain that will directly ferment all biomass sugars, tolerate inhibitors and alcohols, grow at low pH and high temperature ranges and have a high specific growth rate (Zaldivar et al., 2001).

Recent work in genetic engineering on *Saccharomyces cerevisiae* has shown great promise for commercialization. Several genes for cellulolytic enzymes (ß-glucosidase I, endoglucanase II, and cellobiohydrolase II) from *Trichoderma reesei* were successfully inserted into a strain of *Saccharomyces cerevisiae* (Fujita et al., 2004). The recombinant yeast yielded 0.45 g/g (in grams of ethanol produced per gram of carbohydrate consumed), which corresponded to 88.5% of the theoretical yield. *Escherichia coli* strain KO11 is another recombinant microorganism that shows great promise. Strain KO11 can produce ethanol from xylose at 94% of the theoretical yield (1.667 mmol of ethanol produced per mmol of xylose consumed) (Underwood et al., 2002). All of these advancements in direct fermentation of biomass have allowed BC International Corp. to build the first US 30-million-gallon biomass-to-ethanol plant in Jennings, LA, which is anticipated to be operational by the end of 2006.

A potential alternative process for converting biomass-lignocellulosic feedstock- to ethanol is through the indirect fermentation of the gasification of biomass to synthesis gas (CO_2, CO, H_2) followed by the subsequent bioconversion of synthesis gas to ethanol. Biomass gasification is an established

process that produces a valuable gas from a renewable solid (Gil et al., 1997). The subsequent heat from the gasification process can be used in distillation of the final product. Previously, the bioconversion of the synthesis gas via a microbial catalyst was considered an unproven alternative (Grethlein and Jain, 1992); however, the use of biological catalysts would offer low operating temperatures and pressures, high yields, high end-product specificity, low energy costs and tolerance to sulfur species in the synthesis gas (Grethlein and Jain, 1992; Klasson et al., 1992). An additional benefit is the potential impact this process could have on the greenhouse gas balance. This process has the potential to reduce air pollution since lignocellulosic materials such as switchgrass, elephant grass, and prairie bluestem are carbon neutral, consuming as much CO_2 in growth as they produce in combustion.

Acetogenic bacteria have shown the greatest promise for converting synthesis gas into ethanol and other important solvents. This group of bacteria was physiologically defined by its ability to produce acetic acid as the sole metabolic end-product from a variety of substrates ranging from carbohydrates to synthesis gas. The classification of acetogenic bacteria evolved to a more specific definition which includes bacteria that grow by producing acetic acid via the carbon monoxide dehydrogenase (CODH) pathway, often referred to as the acetyl-CoA Wood/Ljungdahl pathway (Drake, 1994; Drake et al., 2002). Most acetogens are phylogenetically classified as clostridial species (Tanner and Woese, 1994), with several clostridial species known to utilize CO as a substrate. These organisms include *Acetobacterium woodii* (Ljungdahl, 1983), *Clostridium*

aceticum (Ljungdahl, 1983), *Moorella thermoaceticum* (Daniel et al., 1990), *Eubacterium limosum* (Ljungdahl, 1986), and *Acetogenium kivui* (Daniel et al., 1990).

The microbial catalysts of choice for the bioconversion of synthesis gas to acids and alcohols include acetogens such as *Butyribacterium methylotrophicum* (Grethlein et al., 1990; Drake et al., 2002), *Clostridium autoethanogenum* (Abrini et al., 1994), and *Clostridium ljungdahlii* (Barik et al., 1988; Tanner et al., 1993). The physiological characteristics of these three strains were used to guide enrichment and screening of potential acetogens in the bioconversion of synthesis gas to acids and alcohols. The selection of *B. methylotrophicum* was based on its metabolic versatility (Grethlein and Jain, 1992). *B. methylotrophicum* grows on 100% CO and produces ethanol, acetate, butyrate, and n-butanol as end products (Grethlein et al., 1991). When grown using different carbon sources, *B. methylotrophicum* produced distinctive end-products. Growth with H_2:CO_2 produced acetate, while growth on methanol (in the presence of acetate) produced butyrate at a 4:1 methanol:butyrate ratio (Grethlein and Jain, 1992). pH also had an affect on acid production in *B. methylotrophicum*. At pH 6.8, *B. methylotrophicum* produced acetate in a 32:1 acetate:butyrate molar ratio, whereas at pH 6.0, the acetate to butyrate ratio was 1:1 (Grethlein and Jain, 1992). At lower pH, *B. methylotrophicum* produced minor butanol and ethanol end-products.

On the other hand, *C. autoethanogenum* and *C. ljungdahlii* (Barik et al., 1988; Tanner et al., 1993) were primarily selected for their ability to produce high

concentrations of ethanol from synthesis gas. Both *C. autoethanogenum* and *C. ljungdahlii* have the ability to convert $CO:CO_2$ to ethanol and acetate. The type strain of *C. autoethanogenum* typically produces ethanol concentrations of approximately 3-5 mM (Abrini et al., 1994), where as type strain of *C. ljungdahlii* can produce ethanol concentrations as high as 2 mM in batch studies (Tanner et al., 1993) or 500 mM when grown in continuous culture (Phillips et al., 1993).

As with *B. methylotrophicum*, pH has a profound effect on acid and alcohol production when *C. ljungdahlii* is grown on carbon monoxide. Acid production is favored at pH's greater than 5.0 with an acetate to ethanol molar ratio of 20:1, whereas alcohol production is favored at pH less than 5.0 with a product molar ratio of 1:9 (Gaddy and Clausen, 1992).

Where pH has been shown to affect solvent production in acetogenic bacteria, other physiochemical factors may also contribute in altering the acid:alcohol ratio. The maximization of ethanol production in the bioconversion of synthesis gas is a prime component for the industrialization of this process. Research in this area has been expanded to include the butyric acid bacteria (Worden et al., 1991). While little is known regarding the factors influencing acetogenic production of solvents from synthesis gas, extensive research has been conducted on *Clostridium acetobutylicum*, a butyrate producing bacterium (Jones and Woods, 1986; Adler and Crow 1987; Grupe and Gottschalk 1992). Although *C. acetobutylicum* has not been shown to utilize CO, research on factors that affect solvent production have been studied in detail (Jones and Woods, 1986; Adler and Crow 1987; Worden et al., 1991; Grupe and Gottschalk 1992). For

example, decreasing the pH from 6.0 to 4.5 or adding butyrate or acetate (50 mM each) to the medium played an important role in the production of alcohols (Adler and Crow 1987; Grupe and Gottschalk 1992). In each case, butanol and acetone increased 2 to 3 fold from original conditions (Grupe and Gottschalk 1992). Grupe and Gottschalk (1992) proposed that two metabolic signals occurred during the shift from acidogenesis to solventogenesis. When the intracellular ATP concentration reached a minimum, before the onset of solventogenesis, the change in ATP and ADP ratio initiated the generation of signal one. This leads to the induction of acetoacetate decarboxylase and CoA-transferase to convert acetyl-CoA and acetate to acetone and CO_2. Because of acetone formation, inadequate amounts of acetyl- CoA act as H^+ acceptors. Thus, the redox balance cannot be stabilized during glycolysis, and signal two is generated when a pool of NADH plus NADPH drastically increases. Signal two induces the synthesis of butyraldehyde and butanol dehydrogenases, and acetone and butanol formation can proceed (Grupe and Gottschalk 1992).

Nutrients are another factor which affected the regulation of solvent production. Limiting inorganic nitrogen (NH_4^+), phosphorous (PO_4^{3-}) and sulfur (SO_4^{2-}) have stimulatory affects on solvent production (butanol) in *C. acetobutylicum* (McNeil and Kristiansen, 1987; Bahl et al. 1982; Bahl and Gottschalk 1984). In each incidence, solvent production peaked at 0.5 fold from standard concentrations when each nutrient was limited (McNeil and Kristiansen, 1987). Other factors that affected the regulation of solvent production in *C. acetobutylicum* included trace metals. Iron or other essential metals serve as

important components in biological proteins and systems (Mills, 1997). The elimination of ferrous iron in the medium decreased the production of solvents by 6.1% from the standard concentrations in *C. acetobutylicum* (McNeil and Kristiansen, 1987). Details of nutrient and cofactor additions are discussed in more detail with *Clostridium carboxidivorans* in Chapter 2.

Limiting factors to using *Butyribacterium methylotrophicum*, *Clostridium autoethanogenum* and *Clostridium ljungdahlii* in an industrial process for synthesis gas conversion are the low product yields and, hence, the final concentrations obtained. It has been proposed that for industrial synthesis gas conversion to be economically feasible, 868 mM (40 g/l) ethanol needs to be made. Clearly, a need exists for the discovery of alternative acidogenic or solventogenic microbial catalysts.

The characteristics on these three strains were used to guide the isolation and metabolic potential of acetogens in the work presented in this dissertation on the Conversion of Agricultural Biomass to Liquid Fuels. Prior to metabolic characterization, isolates were screened for the ability to produce ethanol from synthesis gas and were examined phenotypically to ensure that they were different from *B. methylotrophicum*, *C. autoethanogenum*, and *C. ljungdahlii*. Of the microorganisms isolated for ethanol production strain P7 was determined to be the most viable candidate for continued study. Strain P7 was selected based on its durability and robustness to tolerate environmental stresses (e.g., low pH ~4.2-4.5; oxygen; minimum nutrients). Moreover, strain P7 produced a significant ethanol concentration in comparison to the type strain of *C. ljungdahlii* (Tanner

et al., 1993). Phenotypic characteristics such as sole substrate-utilization, growth rate on synthesis gas, temperature and pH optima, and yield of end-products concluded that strain P7 is different from *B. methylotrophicum* (Grethlein et al., 1990), *C. autoethanogenum* (Abrini et al., 1994), and *C. ljungdahlii* (Tanner et al., 1993).

The type strain, strain P7^T (=ATCC BAA-624^T=DSM 15243^T), is a novel *Clostridium* species enriched from an agricultural settling lagoon using CO as the substrate. Strain P7 has been rename *Clostridium carboxidivorans* strain P7^T [car.bo.xi.di.vo'rans. N.L. neut. n. carboxidum, carbon monoxide, L. part. adj. vorans, devouring, N.L. part. adj. *carboxidivorans*, carbon monoxide devouring] for its ability to utilize carbon monoxide.

Chapter 1 deals with the physiochemical characterization of *C. carboxidivorans* strain P7. Acetate, ethanol, butanol and butyrate were the metabolic end-products of growth on CO:CO_2. Phylogenetic analysis was performed to characterize *C. carboxidivorans* from other clostridial species , and the effects of physiochemical variables such as temperature, pH, nutrient requirements and tolerance to oxygen, methanol, and ethanol on the metabolism of CO were examined. This chapter was written in the style of the *International Journal of Systematic and Evolutionary Microbiology* to which the article was accepted.

Chapter 2 focuses on optimizing *C. carboxidivorans* for the conversion process of synthesis gas to ethanol. Since the general principles of strain screening, identification and development for industrial purposes are well

established, especially for the production of butanol and acetone in *Clostridium acetobutylicum* (Monot, et al., 1982; McNeil and Kristiansen, 1987; Adler and Crow 1987; Grupe and Gottschalk 1992), the optimization of *C. carboxidivorans* for ethanol production from CO was followed by using *C. acetobutylicum* as a model. Studies on the effect of metabolic inhibitors (e.g., trifluoroacetate, fluoroacetate, chlorobutyrate) to decrease the production of one product while increasing the production of the desired product were conducted. Selection of strains with higher ethanol yields and increasing tolerance to ethanol was also conducted. Finally, growth on a defined medium to decrease cost by reducing the provision of nutrients in the medium was also investigated. To date, *C. carboxidivorans* has been optimized to produce 10 grams per liter of ethanol from an initial culturing of 0.7 grams per liter of ethanol. The focus of this chapter is to provide a model for the study and exploration of future microbial catalysts and to propose *C. carboxidivorans* as a potential industrial candidate for the fermentation of synthesis gas to ethanol. This chapter was written in the style required by *Applied Microbiology and Biotechnology*.

Besides the primary research on the microbial conversion of synthesis gas to ethanol, a variety of research projects with an emphasis on applied microbiology in anaerobic systems was conducted. One project was on the microbiota of *Lepomis macrochirus* (bluegills) from treated wastewater and rural lakes. Data generated in this study demonstrated the ecological impact humans and animals can have on the environment.

Appendix 1 is an extension of a finding made during the analysis of the multiple-antibiotic resistance data from a comparative study on the microbiota of the intestinal tract of bluegills from treated sewage water to that of bluegills from clean rural lakes. The overall resistance to antibiotics among native bacteria was unexpectedly high in the clean rural lakes. The two lakes in question are in sparsely populated counties with no identifiable antibiotic inputs. Ciprofloxacin resistance was present in bacterial isolates from these two lakes in high numbers. Though the results were surprising, the study suggests that a natural reservoir of resistance against fluoroquinolones exists, a fact which must be considered if widespread use of these antibiotics is undertaken in response to treating patients with infections of multidrug-resistant bacteria and for broader systemic infections. Appendix 1 was written in the style required by the *Journal of Antimicrobial Chemotherapy*.

REFERENCES

Abrini, J., Naveau, H., and Nyns, E-J. 1994. *Clostridium autoethanogenum*, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. Arch. Microbiol. 161: 345-351.

Adler, H. I. and Crow W. 1987. A technique for predicting the solvent-producing ability of *Clostridium acetobutylicum*. Appl. Environ. Microbiol. 53: 2496-2499.

Bahl, H., Andersch, W., and Gottschalk, G. 1982. Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two-stage phosphate limited chemostat. Eur. J. Appl. Microbiol. Biotechnol. 15:201-205.

Bahl, H. and Gottschalk, G. 1984. Parameters affecting solvent production by *Clostridium acetobutylicum* in continuous culture. In: Wang DIC, Scott CD (eds) Sixth Symposium on Biotechnology for Fuels and Chemicals, Vol 4, John Wiley & Sons, New York, pp 215-223.

Barik, S., Prieto, S., Harrison, S. B., Clausen, E. C., and Gaddy, J. L. 1988. Biological production of alcohols from coal through indirect liquefaction. Appl. Biochem. Biotechnol. 18: 363-378.

Claassen, P. A. M., Van Lier, J. B., Lopez-Contreras, A. M., Van Niel, E. W. J., Sijtsma, L., Stams, A. J. M., De Vries, S. S., and Weusthuis R. A. 1999. Utilization of biomass for the supply of energy carriers. Appl. Microbiol. Biotech. 52: 741-755.

Dale, B.E. 2003. Greening the chemical industry: research and development priorities for biobased industrial products. J. Chem. Technol. Biotechnol.78: 1093-1103.

Daniel, S. L., Hsu, T., Dean, S. I., and Drake, H. L. 1990. Characterization of the H_2- and CO-dependent chemolithotrophic potentials of the acetogens *Clostridium thermoaceticum* and *Acetogenium kivui*. J. Bacteriol. 172: 4464-4471.

Drake, H. L. 1994. Acetogenesis, Acetogenic Bacteria, and the Acetyl-CoA "Wood/Ljungdahl" Pathway: Past and Current Perspectives. *In Acetogenesis*, H. L. Drake (ed), pp. 3-60. New York; Chapman & Hall.

Drake, H. L., K. Küsel, and C. Matthies. 2002. Ecological consequences of the phylogenetic and physiological diversities of acetogens. Antonie Van Leeuwenhoek. 81: 203-213.

Fujita, Y., Ito, J., Ueda, M., Fukuda, H., and Kondo, A. 2004. Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. Appl. Environ. Microbiol. 70: 1207-1212.

Gaddy, J. L., and Clausen, E. C. 1992. *Clostridium ljungdahlii,* an anaerobic ethanol and acetate producing microorganism. US Patent 5,173,429.

Gil, J., Aznar, M., Caballero, M., Frances, E., and Corella, J. 1997. Biomass gasification in fluidized bed at pilot scale with steam-oxygen mixtures. Product distribution for every different operating conditions. Energy Fuels. 11: 1109-1118.

Grethlein, A. J., Worden, R. M., Jain, M. K., and Datta, R. 1990. Continuous production of mixed alcohols and acids from carbon monoxide. Appl. Biochem Biotechnol. 24/25: 875-884.

Grethlein, A. J., Worden, R. M., Jain, M. K. and Datta, R. 1991. Evidence for production of n-butanol from carbon monoxide by *Butyribacterium methylotrophicum*. J. Ferm. Bioeng. 72: 58-60.

Grethlein, A. J. and Jain, M. K. 1992. Bioprocessing of coal-derived synthesis gases by anaerobic bacteria. Trends Biotechnol. 10: 418-423.

Grupe, H. and Gottschalk, G. 1992. Physiological events in *Clostridium acetobutylicum* during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. Appl. Environ. Microbiol. 58: 3896-3902.

Ingram, L. O., Gomez, P. F., Lai, X., Moniruzzaman, B. E., Wood, L. P., Yomano, S. W., and Work, S. W. 1998. Metabolic engineering of bacteria for ethanol production. Biotechnol. Bioeng. 58: 204-214.

Ingram, L. O., Aldrich, H. C., Borges, A. C. C., Causey, T. B., Martinez, A., Morales, F., Saleh, A., Underwood, S. A., Yomano, L. P., York, S. W., Zaldivar, J., and Zhou, S. 1999. Enteric bacterial catalysts for fuel ethanol production. Biotechnol. Prog. 15: 855-866.

Jones, D. T., and Woods, D. R. 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. 50: 484-524.

Klasson, K.T., Ackerson, C. M. D., Clausen, E. C., and Gaddy, J.L. 1992. Biological conversion of synthesis gas into fuels. Int. J. Hydrogen Energy. 17: 281-288.

Ladisch, M.A. and Svarczkopf, J. A. 1991. Ethanol production and the cost of fermentable sugars from biomass. Bioresour. Technol. 36: 83-95.

Ljungdahl, L.G. 1983. Formation of acetate using homoacetate fermenting anaerobic bacteria. Org. Chem. Biomass, pp. 219-248.

Ljungdahl, L. G. 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. Annu. Rev. Microbiol. 40: 415-450.

Lynd, L. R. 1996. Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment and policy. Ann. Rev. Energy Environ. 21: 403-465.

McNeil, B. and Kristiansen, B. 1987. The effect of medium composition on the acetone-butanol fermentation in continuous culture. Biotechnol. Bioeng. 29: 383-387.

Mielenz, J. R. 2001. Ethanol production from biomass: technology and commercialization status. Curr. Opin. Microbiol. 4: 324-329.

Mills, A. L. 1997. Metal requirements and tolerance. In: Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD, Walter MV (eds) Manual of Environmental Microbiology. American Society of Microbiology, DC, pp. 349-357.

Monot, F., Martin, J-R., Petitdemange, H., and Robert, R. 1982. Acetone and butanol production by *Clostridium acetobutylicum* in a synthetic medium. Appl. Environ. Microbiol. 44: 1318-1324.

National Energy Policy. 2001. (www.whitehouse.gov/energy/National-Energy-Policy.pdf.

Phillips, J. R., Klasson, K. T., Clausen, E. C., and Gaddy, J. L. 1993. Biological Production of Ethanol from Coal Synthesis Gas. Appl. Biotechnol. Biochem. 39/40: 559-567.

Tanner, R. S. and C. R. Woese. 1994. A phylogenetic assessment of the acetogens. *In Acetogenesis*, H. L. Drake (ed), pp. 255-269. New York; Chapman & Hall.

Tanner, R. S., Miller, L. M. and Yang, D. 1993. *Clostridium ljungdahlii* sp. nov., an acetogenic species in clostridial rRNA homology group I. J. Syst. Bacteriol. 43: 232-236.

Underwood, S. A., Zhou, S., Causey, T. B., Yomano, L. P., Shanmugam, K. T., and Ingram, L. O. 2002. Genetic changes to optimize carbon partitioning

between ethanol and biosynthesis in ethanologenic *Escherichia coli*. Appl. Environ. Microbiol. 68: 6263-6272.

Worden, R. M., Grethlein, A. J., Jain, M. K. and Datta, R. 1991. Production of butanol and ethanol from synthesis gas via fermentation. Fuel 70: 615-620.

Zaldivar, J., Nielsen, J., and Olsson, J. 2001. Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process intergration. Appl. Microbiol. Biotchnol. 56: 17-34.

Chapter 1. *Clostridium carboxidivorans* sp. nov., a solvent-producing clostridium isolated from an agricultural settling lagoon, and reclassification of *Clostridium scatologenes* strain SL1 as *Clostridium drakei* sp. nov.

Abstract

A new solvent-producing anaerobic clostridium, strain P7^T, was isolated from sediment from an agricultural settling lagoon after enrichment with CO as the substrate. The metabolism of this Gram-positive motile, sporeforming rod was primarily acetogenic. Acetate, ethanol, butyrate and butanol were the end products of metabolism. Strain P7^T grew on CO, H_2:CO_2, glucose, galactose, fructose, xylose, mannose, cellobiose, trehalose, cellulose, starch, pectin, citrate, glycerol, ethanol, propanol, 2-propanol, butanol, glutamate, aspartate, alanine, histidine, asparagine, serine, betaine, choline or syringate as a substrate. Methanol, formate, D-arabinose, fucose, lactose, melibiose, amygdalin, gluconate, lactate, malate, arginine, glutamine or vanillate did not support growth. Nitrate reduction, production of indole, gelatin hydrolysis and esculin hydrolysis were not observed. Analysis of the 16S rRNA gene sequence of the isolate showed that it was closely related to *Clostridium scatologenes*^{T} (99.7% sequence homology) and clostridial strain SL1^T (99.8% sequence homology). Strain SL1^T had been classified as a strain of *C. scatologenes*^{T}. However, DNA-DNA reassociation analysis showed that both strain P7^T and strain SL1^T were novel clostridial

species. We propose that strain P7^T be named *Clostridium carboxidivorans* sp. nov. (type strain = ATCC BAA-624^T =DSM 15243^T) and that strain SL1^T be reclassified *as Clostridium drakei* sp. nov. (type strain = ATCC BAA-623^T = DSM 12750^T).

Introduction

The classic presentation of the acetogenic phenotype is the anaerobic reduction of CO_2 to acetate, with the implication that the microorganism is using the "Wood-Ljungdahl" pathway (Drake, 1994). Other C1 compounds are utilized by acetogens, including formate, methanol and CO. Some acetogens can produce additional end products of metabolism. *Acetobacterium woodii* (Buschhorn *et al.*, 1989) and *Clostridium* strain PETC (Vega *et al.*, 1989), later identified as *Clostridium ljungdahlii* (Tanner *et al*, 1993), can produce ethanol from C1 substrates. *Eubacterium limosum* produced the C4 product butyrate from H_2:CO_2 (Genthner *et al.*, 1981) and the closely related "*Butyribacterium methylotrophicum*" produced butanol from CO (Grethlein *et al.*, 1991).

The addition of other metabolic capabilities in addition to the reduction of C1 substrate to acetate was discovered by Kusel *et al.* (2000). A *Clostridium* strain, $SL1^T$, was isolated from an acidic sediment using H_2 as the energy source and presumptively identified as an acetogen. rRNA gene sequence analysis showed strain $SL1^T$ was a strain of *Clostridium scatologenes*^{T}, which is usually cultured on fermentable carbohydrates, forming acetate and butyrate as the main end products of fermentation. *C. scatologenes*^{T} was also shown to utilize H_2:CO_2 or CO, and had key enzyme activities for the acetogenic pathway, leading to the conclusion that *C. scatologenes*^{T} was an acetogen (Kusel *et al.*, 2000).

A *Clostridium* strain, designated P7^T, was enriched from an agricultural settling lagoon using CO as the substrate. This was done in a search for bacteria with the potential to ferment synthesis gas ($CO:CO_2:H_2$) and produce ethanol as a biofuel (Worden *et al.*, 1991). This strain, like strain SL1^T (Kusel *et al.*, 2000), was closely related to *C. scatologenes*^{T}. Further investigation showed that both P7^T and SL1^T were new species of *Clostridium*.

Materials and Methods

Enrichment and isolation. *Clostridium* strain P7^T was isolated by Rossukon Laopaiboon. Sediment from an agricultural settling lagoon at Oklahoma State University, Stillwater, OK was incubated with an acetogen medium (ATCC medium # 1754; Tanner *et al.*, 1993) at 37 ºC with an initial pH of 5 and an atmosphere of CO:N_2:CO_2 (70:24:6) at a gauge pressure of 230 kPa. Enrichments were monitored by GC for ethanol and acetate production. Strain P7^T was isolated from the enrichment using roll tubes (Hungate, 1969).

Microorganisms and culture conditions. *Clostridium scatologenes* ATCC 25775^T was obtained from the American Type Culture Collection. *Clostridium scatologenes* strain SL1^T (Kusel *et al.*, 2000) was obtained from the laboratory of Harold L. Drake at the University of Bayreuth. The basal medium for routine culture contained (per liter): 25 ml Mineral Solution (Tanner, 2002 [a source of sodium, ammonium, potassium, phosphate, magnesium, sulfate and calcium]); 10 ml Vitamin Solution (Tanner, 2002); 10 ml Trace Metal Solution (Tanner, 2002); 1 g yeast extract (# 0127-17-9, Difco Laboratories); 10 g 2-(*N*-morpholino)ethanesulfonic acid (MES; M-5287, Sigma-Aldrich), with the pH adjusted to 6·1; 6 ml cysteine·sulfide reducing agent (Tanner, 2002). Medium was prepared using strict anaerobic technique (Balch & Wolfe, 1976). Substrate (5 g per liter) utilization cultures were grown under an atmosphere of N_2:CO_2

(80:20) at a gauge pressure of 70 kPa. For growth with H_2 or CO, the atmosphere was H_2:CO_2 (80:20) or CO:N_2:CO_2 (70:24:6) at a gauge pressure of 230 kPa.

The pH range and optimum for growth of strain P7^T, strain SL1^T and *C. scatologenes*^{T} was examined from pH 4 to pH 8 with fructose as the substrate and a Good's buffer (20 g per liter) HOMOPIPES (pKa 4.6), MES (pKa 6.0), TES (pKa 7.2) or TAPS (pKa 8.1) as appropriate (Tanner, 2002). The temperature range and optimum for growth was examined from 4 ºC to 45 ºC. The pH and temperature ranges for strain P7^T were also examined with CO as the substrate. Further phenotypic characterization was performed using the procedures described in Holdeman *et al.* (1977) and Smibert & Krieg (1994).

Microscopy. Cells in exponential growth phase in a medium with yeast extract (5 g per liter) were fixed with 1% glutaraldehyde, spread onto carbon-coated Formvar grids, and stained with phosphotungstate (0.5 %). Transmission electron microscopy was performed using a JOEL JEM 2000 FX transmission electron microscope.

Phylogenetic/genetic analysis. DNA was isolated from cells of strain P7^T, strain SL1^T and *C. scatologenes*^{T} by chromatography on hydroxyapatite according to Cashion *et al.* (1977) or by using a modified Marmur procedure (Ludwig, 1991). The mol% G+C content was determined using the method of Mesbah *et al.*

(1989). The mol% G+C was also determined in the laboratory of Dr. Peter Schumann at the DSMZ (Braunschweig, Germany).

DNA was used as a template for PCR amplification of the 16S rRNA gene using the amplification primers fD1 and rP2 as described by Weisberg *et al.* (1991). The PCR amplification products were sequenced with an automated sequencer, and the resulting sequence assembled to produce 1,015-base contiguous DNA sequences corresponding to *E. coli* positions 23-1055 (Brosius *et al.*, 1978). These contiguous sequences were aligned by hand, based on the secondary structure of the 16S rRNA molecule (Gutell *et al*., 1994), to the most closely related sequences from both the Ribosomal Database Project and GenBank/EMBL databases. The phylogenetic positions of strain $P7^T$ and $SL1^T$ were analyzed using distance matrix (Felsenstein, 1993), maximum likelihood (Olsen *et al*., 1994) and parsimony (Swofford, 2000) methods. A heuristic search was conducted first (using the standard program defaults), followed by a bootstrap analysis (Felsenstein, 1985) to assess the branch points of the resulting phylogenetic trees. A consensus tree was generated by bootstrapping at the greater-than-50% confidence limit, with 1000 replications.

A DNA-DNA reassociation analysis was performed in the laboratory of Dr. Peter Schumann (DSMZ) as described in De Ley *et al*. (1970) with the modifications described by Escara & Hutton (1980) and Huss *et al*. (1983) using a model 2600 spectrophotometer equipped with a model 22527-R thermoprogrammer and

plotter (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992).

Repetitive DNA PCR fingerprinting was performed using the BOXA1R primer, which was obtained from Invitrogen (Carlsbad, California), and the protocol from Versalovic *et al*. (1994). PCR mixtures contained 2.5 µl 10X buffer B (500 mM KCl and 100 mM Tris·HCl), 2µl $MgCl_2 \cdot 6H_2O$ (25 mM), 0.25 µl Taq polymerase (Fisher Scientific), 0.5 µl of each deoxynucleoside triphosphate (10mM, Promega, Madison, Wisconsin), 1 µl primer, and sample DNA in a final volume of 25µl. The PCR reaction was run on a Robocycler Gradient 40 Temperature Cycler (Stratagene, Cedar Creek, Texas) using a protocol of an initial denaturation step (94 ºC, four min, one cycle), 30 reaction cycles (94 ºC for one min, 50 ºC for one min, 72 ºC for eight min), and a final extension step (72 ºC for eight min). PCR product (10 µl) was run on a 5% polyacrylamide vertical gel with a 100 base pair ladder (Fisher Scientific) for 17 hr at 26 ºC and 120 mAmps. The ethidium bromide-stained gel image was analyzed using a NucleoCam Digital Image Documentation System (Nucleo Tech Corp., San Mateo, California).

Analytical procedures. Growth in liquid cultures was measured at 600 nm using a Spectronic 20D spectrophotometer (Milton Roy Co., Rochester, New York; Balch & Wolfe, 1976). Fructose was measured using the phenol-sulfuric acid carbohydrate assay (Dubois *et al*., 1956). Acetate, ethanol, butyrate and butanol were measured using a 3400 Varian GC (Varian, Walnut Creek, California)

equipped with a FID detector and a 6 foot glass column packed with Carbopack B DA 80/20 4% Carbowax 20 M resin (Supelco, Bellefonte, Pennsylvania). The concentration of acetate and butyrate was confirmed using ion exclusion HPLC on an Aminex HPX-87H organic acid analysis column (Bio-Rad, Richmond, California), and the concentration of ethanol confirmed using an alcohol dehydrogenase ethanol assay (Kit 333-B, Sigma-Aldrich). Gasses were measured using a GC equipped with a TCD detector (Varian), and a Porapak Super Q column (Alltech, Deerfield, Illinois) or a molecular sieve 5A column (Alltech).

Results and Discussion

Morphology of strain P7

Colonies of P7^T grown with CO appeared white and opaque, with lobate edges, and were 2-4 mm in diameter after one to two weeks of incubation. Electron and phase-contrast microscopy revealed that cells of strain P7^T were Gram-positive, rod-shaped (0.5 µm in diameter and, most often, 3 µm in length) bacteria occurring most often singly or in pairs (Supplementary Fig. 1). Cells can be motile and peritrichously flagellated, but active motility was not always observed. Spores were rarely observed, but, when present, were subterminal to terminal with some cell swelling. This morphology differs from that reported for strain SL1^T with regards to the Gram reaction and presence of spores (Kusel *et al*., 2000).

Growth properties and substrate utilization

The growth rate, temperature range and pH range for strain P7^T, strain SL1^T and *C. scatologenes*^{T} are given in Table 1. All three bacteria had similar temperature and pH ranges and optima. The growth rates under optimal conditions with fructose or CO as substrate for the three clostridia were in the same range. A doubling time of 2.4 h was observed for strain SL1^T at 38 ºC, an initial culture pH of 6.2, and with fructose as the substrate. A doubling time for SL1^T with fructose as the substrate and at 30 ºC was 5.3 h (Kusel *et al*., 2000). The doubling time of

C. scatologenes^T (17.3 h) on H_2:CO_2 was considerably longer than that for strain P7^T (5.8 h) or strain SL1^T (3.5 h). All three *Clostridium* species utilized CO, H_2:CO_2, ribose, xylose, fructose, glucose, galactose, L-arabinose, mannose, rhamnose, sucrose, cellobiose, melezitose, cellulose, starch, inositol, glycerol, ethanol, propanol, pyruvate, citrate, serine, alanine, histidine, glutamate, aspartate, asparagine, casamino acids, betaine and choline as growth substrates. Formate, methanol, D-arabinose, lactose, raffinose, melibiose, amygdalin, succinate, ferulate, vanillate and trimethoxybenzoate did not support the growth of these clostridia.

A differential pattern of substrate utilization for the three *Clostridium* species was observed with pectin, fucose, maltose, trehalose, sorbitol, mannitol, gluconate, lactate, fumarate, malate, 2-propanol, butanol, glutamine, arginine and syringate (Table 1). For example, *C. scatologenes*^{T} grew with maltose, sorbitol, or glutamine, but these did not support the growth of strain P7^T or strain SL1^T. Strain P7^T did not grow on fucose, gluconate, lactate or arginine, but these substrates were utilized by strain SL1^T and *C. scatologenes*^{T}. Strain SL1^T did not grow on pectin and grew on malate, while the opposite result was observed for strain P7^T and *C. scatologenes*^{T}. These three *Clostridium* species could be distinguished based on growth with these 15 substrates.

The substrate utilization results for SL1^T are mainly in accord with those reported in Kusel *et al*. (2000). However, strain SL1^T was reported to utilize formate,

vanillate and ferulate in the prior report, and it was also reported that *C. scatologenes*^{T} utilized vanillate (Kusel *et al.*, 2000). Other results found for *C. scatologenes*^{T} differ somewhat from prior work. In Holdeman *et al*. (1977), *C. scatologenes*^{T} did not produce acid from (implying lack of growth) galactose, maltose, sucrose, cellobiose, melezitose, starch, sorbitol or inositol, but in this study these all served as growth substrates. A later report (Cato *et al*., 1986) indicated that *C. scatologenes*^{T} is negative for utilization of galactose, maltose, sucrose, melezitose, starch or sorbitol, but these functioned as growth substrates in this study. All of the substrate utilization assays here were performed independently three times and each result set for the three *Clostridium* species were identical. To some degree the differences in reported substrate utilization reflect legitimate differences in performance of the bacteria, especially if slightly different culture conditions are used.

All three *Clostridium* species were methyl red positive in an MRVP assay. All yielded negative results for the Voges-Proskauer reaction, esculin hydrolysis, gelatin hydrolysis, nitrate reduction and production of indole. Strain P7^T was also negative for catalase, oxidase and urease activities.

End products of metabolism were examined in this study. Strain P7^T converted 600 mmoles of CO (equal number of carbons to 100 mmoles fructose) to CO_2 (264 mmoles), ethanol (96 mmoles), acetate (12 mmoles) and butanol (24 mmoles). Strain P7^T converted 100 mmoles of fructose to CO_2 (280 mmoles),

ethanol (23 mmoles), acetate (81 mmoles) and butanol (4 mmoles). It was not unusual to detect butyrate as an end product in other trials. Butyrate was also reported as a minor end product from strain SL1^T (Kusel *et al*., 2000). Under similar conditions (initial culture pH of 6.2 and incubation at 38 ºC), strain SL1^T fermented 100 mmoles of fructose to CO_2 (120 mmoles), ethanol (50 mmoles), acetate (70 mmoles) and butanol (50 mmoles), while CO_2 (100 mmoles), acetate (200 mmoles) and ethanol (50 mmoles) were produced by *C. scatologenes*^{T}. These are different from the ratio of end products from fructose by SL1^T and *C. scatologenes*^{T} reported in Kusel et al. (2000). However, end product ratios in clostridia can change with alteration of pH and other growth conditions (Adler & Crow, 1987).

rRNA gene sequence analysis and molecular characterization

The phylogenetic relationship of strain P7^T to related *Clostridium* species is shown in Fig. 1. The sequence for strain P7^T was deposited with GenBank under accession number AY170379, and the sequence for strain SL1^T was deposited by Harold Drake's laboratory under accession number Y18813. The sequence of P7^T was very similar to that of strain SL1^T (99.8%) and *C. scatologenes*^{T} (99.7%). The sequence of strain SL1^T was also very similar to that of *C. scatologenes*^{T} (99.7%), which had been reported earlier (99.6%; Kusel *et al*., 2000). All three strains are in cluster I (subcluster Ic) of *Clostridium* group I (Collins *et al*., 1994; Stackebrandt & Hippe, 2001). *Clostridium histolyticum*, *Clostridium limosum*

and *Clostridium proteolyticum* are in cluster II of *Clostridium* group I, and the remaining species are in cluster I.

The mol% G+C content of the DNA from P7^T was 31% (nuclease digest), similar to the number determined at the DSMZ (32%). The mol% G+C content of the DNA from strain SL1^T was 32% (nuclease digest), similar to the value reported earlier (30%) in Kusel *et al*. (2000). The mol% G+C content of the DNA from *C. scatologenes*^{T} was 31% (nuclease digest), somewhat different from the number reported previously (27%; thermal melting point) in Johnson & Francis (1975).

The results from the 16S rRNA gene sequence analysis and determination of G+C content showed that these three *Clostridium* species were closely related. The taxonomic status of these was further investigated using DNA-DNA reassociation. The DNA-DNA reassociation values of strain P7^T to strain SL1^T was 31.8%, of strain P7^T to *C. scatologenes*^{T} was 50.2%, and of strain SL1^T to *C. scatologenes*^{T} was 53.0%. Using the threshold value of 70% for the definition of species (Wayne et al., 1987), these three clostridia are each a different species. It had been shown earlier that strains with very similar 16S rRNA sequences could be demonstrated to be distinct species when analyzed by DNA-DNA reassociation (Fox et al., 1992), and that strains with a 16S rRNA sequence similarity greater than 97% require use of a DNA-DNA reassociation analysis to define speciation (Stackebrandt & Goebel, 1994).

As noted above and in Table 1, these three *Clostridium* species could also be separated based on substrate utilization patterns and the slow growth of *C. scatologenes*^{T} on H_2:CO_2, in addition to separation based on DNA-DNA reassociation results. Distinction among the three *Clostridium* species was also examined using repetitive DNA PCR fingerprinting (rep-PCR). This technique should not be used to decide if a bacterium is a novel species, but is presented here as another method available to a greater number of laboratories, compared to DNA-DNA reassociation, to discriminate among the three clostridia. The result of the gel electrophoresis of the PCR products from DNAs with the BOXA1R primer is available (Supplementary Fig. 2). All three *Clostridium* species had a band of approximately 1,190 base pairs in size and a band of approximately 400 base pairs in common. Strain SL1^T and *C. scatologenes*^{T} had a band of approximately 1,350 base pairs in common. *C. scatologenes*^{T} also had PCR product bands of 580 base pairs, 345 base pairs and 250 base pairs. Strain SL1^T showed unique gel bands of >2,000 base pairs and 720 base pairs in size. Strain P7^T had a unique band of 830 base pairs.

The sum of the above results demonstrates that strain P7^T and strain SL1^T should be considered as distinct species of *Clostridium*. We propose the name *Clostridium carboxidivorans* for P7^T, based on its ability to readily utilize CO as a substrate. We propose the name *Clostridium drakei* for SL1^T, in recognition of the contributions that Harold L. Drake has made to the microbiology of the acetogens. In both instances, this expands the repertoire of species of acetogens

that are members of the genus *Clostridium*, and that can produce C4 compounds in addition to acetate as end products of metabolism. This also adds, in addition to *C. ljungdahlii*, to the known acetogenic species of *Clostridium* species which can produce ethanol as a product from C1 substrates in addition to acetate, and expands upon this solvent-producing ability in that these new species can produce butanol, as well.

Description of *Clostridium carboxidivorans* sp. nov.

Clostridium carboxidivorans [car.bo.xi.di.vo'rans. N.L. neut. n. carboxidum, carbon monoxide, L. part. adj. vorans, devouring, N.L. part. adj. *carboxidivorans*, carbon monoxide devouring]. Gram-positive, motile rods (0.5 x 3 µm) occurring singly and in pairs. Cells rarely sporulate, but spores are subterminal to terminal with slight cell swelling. Obligate anaerobe with optimum growth temperature of 38 ºC and optimum pH 6.2. Grows autotrophically with H_2:CO_2 or CO, and chemoorganotrophically with ribose, xylose, fructose, glucose, galactose, L-arabinose, mannose, rhamnose, sucrose, cellobiose, trehalose, melezitose, pectin, starch, cellulose, inositol, mannitol, glycerol, ethanol, propanol, 2-propanol, butanol, citrate, serine, alanine, hisitidine, glutamate, aspartate, asparagine, casamino acids, betaine, choline, and syringate. Methanol, D-arabinose, fucose, maltose, lactose, raffinose, melibiose, amygdalin, sorbitol, gluconate, lactate, malate, succinate, and arginine did not support growth. Acetic acid, ethanol, butyrate and butanol are the end products of metabolism. Cultures are methyl red positive, but negative for the Voges-Proskauer reaction, esculin hydrolysis,

gelatin hydrolysis, nitrate reduction, indole production, catalase, oxidase and urease. The mol% G+C content is 31-32%.

The type strain, strain P7^T (=ATCC BAA-624^T=DSM 15243^T), was isolated from an agricultural settling lagoon in Oklahoma. 16S rRNA gene sequence analysis showed *C. carboxidivorans* was very closely related to *C. scatologenes*^{T} and *C. drakei*, but DNA reassociation analysis showed that *C. carboxidivorans* is a distinct species, with a reassociation value of 50% to *C. scatologenes*^{T} and a reassociation value of 32% to *C. drakei*.

Description of *Clostridium drakei* sp. nov.

Clostridium drakei [drak'e.i. N.L. gen. n. *drakei*, of Drake, in recognition of Harold L. Drake's contributions to our understanding of the physiology and ecology of acetogens]. Original description in Kusel et al. (2000). Gram-negative, motile rods (0.6 x 3-4 µm). Forms terminal spores. Obligate anaerobe with an optimum growth temperature of 30-37 ºC and optimum pH 5.5-7.5. Grows autotrophically with H_2:CO_2 or CO, and chemoorganotrophically with ribose, xylose, fructose, glucose, galactose, fucose, L-arabinose, mannose, rhamnose, sucrose, cellobiose, melezitose, starch, cellulose, inositol, mannitol, gluconate, glycerol, ethanol, propanol, 2-propanol, butanol, citrate, malate, fumarate, lactate, serine, alanine, histidine, glutamate, aspartate, asparagine, arginine, casamino acids, betaine, choline and syringate. Methanol, D-arabinose, maltose, lactose, trehalose, raffinose, melibiose, amygdalin, sorbitol and succinate

did not support growth. Acetic acid, ethanol, butyrate and butanol are the end products of metabolism. Cultures are methyl red positive, but negative for the Voges-Proskauer reaction, esculin hydrolysis, gelatin hydrolysis, nitrate reduction and indole production. The mol% G+C content is 30-32%.

The type strain, strain SL1^T (=ATCC BAA-623^T=DSM 12750^T), was isolated from sediment collected from an acidic coal mine pond in east central Germany. 16S rRNA gene sequence analysis showed *C. drakei* was very closely related to *C. scatologenes*^{T} and *C. carboxidivorans*, but DNA reassociation analysis showed the *C. drakei* is a distinct species, with a reassociation value of 53% to *C. scatologenes*^{T} and a reassociation value of 32% to *C. carboxidivorans*.

References

Adler, H. I. & Crow, W. (1987). A technique for predicting the solvent-producing ability of *Clostridium acetobutylicum*. *Appl Environ Microbiol* **53,** 2496-2499.

Balch, W. E. & Wolfe, R. S. (1976). New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. *Appl Environ Microbiol* **32,** 781-791.

Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **75,** 4801-4805.

Buschhorn, H., Durre, P. & Gottschalk, G. (1989). Production and utilization of ethanol by the homoacetogen *Acetobacterium woodii*. *Appl Environ Microbiol* **55,** 1835-1840.

Cashion, P., Hodler-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for base ratio determination of bacterial DNA. *Anal Biochem* **81,** 461-466.

Cato, E. P., George, W. L. & Finegold, S. M. (1986). Genus *Clostridium* Prazmowski 1880, 23^{A}L. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1141-1200. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44,** 812-826.

De Ley, J., Cattior, H. & Reynaerts (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12,** 133-142.

Drake, H. L. (ed.) (1994). *Acetogenesis*. New York: Chapman and Hall.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal Chem* **28,** 350-356.

Escara, J. F. & Hutton, J. R. (1980). Thermal stability and renaturation of DNA in dimethylsulphoxide solutions: acceleration of renaturation rate. *Biopolymers* **19,** 1315-1327.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39,** 783-791.

Felsenstein, J. (1993). *PHYLIP (Phylogenic Inference Package),* version 3.5c. Seattle: University of Washington.

Fox, G. E., Wisotzkey, J. D. & Jurtshuk, Jr., P. (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42,** 166-170.

Genthner, B. R. S., Davis, C. L. & Bryant, M. P. (1981). Features of rumen and sewage sludge strains of *Eubacterium limosum*, a methanol- and H_2-CO_2-utilizing species. *Appl Environ Microbiol* **42,** 12-19.

Grethlein, A. J., Worden, R. M., Jain, M. K. & Datta, R. (1991). Evidence for production of n-butanol from carbon monoxide by *Butyribacterium methylotrophicum*. *J Ferm Bioeng* **72**, 58-60.

Gutell, R. R., Larsen, N. & Woese, C. R. (1994). Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol Rev* **58,** 10-26.

Holdeman, L. V., Cato, E. P. & Moore, W. E. C. (1977). *Anaerobe Laboratory Manual*, 4th edn. Blacksburg, Virginia: Virginia Polytechnic Institute and State University.

Hungate, R. E. (1969). A roll tube method for cultivation of strict anaerobes. In *Methods in Microbiology*, vol. 3B, pp. 117-132. Edited by J. R. Norris & D. W. Ribbons. New York: Academic Press.

Huss, V. A. R., Festl, H. & Schleifer, K.-H. (1983). Studies on the spectrometric determination of DNA hybridization from renaturation rates. *J Syst Appl Microbiol* **4,** 184-192.

Jahnke, K.-D. (1992). Basic computer program for evaluation of spectroscopic DNA renaturation data from GILFORD System 2600 spectrometer on a PC/XT/AT type personal computer. *J Microbiol Methods* **15,** 61-73.

Johnson, J. L. & Francis, B. S. (1975). Taxonomy of the clostridia: ribosomal ribonucleic acid homologies among the species. *J Gen Microbiol* **88,** 229-244.

Kusel, K., Dorsch, T., Acker, G., Stackebrandt, E. & Drake, H. L. (2000). *Clostridium scatologenes* strain SL1 isolated as an acetogenic bacterium from acidic sediments. *Int J Syst Evol Microbiol* **50,** 537-546.

Ludwig, W. (1991). DNA sequencing in bacterial systematics. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 69-95. Edited by E. Stackebrandt & M. Goodfellow. New York: John Wiley & Sons.

Mesbah, M., Premachandran, U. & Whitman, B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159-167.

Olsen, G. J., Matsuda, H., Hagstrom, R. & Overbeek, R. (1994). fastDNAmL: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Appl Biosci* **10,** 41-48.

Smibert, R. M. & Krieg (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607-654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, D.C.: American Society for Microbiology.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44,** 846-849.

Stackebrandt, E. & Hippe, H. (2001). Taxonomy and systematics,. In *Clostridia*, pp. 19-48. Edited by H. Bahl & P. Dürre, New York City, NY: Wiley-VCH.

Swofford, D. L. (2000). *PAUP 4.0*, beta version 4.0b4a. Sunderland, Massachusetts: Sinauer Associates, Inc.

Tanner, R. S. (2002). Cultivation of bacteria and fungi. In *Manual of Environmental Microbiology*, 2nd ed., pp. 62-70. Edited by C. J. Hurst, R. L. Crawford, G. R. Knudsen, M. J. McInerney & L. D. Stetzenbach. Washington, D.C.: ASM Press.

Tanner, R. S., Miller, L. M. & Yang, D. (1993). *Clostridium ljungdahlii* sp. nov., an acetogenic species in clostridial rRNA homology group I. *Int J Syst Bacteriol* **43,** 232-236.

Vega, J. L., Prieto, S., Elmore, B. B., Clausen, E. C. & Gaddy, J. L. (1989). The biological production of ethanol from synthesis gas. *Appl Biochem Biotechnol* **20/21,** 781-797.

Versalovic, J., Schneider, M., de Bruijn, F. J. & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* **5,** 25-40.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P. & Truper, H. G. (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37,** 463-464.

Weisberg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173,** 697-703.

Worden, R. M., Grethlein, A. J., Jain, M. K. & Datta, R. (1991). Production of butanol and ethanol from synthesis gas via fermentation. *Fuel* **70,** 615-619.

Table 1. Characteristics of strain P7^T, strain SL1^T and *Clostridium scatologenes*^{T}

Temperature and pH range/optimum determined with fructose as the substrate. +, growth; -, no growth.

Characteristics	P7^T	SL1^T	*C. scatologenes*^{T}
Growth rate:			
Fructose	0.32 h^{-1}	0.29 h^{-1}	0.39 h^{-1}
$CO:CO_2$	0.16 h^{-1}	0.12 h^{-1}	0.09 h^{-1}
$H_2:CO_2$	0.12 h^{-1}	0.20 h^{-1}	0.04 h^{-1}
Temperature range	24 - 42 ºC	18 - 42 ºC	18 - 42 ºC
Temperature optimum	37 - 40 ºC	30 - 37 ºC	37 - 40 ºC
pH range	4.4 -7.6	4.6 - 7.8	4.6 - 8.0
pH optimum	5.0 - 7.0	5.4 - 7.5	5.4 - 7.0
Utilization of:			
Pectin	+	-	+
Fucose	-	+	+
Maltose	-	-	+
Trehalose	+	-	-
Sorbitol	-	-	+
Mannitol	+	+	-
Gluconate	-	+	+

Lactate	-	+	+
Fumarate	-	+	+
Malate	-	+	-
2-Propanol	+	+	-
Butanol	+	+	-
Glutamine	-	-	+
Arginine	-	+	+
Syringate	+	+	-

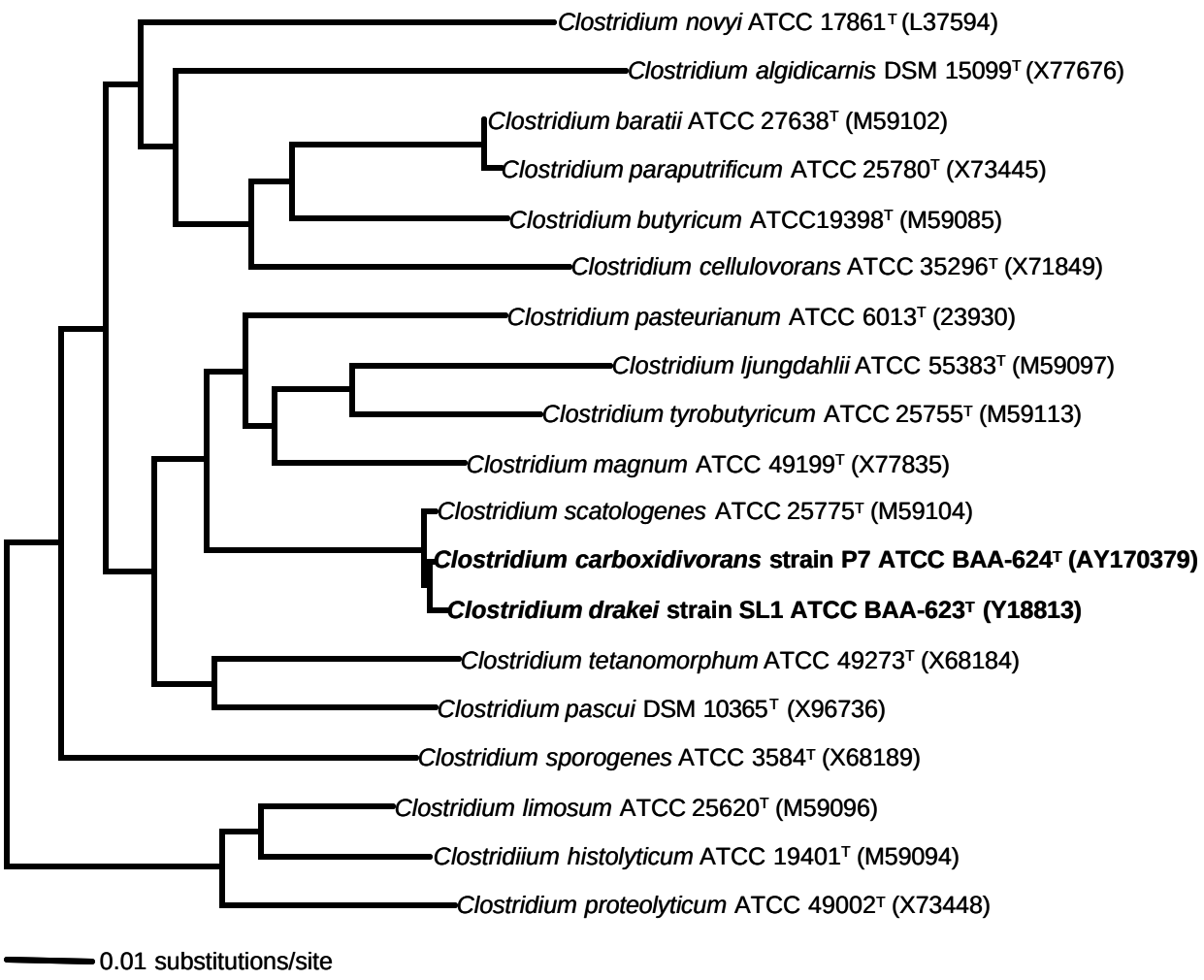
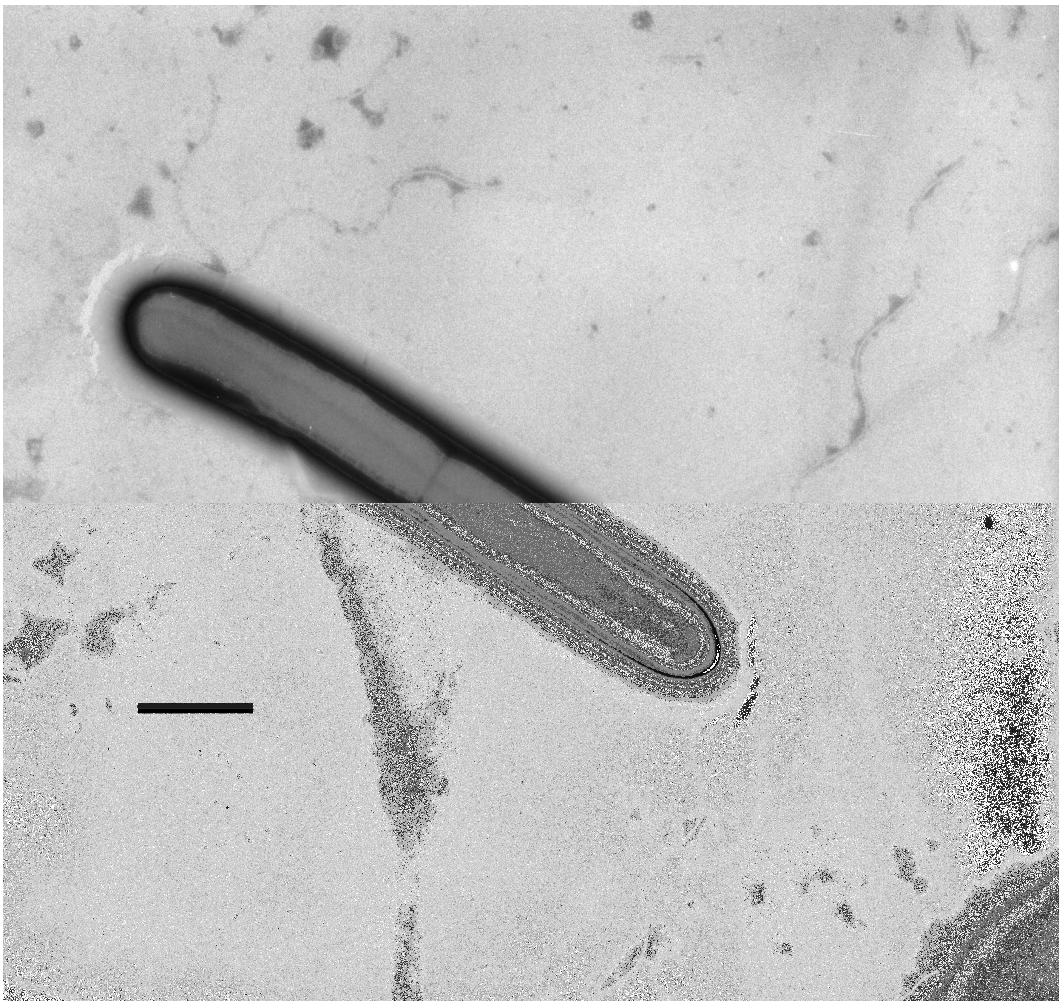
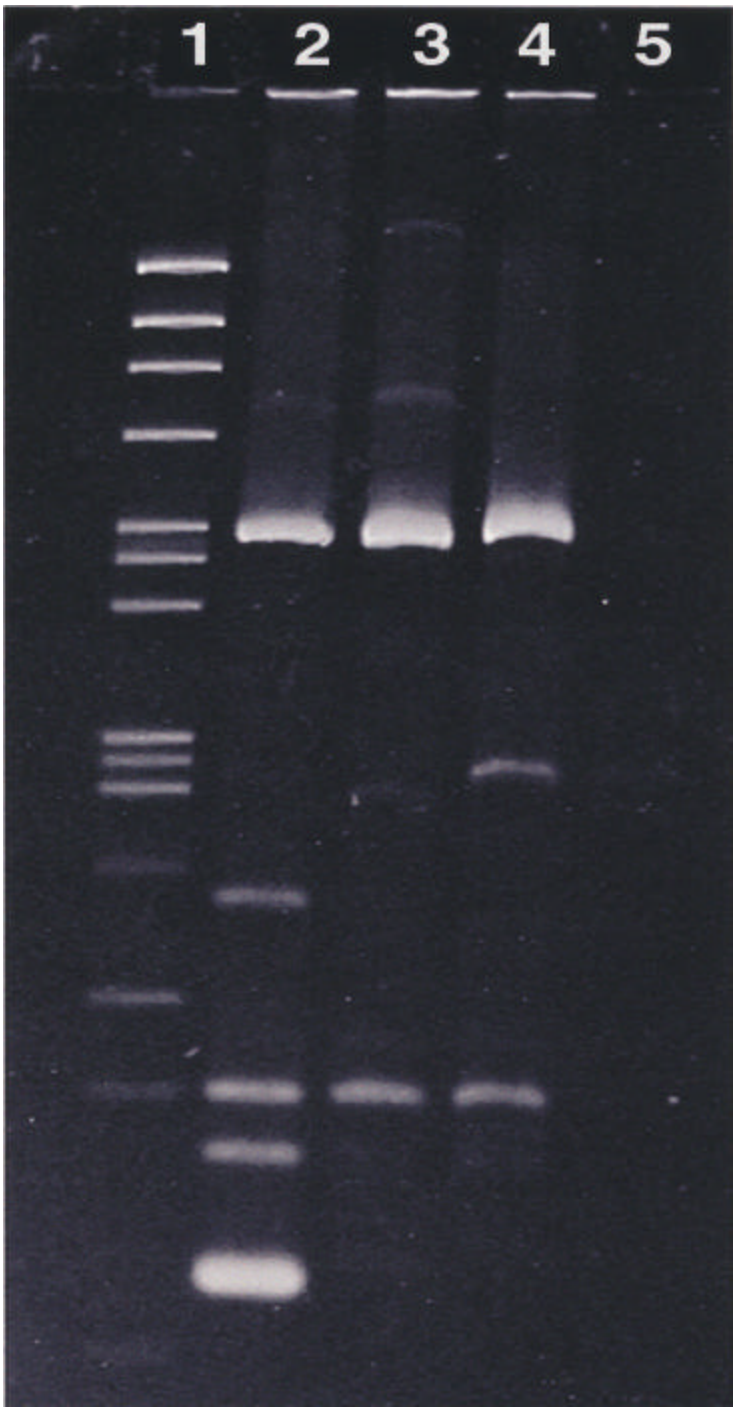


Fig. 1. The phylogenetic tree for clostridial strain P7^T, as determined from a 16S rRNA gene sequence analysis. The total horizontal distance between species indicates the difference between their sequences. The bar indicates a difference of 0.01 substitutions per site (= 1% difference).



Supplementary Fig. 1. Transmission electron micrograph of a pair of negatively stained cells of strain P7^{T}. Bar, 1 µm.



Supplementary Fig. 2. BOX-PCR fingerprints of *C. scatologenes*^{T} (lane 2), strain SL1^{T} (lane 3) and strain P7^{T} (lane 4). A DNA standard (2,072 – 400 bp) is shown in lane 1 and a gel control is shown in lane 5.

Chapter 2. Production of acids and alcohols from carbon monoxide by *Clostridium carboxidivorans*, strain P7

Abstract

Biofuels, such as ethanol from biomass have the potential to reduce petroleum imports, decrease greenhouse gas emissions and improve rural economics. Biomass can be fermented indirectly after pyrolysis with air to synthesis gas (N_2:CO:CO_2:H_2). *Clostridium carboxidivorans* can ferment synthesis gas to ethanol, butanol, acetic acid, and butyric acid. The effects of physiochemical variables and medium components on the conversion of CO to ethanol by *C. carboxidivorans* were examined. *C. carboxidivorans* grew in the presence of 32,000 µl/l of oxygen with fructose, 29,000 µl/l of oxygen with CO:CO_2 and 480 µl/l of oxygen with H_2:CO_2 as the substrate. Initial growth on CO was dependent on having 11,000 µl/l CO_2 in the gas phase. *C. carboxidivorans* required pantothenic acid, para-amino benzoic acid, and biotin for growth. Growth on gaseous substrates was stimulated with the addition of proline and/or isoleucine (5 mg/L each). The most important factors for induction of *C. carboxidivorans* solvent production, appeared to be a low pH of 5.0 and increased iron concentration (10X) in the medium. By augmented and eliminated nutrient components, specific physiochemical parameters, and the addition of metabolic inhibitors, approximately 220 mM ethanol was produced during *C. carboxidivorans* growth on CO. The potential utility of *C. carboxidivorans* for

the commercial production of organic acids and alcohols from synthesis gas warrants further investigation.

Introduction

Economic and environmental considerations have directed attention to the development of alternative non-petroleum-based sources of energy such as biofuels. The use and production of biofuels would decrease the amount of fossil fuel imported each year. In addition, biofuels would decrease and balance the amount of greenhouse gas emissions (Hohenstein and Wright 1994) since biomass-derived fuels burn more cleanly and consume as much carbon in growth as they produce in combustion. Biomass, such as switchgrass, wood chips, and paper wastes, can be pyrolyzed to synthesis gas containing mixed concentrations of CO, H_2 and CO_2. Aerobic and anaerobic bacterial growth with CO and H_2:CO_2 has been well documented (Mörsdorf et al. 1992; Mielenz 2001); solvents are often the end products.

The advantages of using a biological conversion of synthesis gas include the general resistance to sulfides observed with bacteria and reactions at lower pressure and temperatures than required for chemical catalysis, which could reduce operating and capital expenditures (Grethlein and Jain 1992). Major limiting factors to using bacteria in an industrial process for synthesis gas conversion are the low product yield and rates of production obtained. Improvements of synthesis gas and fermentation technologies may alleviate some of these difficulties; however, it is clear that alternative acidogenic or solventogenic microbial catalysts should be explored.

Clostridium carboxidivorans is a Gram-positive, motile, spore-forming, rod-shaped, obligate anaerobic bacterium isolated from an agricultural settling lagoon sediment for its ability to convert synthesis gas to ethanol (Liou et al. in press). Under routine growth conditions on fermentable carbohydrates or synthesis gas, *C. carboxidivorans* produces C_2 and C_4 end products. This strain, like *Clostridium ljungdahlii* (Tanner et al. 1993), produced acetate and ethanol as end products of metabolism. However, like *Clostridium scatologenes* (Kusel et al. 2000), it also produced butyrate and butanol as end products.

The effect of nutrients, medium formulation and culture conditions on solvent production by *Clostridium acetobutylicum*, *Clostridium beijerinckii*, and *Clostridium saccharobutylicum* has been investigated (Roos et al. 1984; McNeil and Kristiansen 1987; Shaheen et al. 2000). Solvent production by these species was affected by either the elimination or augmentation of NH_4^+, PO_4^{3-}, Mg^{2+} and Fe^{2+}, and by varying the pH conditions or the kind of fermentable carbohydrates. Other factors that affected solvent production were metabolic inhibitors that either shifted the end products from acids to solvents or stimulated solvent production (Singh et al. 1991; Lohmeier-Vogel and Hahn-Hägerdal 1985). Each species responded differently to varying culture conditions or manipulations, suggesting that one or the other might be preferred for certain industrial application.

In this work, ethanol production by *C. carboxidivorans* with synthesis gas as the substrate was investigated. The selection of this novel isolate was based on its robustness, especially for its viable culture stability even after storage at room temperature for 12 months. Nutrient manipulations and use of metabolic

inhibitors contributed to increased solvent production. Overall, the most important factors for *C. carboxidivorans* solvent induction appeared to be a low pH of 5.0 and increased iron concentration in the medium. The investigation has identified *C. carboxidivorans* as another potential candidate for the fermentation of synthesis gas to ethanol.

Materials and methods

Microorganism and culture conditions

Clostridium carboxidivorans strain P7 (ATCC BAA-624^T = DSM 15243^T) was maintained in the laboratory of R. S. Tanner at the University of Oklahoma. Stock cultures of *C. carboxidivorans* were maintained in 500 ml crimp-sealed serum bottles and culture tubes (Balch and Wolfe 1976) containing growth medium. Cultures were grown at 37^{o}C in shaking incubators set at 100 rpm and transferred weekly to fresh serum bottles and culture tubes. Stock cultures of *Clostridium thermocellum* (ATCC 27405^T = DSM 1237^T) were also maintained in culture tubes containing growth medium. *C. thermocellum* were grown at 55^{o}C and transferred weekly to culture tubes.

The medium used for maintaining growth and performing physiological experiments was prepared by using strict anaerobic techniques (Balch and Wolfe 1976) and contained (per liter): Mineral Solution (25 ml), yeast extract (1 g) (#0127-17-9, Difco Laboratories), Trace Metal Solution (10 ml), Vitamin Solution (10 ml), 10 g of 2-(*N*-morpholino) ethanesulfonic acid (MES) (Sigma-Aldrich Co., St. Louis, MO) as buffer (final pH 6.0-6.2) and 6 ml of cysteine·sulfide reducing agent (Tanner, 2002) with the exception of iron, molybdenum, calcium, boric acid (H_3BO_3), sodium formate (HCOONa), sulfate, phosphate, and ammonium in the augmentation/elimination experiments. A N_2:CO_2 (80:20, pressurized to 70 kPa) headspace was used for growth with organic substrates (5 g per liter), and either CO:N_2:CO_2 or CO:H_2:CO_2 (70:24:6,

pressurized to 210 kPa) was used for growth on gaseous substrates (Tanner et al. 1993) with the exception of the CO_2 requirement experiment.

pH

The optimum pH for solventogenesis was examined with CO as the substrate from pH 4 to pH 8 in medium containing a Good's buffer (20 g per liter), HOMOPIPES (pKa 4.6), MES (pKa 6.0), TES (pKa 7.2) or TAPS (pKa 8.1), as appropriate (Tanner, 2002).

Nutrients

Normal concentrations of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (20 µM) and $Na_2MoO_4 \cdot 2H_2O$ (0.05 µM) in the medium were eliminated (0X) or augmented (5X, 10X and 15X the original concentration). NaCl (14 mM), $CaCl_2 \cdot 2H_2O$ (272 µM), $MgSO_4 \cdot 7H_2O$ (811 µM), KH_2PO_4 (735 µM), and NH_4Cl (18 mM) were eliminated or augmented 10X the original concentration. H_3BO_3 (16 mM, 80 mM, 162 mM) and HCOONa (1.5 mM, 15mM, 30 mM) at varying concentrations were augmented in the medium. All experiments were conducted in a final volume of 10 ml in triplicate.

Metabolic inhibitors

1 M methanol and 1 M 4-chlorobutyric acid stocks were diluted to a range of concentrations (5 – 70 mM) in final volumes of 10 ml in triplicate. Ethanol (200 proof) was diluted to a range of concentrations (0.33 – 1.74 M). For ethanol

adaptation, sterile ethyl alcohol was serially transferred into medium using increasing concentrations (0.52 – 2.17 M). Fluoroacetate and trifluoroacetate were diluted to a range of concentrations (5 – 60 mM) in final volumes of 10 ml in triplicate. All stocks were prepared under strict anaerobic techniques.

Gases

The composition of the headspaces used in the CO_2 requirement experiment was $CO:H_2$, $CO:N_2$, $CO:CO_2$ (80:20, pressurized to 210 kPa), or $CO:H_2:CO_2$ (75:20:5, pressurized to 210 kPa) in triplicate. Under $CO:CO_2$, the composition of the headspaces was 100**:**0, 99.4**:**0.6, 98.8**:**1.2, 98.2**:**1.8, 97**:**3, 94.3**:**5.7 (pressurized to 240 kPa) in triplicate.

In the oxygen tolerance experiment, the composition of the headspaces was $CO:N_2:CO_2$ or $N_2:CO_2$ with O_2 additions of 0 to 67,034 µl/l or the composition of the headspace was $H_2:CO_2:N_2$ with O_2 additions of 0 to 4,840 µl/l, all in triplicate.

Analytical procedures

Growth was quantitated by measuring optical density using a Spectronic 20D spectrophotometer (Milton Roy Company, Rochester, NY) at 600 nm. Acetate, ethanol, butyrate, and butanol were measured using a 3400 Varian Gas Chromatograph (Varian, Sugar Land, TX), equipped with a FID and a 6 ft glass column packed with Carbopack B DA 80/20 4 % Carbowax 20 M resin (Supelco, Bellefonte, PA) Synthesis gas and oxygen headspace were measured with a GC

equipped with a thermal conductivity detector (Varian, Sugar Land, TX) and a 6 ft x 2 mm ID, glass Porapak Super Q 80/100 column (Alltech, Deerfield, IL) set at 75^{o}C with 20 ml/min helium flow rate or a 6 ft x 1/8" OD, stainless steel Molecular Sieve 5A column (Alltech) set at 65^{o}C with 30 ml/min helium flow rate. Throughout each experiment, the final pH was verified by an AccutupH+ pH probe from Fisher Scientific.

Results

Culture conditions

C. carboxidivorans grew chemolithotrophically with $CO:H_2:CO_2$, $CO:N_2:CO_2$, or $H_2:CO_2$ as well as chemoorganotrophically with some simple carbohydrates such as glucose and fructose (Liou et al. in press). *C. carboxidivorans* reached an optical density greater than 3.0 at 600 nm within 10 hours using $CO:CO_2$ as the substrate. Batch cultures of *C. carboxidivorans* feeding on bottled synthesis gases showed simultaneous utilization of CO and H_2 as substrates (Figure 1). The temperature for growth with CO as the substrate was tested from 4 ^oC – 45 ^oC. Growth occurred between 30 ^oC and 42 ^oC, and the optimum temperature was 37 ^oC - 39^oC.

pH

The effect of pH on the growth and metabolism of *C. carboxidivorans* was examined over several pH values ranging from 4.0 to 8.0 in order to determine both the optimum initial pH to simulate growth as well as the optimum pH for solvent production under synthesis gas. The optimum pH for growth was between 6.0 and 6.2. Growth did not occur at an initial culture pH of 4.0 or 8.0, and minimal growth occurred at pH values of 4.2 or 7.5. Cultures with an initial pH of 4.5 to 5.7 or 6.5 to 7.5 had longer lag time and longer doubling time (approx. 10 hours to 20 hours) compared with growth at pH 6.0 to 6.2. *C. carboxidivorans* mainly produced acids at pH 6.0 and solvents at pH 5.0 (Table

1). Maximum solventogenesis occurred at pH 5.0 (Table 1), at which *C. carboxidivorans* produced 32 mM of ethanol and 13 mM of butanol under substrate limiting conditions. The optimum culture pH for production of acetate and butyrate was between 5.5 and 6.0 (Table 1). *C. carboxidivorans* produced 78 mM acetate and 15 mM butyrate in substrate limiting conditions with CO as the substrate. Additionally, experiments with *C. carboxidivorans* growing in an unbuffered medium of pH 6.0 to 6.2 resulted in a final pH between 3.9 and 4.2.

Nutrients

Growth of *C. carboxidivorans* did not occur in the absence of either Vitamin or Trace Metal Solution. Reliable and reproducible growth on gaseous substrates required the addition of either yeast extract (1 g per liter) or Casamino Acids (1 g per liter), whereas growth on glucose or fructose did not required the addition of yeast extract or Casamino Acids. Growth of *C. carboxidivorans* required the addition of pantothenic acid, para-amino benzoic acid (PABA), and biotin, as essential vitamins, whereas B_{12} or folic acid were not required. *C. carboxidivorans*, growing under CO-limited conditions, typically produced acetate (30 mM), ethanol (21 mM), butanol (5 mM) and butyrate (7 mM). Cell density typically reached an OD_{600nm} value between 0.7 and 1.2, and the final pH typically ranged from 4.8 to 5.0. Specific nutrients required to simulate CO metabolism without the addition of yeast extract or Casamino Acids for *C. carboxidivorans* under gaseous substrates were 5 mg per liter of proline and isoleucine.

Ammonium or sodium as provided by the mineral solution was not required for growth on gaseous substrates or simple carbohydrates. Increased or decreased concentrations of ammonium, sodium, molybdenum and calcium in the medium showed no increases in solvent or acid production. Product formation over time, cell growth measured by the absorbance at 600 nm and pH were similar to their typical end product concentrations. Limiting phosphate in the medium reduced ethanol production by 6.5%, butanol production by 36.4%, acetate production by 13.4% and butyrate production by 2.2% from normal concentrations. Elevated levels of phosphate increased acetate production by 32.3% from the typical concentration; however, ethanol production was reduced by 22%, butanol production by 12% and butyrate production by 3.3% from normal concentrations. The elimination or elevation of sulfate from the normal concentration had no effect on solvent production. However, when sulfate was eliminated from the medium, acetate and butyrate production was reduced by 75.5% and 100%, respectively, and when sulfate was elevated in the medium, acetate and butyrate production was increased by 125% and 350%, respectively. The addition of H_3BO_3 in the medium had an insignificant effect on the production of ethanol and acetate; however, the production of C_4 products was significantly reduced by an average of 50% with the addition of 80 mM H_3BO_3.

The deletion of ferrous iron from the medium eliminated the production of ethanol and inhibited acetate, butyrate and butanol production. Ethanol production from CO was increased 100% when the concentration of ferrous iron in the medium was increased 10X from the normal concentration. Interestingly,

production of acetate and butyrate was decreased approximately 17% and 14%, respectively, by the presence of elevated ferrous iron concentration greater than ten-fold times the normal concentration.

No or insignificant effects in the single variable experiments for the optimization of solventogenesis in CO metabolism demonstrates that ethanol production was limited by an entirely different variable rather than the one under investigation.

Co-substrates and metabolic inhibitors

Formate and methanol may be potential co-substrates for ethanol production. Methanol was added to growing cultures of *C. carboxidivorans* under CO at concentrations ranging from 5 to 70 mM. Methanol inhibited the growth of *C. carboxidivorans* at concentrations greater than 30 mM when grown with $CO:N_2:CO_2$ and 45 mM on fructose. Analysis of the samples grown on fructose or $CO:N_2:CO_2$ showed no significant conservation of CO, stimulation of growth or increased solvent production. Formate was taken up; however, no effects on final production of end products were observed with the addition of 1.5 mM, 15 mM or 30 mM formate in the medium.

The adaptation of *C. carboxidivorans* to tolerate high concentrations of ethanol and the affinity of *C. carboxidivorans* to ethanol as a growth substrate were investigated. Cultures of *C. carboxidivorans* grown on $CO:N_2:CO_2$ were amended with 0 to 1.74 M ethanol. An average of 108 mM ethanol was consumed after $CO:N_2:CO_2$ was utilized as the substrate (Table 2). The addition

of ethanol at concentrations greater than 0.33 M inhibited the formation of butyrate and butanol (Table 2). *C. carboxidivorans* was adapted and screened with increasing concentrations of ethanol. Ethanol inhibited the growth of *C. carboxidivorans* at 0.54 M initially. After a series of serial transfers of *C. carboxidivorans* into medium with increasing concentrations of ethanol, *C. carboxidivorans* was shown to metabolize CO in the presence of 1.74 M ethanol (Table 2).

Trifluoroacetate (TFA) or fluoroacetate (FA) were examined for their potential as a C_2 inhibitor. TFA or FA reduced the production of C_4 products. Production of C_4 products was inhibited by 60 mM of TFA or FA. The addition of TFA reduced butanol and butyrate concentrations on average by 39% and 15%, respectively. FA reduced butanol and butyrate concentrations by an average of 33% and 84%, respectively. The addition of TFA or FA to the medium reduced the amount of acetate produced by 24% on average. However, ethanol production was unaffected.

Chlorobutyrate is a C_4 analog of FA. The effect on growth of *C. carboxidivorans* was minimal at 100 mM chlorobutyrate. Chlorobutyrate reduced solvent production and increased acid production. The addition of chlorobutyrate on average reduced ethanol by 8.6% and butanol by 24%; however, acetate production was increased by 18.6% and butyrate production by 33.3% from normal concentrations. Growth and production of solvents and acids were completely inhibited at 500 mM of chlorobutyrate,.

The ability to withstand oxygen contamination was conducted due to conditions that might exist in scale-up plants. Oxygen tolerance in *C. carboxidivorans* was tested between 0 and 67,034 µl/l of O_2. *C. carboxidivorans* tolerated up to 32,186 µl/l of O_2 under fructose, 29,040 µl/l of O_2 under CO:CO_2 and 484 µl/l of O_2 under H_2:CO_2 as the substrate. At maximum oxygen tolerance levels for *C. carboxidivorans* growth on CO:CO_2 as the substrate, ethanol production was reduced by 78%, butanol production by 55.5%, acetate production by 41% and butyrate production by 36% from normal concentrations. Growth of *C. carboxidivorans* on H_2:CO_2, as the substrate, at maximum oxygen tolerance levels, ethanol production was reduced by 40%, butanol production by 100%, acetate production by 70% and butyrate production by 100% from normal concentrations. Oxygen contamination above the tolerance maxima inhibited growth and end-product formation under all growing conditions.

CO_2 requirement

The amount of CO_2 required for growth under different gaseous or simple carbohydrate substrates was examined. Carbon dioxide was required for growth with different gaseous substrate mixtures, even though *C. carboxidivorans* produces CO_2 as a metabolic end product (Liou et al. in press). An initial 11,000 µl/l of CO_2 was required for growth to occur when CO or H_2 was supplied as a substrate. CO_2 was not required for growth on glucose or fructose substrates.

Direct fermentation

The ability to utilize lignocellulose as a raw material was investigated. *C. carboxidivorans* produced ethanol (1.3 mM) and acetate (7.4 mM) with 1% (w/v) of switchgrass as substrate in medium under N_2:CO_2 headspace. This fermentation was comparable to *Clostridium thermocellum*, which produced 2.4 mM ethanol and 12 mM acetate under identical conditions. Controls without switchgrass as the substrate did not produce ethanol or acetate.

Pilot study

Several batch culture runs were conducted with synthesis gas generated from a pilot-scale fluidized bed air gasifier at Oklahoma State University. Cultures of *C. carboxidivorans* utilized the producer gas; however a long lag phase of approximately 50 hours occurred before the optical density of the cells increased and before CO and H_2 concentration were significantly reduced. Growth on producer gas in batch cultures on average produced 33 mM ethanol, 35 mM acetate, 3 mM butanol and 5 mM butyrate.

By optimizing the solventogenic properties of *C. carboxidivorans*, the final concentration of ethanol was increased from initial culturing. Ethanol was increased from 15 mM (0.7 g/l) initially to 220 mM (10 g/l), along with acetate from 10 mM to 80 mM, and butanol from 2.5 mM to 13.4 mM, while butyrate was decreased from 30 mM to 10.2 mM with CO as a substrate by varying culture conditions and medium compositions (Table 3 and Table 4).

Discussion

Clostridium carboxidivorans was selected based on its robustness (stable and viable after an extend period (9 -12 months) of storage) at room temperature and tolerance of O_2, low pH, minimal nutrient supplementation and significant ethanol concentrations. All of these traits were evidenced by the physiological and biochemical experiments conducted in the indirect fermentation process. The selection of this novel isolate over other potential candidates was also due to its responsiveness to manipulation (Table 3 & 4). The primary focus of the research presented here was to improve the microbial conversion of synthesis gas (CO and other components) to ethanol. Strain development and process improvements, such as nutrient manipulation and adaptation, were employed to better understand and improve the conversion process (Parekh et al. 2000). The optimization of *C. carboxidivorans* resulted in increased production of ethanol from 15.2 mM (0.7 g/l) initially to a final concentration of 220 mM (10 g/l) in continuous cultures.

C. carboxidivorans physiology is similar to other solvent-producing *Clostridium*, such as *C. acetobutylicum*. Much like *C. acetobutylicum* in batch cultures (Grupe and Gottschalk 1992), it is probable *C. carboxidivorans* undergoes an initial period of rapid growth, coupled to production of CO_2, acetate, and butyrate. As the cell density increased and the concentration of available nutrients decreased, acidic end products accumulated, and the medium pH dropped. The culture went into stationary phase, and metabolism shifted to solvent production of ethanol and butanol. During solventogenesis, the carbon

flow switched from acid production to solvent production (Grupe and Gottschalk 1992). This acid to solvent shift was observed with *C. carboxidivorans* in unbuffered medium and end product analysis at solventogenic and acidogenic conditions.

The discovery of the metabolic switch in *C. acetobutylicum* is potentially advantageous from an industrial consideration as the ability to grow optimally and, then, switch to solvent formation means continuous operation and production. The optimal pH for growth in *C. carboxidivorans* was different from the optimal pH for solvent formation, as seen in *C. acetobutylicum* (Adler and Crow 1987; Grupe and Gottschalk 1992). In this investigation, the role of pH in triggering solventogenesis is a part of a multiple-component stimulus involving other factors such as specific nutrient factors (Table 3). Though the evidence was indirect, solventogenesis was controlled by pH, and the products formed were not growth associated.

Other factors that affected the regulation of solvent production were nutrient components (Table 3). Iron or metals serve as important components in biological systems (Mills, 1997). The augmentation of ferrous iron concentration in the medium significantly increased the production of solvents, where as the elimination of ferrous iron from the medium led to a decrease in solvent production, as also seen in *C. acetobutylicum* (McNeil and Kristiansen, 1987). The ferrous iron could be serving as a prosthetic group or cofactor to carbon monoxide dehydrogenase, hydrogenase, or formate dehydrogenase during CO metabolism (Mills, 1997; Ragsdale and Kumar, 1996; Ragsdale, 2004). Ferrous

iron could also be stimulating solvent production due to its involvement with many oxidation-reduction reaction through Ferredoxin (McNeil and Kristiansen, 1987).

Solvent production by *C. acetobutylicum* was increased by limiting concentrations of phosphate or sulfate (Bahl et al. 1982; Bahl and Gottschalk 1984), however, in *C. carboxidivorans*, limiting phosphate concentrations reduced solvent and acid production. Microbial growth requires phosphate, therefore, it was not surprising that limiting phosphate led to a decrease in solvent and acid production. Sources of mineral nutrients such as sulfur are required for growth, and since acid production in *C. carboxidivorans* is growth associated, the addition or elimination of sulfate in the medium explains why acid production was effected (Table 3). The addition or deletion of molybdenum, calcium and boric acid concentrations in the medium had no effect on ethanol production; however, adding boric acid did significantly reduce C_4 products. Since these components are nonessential to the growth of the microorganism, eliminating or increasing the concentrations of these components could be affecting its secondary metabolite and, therefore, explained the effect H_3BO_3 has on the production of C_4 products. The addition of yeast extract to the growth medium for reliable and reproducible growth could also be minimizing the elimination effect of micronutrients, such as molybdenum.

Ethanol production by *C. carboxidivorans* could be increased by using metabolic inhibitors such as fluoroacetate or trifluoroacetate which would allow the bacteria to metabolize more carbon through the ethanol rather than the acetate

pathway (Cappenberg and Prins, 1974; Chidthaisong and Conrad, 2000). Both FA and TFA inhibited production of C_4 products from CO, but the flow of end products to ethanol formation did not occur even though acetate production was decreased. Metabolic inhibitors commonly affect each organism differently, and therefore, unpredicted results ensue.

In addition to increasing the ethanol yield, industrial requirements are such that *C. carboxidivorans* must have a high tolerance for ethanol in the medium. The initial culturing inhibition concentrations of ethanol were in excess of 540 mM (25 g/l); however, as *C. carboxidivorans* adapted to increasing ethanol concentrations, inhibition decreased. The ability of *C. carboxidivorans* to tolerate high concentrations of a metabolite is associated with its potential ability to produce high concentrations of the same compound. The ethanol tolerance in *C. carboxidivorans* was similar to other solvent producing *Clostridium*, like *C. saccharolyticum* (Murray et al. 1983) or *C. acetobutylicum* with butanol tolerance (Hermann et al. 1985; Lin and Blaschek 1983). The results of these selective and adaptive strategies indicated that increased solvent yield could be coupled to increased solvent tolerance since most strains with higher ethanol yield tolerate higher ethanol concentration. In acetogens and methanogens, ethanol could be used as a substrate (Drake, 1994). The results showed that the ethanol produced by *C. carboxidivorans* would be minimally consumed and would not inhibit CO metabolism when desired concentrations of ethanol were reached experimentally.

The addition of methanol to growing cultures of anaerobic bacteria has been previously reported (Grethlein 1989). Methanol, as a co-substrate, has been

used experimentally to spare or conserve carbon contained in CO_2 (Grethlein 1989). The amendment of methanol has also been shown to increase growth rates and product formation (Grethlein and Jain 1992). Some *Clostridium* species, such as *C. formicoaceticum* and *C. aceticum*, are able to utilize alcohols like methanol by using bicarbonate or CO_2 as an electron acceptor for acetate formation (Clarke and Minton 1989). For this study, methanol and formate were examined as substrates to spare the utilization of CO for increased solvent production. The results did not show increased solvent or acid production in *C. carboxidivorans* (Table 4). Methanol was tested as a co-substrate because it has been shown as a growth substrate in other acetogens (Drake 1994). Low concentration of methanol (40 mM (1.3 g/l) with fructose and 30 mM (0.96 g/l) with CO as the substrate) inhibited the growth of *C. carboxidivorans*.

C. carboxidivorans did not grow chemolithoautotrophically without CO_2 present with CO as the substrate. A minimum of 11,000 µl/l (0.01 mM) CO_2, an electron acceptor for acetogens (Drake 1994), was required by *C. carboxidivorans* for growth. Our observations indicate that once initial concentrations of CO_2 were obtained, metabolism and growth proceeded. CO_2 is a major component in synthesis gas, therefore, supplying the initial concentration to initiate metabolism of CO and H_2 will not present additional capital or expenditures in the indirect fermentation process.

In an anaerobic system or anaerobic metabolism, oxygen contamination can lead to fatal termination or instability in product formation (Gupta et al 1994). The generation of producer gas from the gasification of switchgrass produces

mainly N_2, CO, CO_2 and H_2; however, traces of O_2, NO_x, and other C_2 compounds could contaminate the producer gas (Cateni et al. 2000, 2003). Tests on oxygen sensitivity with *C. carboxidivorans* were conducted to simulate conditions with producer gases and in scale-up plants. The differences in the oxygen tolerance between cultures grown on $CO:CO_2$ and $H_2:CO_2$ as the substrate were due to the sensitivity of the enzymes used for metabolizing the substrate. In this study, carbon monoxide dehydrogenase was not as oxygen liable as the hydrogen dehydrogenase. Though not measured directly, we speculate the defense mechanism against toxic byproducts of oxygen metabolism (hydroxyl radical, superoxide anion, and hydrogen peroxide) was sufficient for the complete removal of toxic products derived from oxygen. *C. carboxidivorans*' oxygen tolerance in synthesis gas should be sufficient to withstand possible oxygen contamination in the system without shutting down biosynthetic metabolism as evident by the successful growth of *C. carboxidivorans* on producer gas with oxygen contamination of 1000-1300 mg per liter (Cateni et al. 2000, 2003).

C. carboxidivorans ability to metabolize synthesis gas to ethanol, acetate, butanol and butyrate along with high ethanol, methanol, and oxygen tolerance is very desirable. *C. carboxidivorans* is the first known *Clostridium* species capable of both direct and indirect fermentation of lignocellulosic material. *C. carboxidivorans* produced ethanol and acetate in a direct fermentation of switchgrass at about 45% of the activity of *Clostridium thermocellum* (Ng et al. 1981). The capacity of *C. carboxidivorans* to grow at elevated temperatures and without the addition of ammonia, sodium, yeast extract or Casamino Acids makes

it desirable in industrial applications over other microbial catalysts. Experimentation on nutritional factors (Table 3), CO_2, methanol, ethanol, pH, and oxygen has answered many questions on the physiology and solventogenesis pertaining to ethanol production. The compilation of the data has allowed us to increase ethanol production from 15.2 mM (0.7 g/l) to 220 mM (10 g/l) and has made it possible for us to use this novel organism for a pilot study conducted in Oklahoma State University.

Reference

Adler HI, Crow W (1987) A technique for predicting the solvent-producing ability of *Clostridium acetobutylicum*. Appl Environ Microbiol 53:2496-2499

Bahl H, Andersch W, Gottschalk G (1982) Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two-stage phosphate limited chemostat. Eur J Appl Microbiol Biotechnol 15:201-205

Bahl H, Gottschalk G (1984) Parameters affecting solvent production by *Clostridium acetobutylicum* in continuous culture. In: Wang DIC, Scott CD (eds) Sixth Symposium on Biotechnology for Fuels and Chemicals, Vol 4, John Wiley & Sons, New York, pp 215-223

Balch WD, Wolfe RS (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl Environ Microbiol 32:781-791

Cappenberg TE, Prins RA (1974) Interrelations between sulfate-reducing and methane-producing bacteria in bottom deposits of a fresh water lake. III.

Experiments with carbon-14-labeled substrates. Antonie Van Leeuwenhoek. 40:457-469

Cateni BG, Bellmer DD, Huhnke RL, Lelo MM, Bowser TJ (2000) Recirculation in a fluideized bed gasifier to minimize oxygen content in synthesis gas from biomass. ASAE 006033. Proceedings of the American Society of Agricultural Engineers, St. Joseph, MI.

Cateni BG, Bellmer DD, Huhnke RL, Bowser TJ (2003) Effect of switchgrass moisture content on producer gas composition and quality from a fluidized bed gasifier. ASAE 036029. Proceedings of the American Society of Agricultural Engineers, St. Joseph, MI.

Chidthaisong A, Conrad R (2000) Specificity of chloroform, 2-bromoethanesulfonate and fluoroacetate to inhibit methanogenesis and other anaerobic processes in anoxic rice field soil. Soil Biol. Biochem. 32:977-988.

Clarke DJ, Minton NP (1989) Clostridia. In: Atkinson T, Sherwood RF (eds) Biotechnology Handbooks 3. Plenum Press, New York, pp. 1-304

Drake HL (1994) Acetogenesis. Chapman & Hall New York

Grethlein AJ (1989) Bioconversion of carbon monoxide for production of mixed acids and alcohols. Master of Science thesis, Department of Chemical Engineering, Michigan State University, East Lansing, MI

Grethlein AJ, Jain MK (1992) Bioprocessing of coal-derived synthesis gases by anaerobic bacteria. Trends Biotechnol 10:418-423

Grupe H, Gottschalk G (1992) Physiological events in *Clostridium acetobutylicum* during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. Appl Environ Microbiol 58: 3896-3902

Gupta M, Gupta A, Suidan MT, Sayles GD, Flora JRV (1994) ORP measurement in anaerobic systems using flow-through cell. J Environ Eng 120: 1639-1645

Hermann M, Fayolle F, Marchal R, Podvin L, Sebald M, Vandecasteele J-P (1985) Isolation and characterization of butanol-resistant mutants of *Clostridium acetobutylicum*. Appl Environ Microbiol 50:1238-1243

Hohenstein WG, Wright, LL (1994) Biomass energy production in the United States: an overview. Biomass Bioenergy 6: 161-173.

Kusel K, Dorsh T, Acker G, Stackebrandt E, Drake HL (2000) *Clostridium scatologenes* Strain SL1 isolated as an acetogenic bacterium from acidic sediments. Int J Syst Evol Microbiol 50: 537-546

Lin Y-L, Blaschek HP (1983) Butanol production by a butanol-tolerant strain of *Clostridium acetobutylicum* in extruded corn broth. Appl Environ Microbiol 45:966-973

Liou JS-C, Balkwill DL, Drake GR, Tanner RS (2005) *Clostridium carboxidivorans* sp. nov., a solvent-producing clostridium isolated from an agricultural settling lagoon, and reclassification of *Clostridium scatologenes* strain SL1 as *Clostridium drakei* sp. nov. Int J Syst Evol Microbiol (in press)

Lohmeier-Vogel E, Hahn-Hägerdal B (1985) The utilization of metabolic inhibitors for shifting product formation from xylitol to ethanol in pentose fermentations using *Candida tropicalis*. Appl Microbiol Biotechnol 21: 167-172

McNeil B, Kristiansen B (1987) The effect of medium composition on the acetone-butanol fermentation in continuous culture. Biotechnol Bioeng 29: 383-387

Mielenz JR (2001) Ethanol production from biomass: technology and commercialization status. Curr. Opin. Microbiol. 4: 324-329.

Mills AL (1997) Metal requirements and tolerance. In: Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD, Walter MV (eds) Manual of Environmental Microbiology. American Society of Microbiology, DC, pp. 349-357

Mörsdorf G, Frunzke K, Gadkari D, Meyer O (1992) Microbial growth on carbon monoxide. Biodegradation 3: 61-82

Murray WD, Wemyss KB, Khan AW (1983) Increased ethanol production and tolerance by a pyruvate-negative mutant of *Clostridium saccharolyticum*. Eur J Appl Microbiol Biotechnol 18:71-74

Ng TK, Ben-Bassat A, Zeikus JG (1981) Ethanol production by thermophilic bacteria: fermentation of cellulosic substrates by cocultures of Clostridium thermocellum and Clostridium thermohydrosulfuricum. Appl Environ Microbiol 41: 1337-1343

Parekh S, Vinci VA, Strobel RJ (2000) Improvement of microbial strains and fermentation processes. Appl Microbiol Biotechnol 54:287-301

Ragsdale SW (2004) Life with carbon monoxide. Crit Rev Biochem Mol Biol 39: 165-195

Ragsdale SW, Kumar M (1996) Nickel-containing carbon monoxide dehydrogenase/acetyl-CoA synthase. Chem Rev 96:2515-2539

Roos JW, McLaughlin JK, Papoutsakis ET (1984) The effect of pH on nitrogen supply, cell lysis, and solvent production in fermentations of *Clostridium acetobutylicum*. Biotechnol Bioeng 27: 681-694

Singh A, Kumar PKR, Schügerl K (1991) Shift in product formation from acetate to ethanol using metabolic inhibitors in *Fusarium oxysporum*. Biotechnol lett 13: 527-532

Shaheen R, Shirley M, Jones DT (2000) Comparative fermentation studies of industrial strains belonging to four species of solvent-producing clostridia. J Mol Microbiol Biotechnol 2: 115-124

Tanner RS, Miller LM, Yang D (1993) *Clostridium ljungdahlii* sp. nov., an acetogenic species in the clostridial rRNA homology group I. Int J Sys Bacteriol 43:232-236

Tanner RS (2002) Cultivation of Bacteria and Fungi. In: Hurst CJ, Crawford RL, Knudsen GR, McInerney MJ, Stetzenbach LD (eds) Manual of Environmental Microbiology. American Society of Microbiology, Washington DC, pp. 52-60

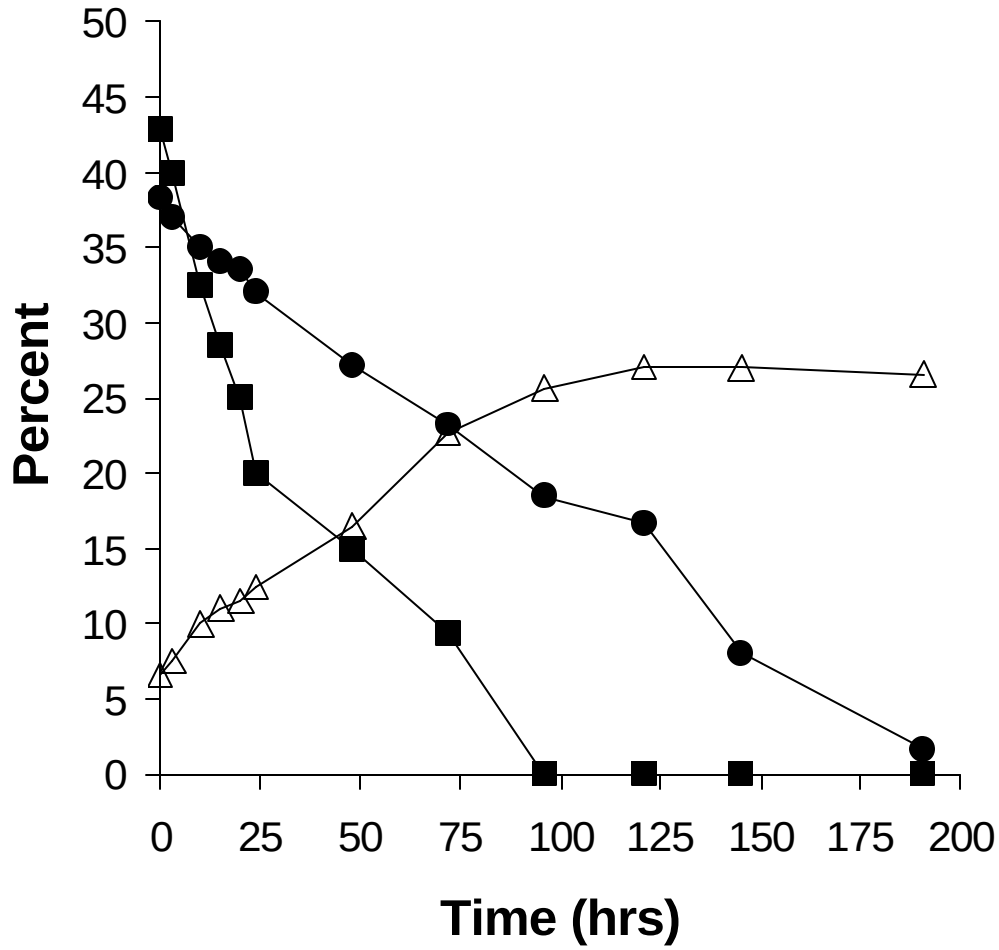


Figure 1. Simultaneous utilization of H_2 and CO, as the substrate by *Clostridium carboxidivorans*. ¦ CO, ? H_2, ? CO_2.

Table 1. Effect of pH on solventogenesis in *C. carboxidivorans* with CO as the substrate

	Acid and Alcohol Produced				
	Acetate	**Ethanol**	**Butanol**	**Butyrate**	
pH[1]	**Concentration (mM)**				**A_{600nm}[2]**
4.5	14.9	15.1	1.5	0.3	0.42
5.0	16.1	32.0	13.4	5.4	0.87
5.5	51.2	11.6	5.6	14.3	1.04
6.0	77.9	6.4	2.3	15.4	1.10

[1]pH was constant throughout growth.

[2]Final OD.

Table 2. Effect of ethanol on CO metabolism by *C. carboxidivorans*

Acid and Alcohol Produced and Consumed*							
Ethanol (mol/l)	**pH[1]**	**Acetate**	**Ethanol***	**Butanol**	**Butyrate**	**A_{600nm}[2]**	
		Concentration (mmol/l)					
0	5.5	33	40	12	3	1.0	++++
0.33	5.5	106	(100)*	3	0	0.63	++++
0.54	5.5	87	(120)*	3	0	0.48	+++
1.10	6.0	5	(0)*	0	0	0.12	+
1.74	6.2	0	(0)*	0	0	0.02	+

[1]Final pH

[2]Final O.D

*Amount consumed

+ + + + ~ 100% CO utilization

+ + + = 50% CO utilization

+ = 10% CO utilization

Table 3. General effects of medium components on the fermentation of CO by *C. carboxidivorans*

	Acids and Alcohols			
	Acetate	**Ethanol**	**Butanol**	**Butyrate**
- Na^+	**-**	**-**	**-**	**-**
- NH_4^+	**-**	**-**	**-**	**-**
- Mo^{2+}	**-**	**-**	**-**	**-**
- Ca^{2+}	**-**	**-**	**-**	**-**
- PO_4^{3-}	**dec**	**dec**	**dec**	**dec**
10X PO_4^{3-}	**inc**	**dec**	**dec**	**dec**
- SO_4^{2-}	**dec**	**-**	**-**	**dec**
10X SO_4^{2-}	**inc**	**-**	**-**	**inc**
- Fe^{2+}	**dec**	**dec**	**dec**	**dec**
10X Fe^{2+}	**dec**	**inc**	**inc**	**dec**

-, no effect

inc, increased product concentration

dec, decreased product concentration

Table 4. General effects of metabolic inhibitors on the fermentation of CO by *C. carboxidivorans*

	Acids and Alcohols			
Inhibitors	**Acetate**	**Ethanol**	**Butanol**	**Butyrate**
Methanol	-	-	-	-
Formate	-	-	-	-
Ethanol	-	-	dec	dec
TFA[1]	dec	-	dec	dec
FA[2]	dec	-	dec	dec
Chlorobutyrate	inc	dec	dec	dec
O_2	dec	dec	dec	dec

[1]Trifluoroacetate

[2]Fluoroacetate

-, no effect

inc, increased product concentration

dec, decreased product concentration

Appendix 1. Fluoroquinolone-resistant bacteria from rural lakes

Abstract

Objective: A reservoir of resistance against ciprofloxacin was discovered during a study of the microbiota of lakes in Oklahoma, USA.

Materials: The microbiota of the water column and of the lower gastrointestinal tract of inhabitant *Lepomis macrochirus* (bluegill) was surveyed at Mountain Lake, Carter County, OK, and American Horse Lake, Blaine County, OK. Isolates from CHROMagar ECC plates and a most-probable-number assay for each fish and water sample were screened for resistance using minimum inhibitory concentrations (MICs) of sixteen antibiotics.

Results: Most all the isolates were resistant to one or more of the sixteen antibiotics, and two of the isolates were resistant to ciprofloxacin. Strain AHW1G3 and MLF3G2 were then post-screened initially with 5 µg/ml of ciprofloxacin and levofloxacin, and then the MIC was determined. The MIC for strain MLF3G2 from the intestinal tract of a bluegill caught from Mountain Lake was 100 µg/ml and 20 µg/ml for ciprofloxacin and levofloxacin, respectively. The MIC for strain AHW1G3 from the water sample taken in American Horse Lake was 400 µg/ml and 2,500 µg/ml for ciprofloxacin and levofloxacin,

respectively. Both of these lakes are rural lakes and are little impacted by human activity (angling).

Conclusion: The overall resistance to antibiotics among native bacteria was unexpectedly high. Reservoirs for antibiotic resistances to ciprofloxacin and levofloxacin were discovered from screening of native bacteria from two rural lakes in Oklahoma. The two lakes are in sparsely populated counties with no identifiable antibiotic inputs. Based on the presence of natural resistance reservoirs, the potential for widespread resistance to ciprofloxacin following extensive use and overuse is probably considerable.

Introduction

Widespread antibiotic resistance in bacterial populations has been associated with humans and human activities, such as intensive animal husbandry or hospital effluent. The presence of multiple antibiotic resistant (MAR) bacteria in clinical settings is especially troubling.[1] Resistance is presumed to be acquired by bacterial populations after exposure to antibiotics, leading to the proposal that strict regulation of antibiotics could control the spread of resistance.[2-3] Antibiotic resistance was almost absent in enterobacteria from feces of wild mammals collected in Finland, consistent with the presumption above.[3] In contrast, a prior study showed extensive antibiotic resistance in coliforms from wild rodents,[4] raising questions about the origins of this resistance, persistence of resistance, and proper management of antibiotic resistance. Resistance genes may exist in natural ecosystems and be transferred to bacteria in humans and other hosts.[5-6] Natural resistance to fluoroquinolones, synthetic antibiotics, is more difficult to understand compared to resistance to penicillin or streptomycin. Although nosocomial pathogens have developed multidrug resistance[7-8], currently, fluoroquinolones have been successful in combating these pathogens. The potential existence of natural reservoirs of fluoroquinolone-resistant bacteria becomes important in light of the use of ciprofloxacin as a recommended treatment for multidrug-resistant pneumococci infections,[7-8] prophylactic for anthrax exposure,[9] and potential transfer from natural reservoirs to pathogens.

Materials and methods

Antibiotic resistances from strain MLF3G2 and AHW1G3 were identified in the study of the microbiota of *Lepomis macrochirus* (bluegill) and associated waters, respectively. The lakes studied were in Blaine Co. (33 inhabitants per square kilometer) and Carter Co. (143 inhabitants per square kilometer), OK, USA (see www.factfinder.census.gov). Land use in the watersheds was agricultural, e.g., low-intensity cattle grazing. Lake water contained <0.2 *E. coli* per ml; identification and counts were done using CHROMagar ECC (Paris). Samples of bluegill intestinal contents and lake water were diluted to extinction in tryptic soy broth in a most-probable-number assay. Isolates recovered, presumably of cells present in high numbers, were identified using a BIOLOG system (Hayward, CA) and were tested for sensitivity to 16 antibiotics (Table 1). Antimicrobial susceptibility was determined using a Bauer-Kirby antimicrobial disc assay according to National Committee for Clinical Laboratory Standards (NCCLS - Approved Standard M2-A7) guidelines. Isolates resistant to the Ciprofloxacin Sensi-Disc (5µg) (Becton Dickinson and Company, Sparks, MD, USA) were further subjected to MICs testing of ciprofloxacin (Bayer, West Haven, CT, USA) and levofloxacin (Ortho-McNeil Pharmaceutical Inc., Raritan, NJ, USA) by the broth microdilution technique according to NCCLS - Approved Standard M7-A4 guidelines. The ciprofloxacin-resistant isolates were also further identified using partial 16S rRNA gene sequence analysis. DNA sequencing of the PCR products were carried out on an Applied Biosystems Model 3730 automatic sequencer at

the Oklahoma Medical Research Foundation, OK. Sequence analyses were aligned and corrected using the computer program Sequencer. Related sequences of the 16S rRNA gene were conducted by using Blastn search program of the National Center for Biotechnology Information.

Results

Genera identified from intestinal contents included *Aeromonas*, *Plesiomonas*, and *Enterococcu*s. Genera identified from water samples included *Citrobacter*, *Pectobacterium*, *Enterobacter*, and *Microbacterium*. Two isolates were resistant to fluoroquinolones, one from a water sample (strain AHW1G3) and one from bluegill intestinal contents (strain MLF3G2) (Table 1). Identification of these strains was confirmed by partial 16S rDNA sequence analysis, and strain AHW1G3 was deposited with ATCC (accession no. BAA-799). The resistant phenotype was unexpected, and the level of resistance was exceptional. The MIC of ciprofloxacin and levofloxacin against strain AHW1G3 was 400 and 2,500 µg/ml, respectively. The MIC of ciprofloxacin and levofloxacin against strain MLF3G2 was 100 and 20 µg/ml, respectively. This is in contrast to that reported for clinical isolates such as *Streptococcus pneumoniae* (MIC of ciprofloxacin for >99% of strains <4 µg/ml[10]), and *Pseudomonas aeruginosa* (MIC of ciprofloxacin and levofloxacin 0.25-1 µg/ml and 0.5-4 µg/ml, respectively).

The magnitude of MAR from the two rural lakes and bluegill intestines was extensive among all isolates collected (n=65) from CHROMagar ECC plates and the most-probable-number assays. 68% of the isolates were resistant to 2 or more of the 16 antibiotics screened, 57% to three or more, and 35% to four or more of the antibiotics screened.

Discussion

The finding of high levels of antibiotic resistance, including in two instances to ciprofloxacin, among the numerically dominant culturable heterotrophs from rural lakes in areas that are sparsely populated, have limited human impacts, and no identified antibiotic input was puzzling, but similar to the observations of Gilliver et al.[4] A low population density per se[3] does not account for antibiotic resistance patterns. How these resistances were established and are maintained remains unknown. However, a natural reservoir of ciprofloxacin resistance exists, a fact which must be considered if widespread use of this antibiotic is undertaken in response to treating patients with multidrug-resistant bacteria and for broader systemic infections.

References

1. Davies, J. (1996). Bacteria on the rampage. *Nature* **383,** 219-20.

2. Levy, S. B. (2002). Factors impacting on the problem of antibiotic resistance. *Journal of Antimicrobial Chemotherapy* **49,** 25-30.

3. Osterblad, M., Norrdahl, K., Korpimaki, E. *et al.* (2001). How wild are wild mammals? *Nature* **409,** 37-38.

4. Gilliver, M. A., Bennett, M., Begon, M *et al.* (1999). Antibiotic resistance found in wild rodents. *Nature* **401,** 233-34.

5. Ferber, D. (1998). Microbiology: new hunt for the roots of resistance. *Science* **280,** 27.

6. Humeniuk, C., Arlet, G., Gautier, V. *et al.* (2002). ß-Lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. *Antimicrobial Agents and Chemotherapy* **46,** 3045-49.

7. Weiss, K., Restieri, C., Gauthier, R., *et al.* (2001). A nosocomial outbreak of fluoroquinolone-resistant Streptococcus pneumoniae. *Clinical Infectious Diseases* **33,** 517-22.

8. Davidson, R., Cavalcanti, R., Brunton, J. L. *et al.* (2002). Resistance to levofloxacin and failure of treatment of pneumococcal pneumonia. *New England Journal of Medicine* **346,** 747-50.

9. Rosovitz, M. J. & Leppla, S. H. (2002). Virus deals anthrax a killer blow. *Nature* **418,** 825-26.

10. Sahm, D. F., Peterson, D. E., Critchley, I. A. *et al.* (2002). Analysis of ciprofloxacin activity against *Streptococcus pneumoniae* after 10 years of use in the United States. *Antimicrobial Agents and Chemotherapy* **44,** 2521-24.

Table 1 Antibiotic resistance profile of aquatic strains resistant to ciprofloxacin

	CRO30	E15	GEN10	K30	STR10	NA30	ST250	CIP5	MIC CIP	MIC LVX
Microbacterium sp. strain AHW1G3	S	S	S	S	S	R	S	R	400 µg/ml	2,500 µg/ml
Enterococcus sp. strain MLF3G2	R	R	R	R	R	R	R	R	100 µg/ml	20 µg/ml

Antibiotic sensitivity (S) and resistance (R) were determined using a Bauer-Kirby antimicrobial disc assay. Antibiotic discs were from Becton, Dickinson and Company, Sparks, MD, USA. Antibiotic discs listed above were: ceftriaxone, 30 µg; erythromycin, 15 µg; gentamicin, 10 µg; kanamycin, 30 µg; streptomycin, 10 µg; nalidixic acid, 30 µg; sulfathiazole, 250 µg; ciprofloxacin, 5 µg. Both strains were sensitive to ampicillin (10 µg), carbenicillin (100 µg), cefaclor (30 µg), chloramphenicol (30 µg), doxycycline (30 µg), oxytetracycline (30 µg), tetracycline (30 µg) and trimethoprim (5 µg) in this assay. The minimal inhibitory concentration (MIC) of ciprofloxacin and levofloxacin was determined in tryptic soy broth. The MIC of these antibiotics against *Escherichia coli* was <0.1 µg/ml.

PERSPECTIVES & FUTURE WORK

Perspective. The work presented in this dissertation has, in part, fulfilled the objectives set for the bioconversion of agricultural biomass to liquid fuels. In chapter 1, *Clostridium carboxidivorans* strain P7 was characterized and identified as a novel solvent-producing *Clostridium* species. Although molecular techniques were utilized to distinguish the species, the classical approach in microbial systematics was an integral part in differentiating *C. carboxidivorans* from other species. The utilization of classical and molecular systematics (i.e., phenotype and phylotype) has led to many new discoveries in taxonomy; however, much of the decoded relationship among prokaryotes stems directly from the molecular approach. It would be short-sighted to say that phenotypic traits do not contribute to defining microbial taxa (Wheelis *et al*., 1992). An example of this is exhibited by the intestinal bacterial community of the termite (Ohkuma and Kudo, 1996). Of 55 bacterial clones sequenced, two-thirds of the analyzed clones had less than 90% sequence identity to any known 16S rRNA gene sequences of cultivated organisms. Furthermore, one-third of the clones showed no sequence similarity to any recognized bacterial phylum in the rRNA database. The phylogenetic approach was limited, and the best course of action was to use phenotypic analysis to determine taxonomy (Ohkuma and Kudo, 1996). The balance between classical and molecular systematics helps to define and direct the understanding of a complete evolutionary paradigm.

Another example of utilizing classical and molecular systematics was exemplified in the characterization of *Clostridium carboxidivorans* strain P7. The 16S rRNA gene sequence of *C. carboxidivorans* strain P7 was very similar to that of *Clostridium scatologenes* strain SL1^T (99.8%) and *C. scatologenes*^{T} (99.7%). The 16S rRNA gene sequence of strain SL1^T was also very similar to that of *C. scatologenes*^{T} (99.7%), which had been reported earlier (99.6%; Kusel *et al*., 2000). All three strains are in cluster I (subcluster Ic) of *Clostridium* group I (Collins *et al*., 1994; Stackebrandt and Hippe, 2001). Based only on 16S rRNA gene sequences, the three strains would likely have been considered the same species and elucidation of *C. carboxidivorans* or strain SL1^T as novel *Clostridium* species would not have occurred. However, classical methods in phenotypic characterization had indicated distinct differences between the species. Although other molecular techniques (e.g., BOX-PCR and DNA:DNA reassociation) did phylogenetically differentiate the species, phenotypical characterization played a critical role in distinguishing *C. carboxidivorans* strain P7^T from other clostridial species such as *C. scatologenes*^{T} and *C. scatologenes* strain SL1^T. Utilizing both classical and molecular systematics to identify and characterize an organism is truly how one can respectively differentiate one species from another.

Future Work. In chapter 2, growth of *C. carboxidivorans* was optimized to increase the yield of ethanol. Knowledge gained from this research will improve the future cultivation and use of acetogens for maximizing conversion of synthesis gas to ethanol. Chapter 2 illustrates how the effects of nutrients and

metabolic inhibitors can influence CO fermentation, thus signifying a means of achieving useful solvent production by the manipulation of these factors. However, further research is necessary. For example, other components such as trace metals should be investigated. Iron and other essential metals serve as important components in biological systems (Mills, 1997); therefore, future experiments should explore the effect individual trace metal components can have on solvent production in *C. carboxidivorans*.

Although future research on media components may lead to increased solvent production in *C. carboxidivorans*, these experiments will not elucidate how CO is utilized to produce alcohols. It is well established how carbohydrates are utilized to produce alcohols in acetogens (Drake et al., 2002); however, the basic metabolic pathway for how CO feeds into the pathway to produce alcohol is still speculative. Therefore, more fundamental research on carbon monoxide metabolism in *C. Carboxidivorans* would seem practical. One starting point would be to determine the activities of key enzymes under acidogenic and solventogenic conditions. Under acidogenic conditions, activities of acetate kinase, butyrate kinase, phosphotransacetylase, and phosphotransbutyrylase should be high, and activities of acetaldehyde dehydrogenase, butyraldehyde dehydrogenase, ethanol dehydrogenase, and butanol dehydrogenase should be low (Andersch et al., 1983). Under solventogenic conditions, the level of activities should be reversed (Andersch et al., 1983). Although these experiments indirectly provide understanding of how CO feeds into the pathway to produce alcohol, determining variations in enzyme activities at different periods of growth will

pinpoint conditions for acid or solvent production more precisely, thereby finding the rate limiting step to increasing ethanol production. It will also illustrate whether regulated enzyme activities are coordinated or independent when utilizing CO to produce alcohols. In addition to providing basic physiological data, this information would further help identify metabolic control mechanisms and influence industrial fermentation strategies.

Other factors for increasing solvent production that should be considered, in conjunction with medium manipulation and strain adaptation, are random mutagenesis and metabolic engineering. Clostridial mutagenesis has been successfully performed using N-methyl-N'-nitro-N-nitrosoguanidine (NTG), a strong alkylating agent (Murray et al., 1983). NTG mutant strains produced up to 4-fold higher ethanol to acetate ratios when compared to the parent strain. As a mutagen, NaBr and $NaBrO_3$ salt (Cueto and Mendez, 1990) release a toxic bromine gas at low pH. The bromine gas selectively kills acid producers and spares cells that are non-acid producers. Strains obtained with this method also produced up to 1-fold higher solvent to acid ratios when compared to the parent strain (Cueto and Mendez, 1990). These methods should be employed for engineering a better solvent producing strain of *C. carboxidivorans*.

As a model for metabolic engineering, genes of minicellulosomes (i.e. miniCipC1-man5K complex) from *Clostridium cellulolyticum* have been successfully inserted into *Clostridium acetobutylicum* to degrade cellulose and related plant cell wall polysaccharides (Mingardon et al., 2005). The recombinant strain of *C. acetobutylicum* secreted mannanase and degraded galactomannan,

thereby indicating successful insertion of the gene and the ability to utilize lignocellulosic materials (Mingardon et al., 2005). Therefore, it might be possible to insert duplicate copies of ethanologenic genes such as alcohol dehydrogenase or genes such as formate dehydrogenase to increase solvent production in *C. carboxidivorans*. Pursuing metabolic engineering to increase the yield and efficiency of *C. carboxidivorans*' ability to utilize synthesis gas can eventually make it optimal for a industrial scale fermentation.

The discovery made during the analysis of the multiple-antibiotic resistance data from the comparative study on the microbiota of the intestinal tract of bluegills from treated sewage water to that of bluegills from clean rural lakes certainly warrants further investigation. In Appendix 1, the overall resistance to antibiotics among native bacteria from the clean rural lakes was noticeably high, and fluoroquinolone-resistant bacteria were present in high numbers. This discovery suggested that a natural reservoir of resistance against fluoroquinolones exists. Continued studies determining the source of this fluoroquinolone resistance are justifiable due to the potential public health issues. Future work should include field studies to identify and sample watersheds and any natural springs or waterways that may feed into these lakes. Direct screening against ciprofloxacin should be conducted on the water and soil in these lakes and on the water sources feeding into these lakes. In addition to illustrating the potential prevalence of this phenotype among the native bacteria, it will address which bacterial species constitute the principal reservoirs of resistance, what proportion of these resistant populations may carry the plasmid, and the pattern of resistance

among different species with various antibiotics. The origin of this resistance could be eluded by addressing these questions (Esiobu et al., 2002) or determining the sources where this resistance is established and maintained (Levy, 2002).

Although the primary research in this dissertation was on the microbial conversion of synthesis gas to ethanol, research conducted in applied environmental microbiology on anaerobic systems such as microbial water ecology enhances the overall research. The richness of microbial diversity present in these anaerobic systems (e.g. lakes, natural waterways) may yield organisms with multiple functions of value, such as high capacity biodegradative or specialized metabolic functions. The isolation of *Clostridium carboxidivorans* strain P7 and bacteria resistant against fluoroquinolones surely exemplifies this value; therefore, further research in applied environmental microbiology on anaerobic systems seems warranted to further explore other multiple functions of value.

REFERENCES

Andersch, W., Bahl, H., and Gottschalk, G. 1983. Levels of enzymes involved in acetate, butyrate, acetone, and butanol formation by *Clostridium acetobutylicum*. Eur. J. Appl. Microbiol. Biotechnol. 18: 327-332.

Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. and Farrow, J. A. E. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44: 812-826.

Cueto, P. H., and Mendez B. S. 1990. Direct selection of *Clostridium acetobutylicum* fermentation mutants by a proton suicide method. Appl. Environ. Microbiol. 56: 578-580.

Drake, H. L., K. Küsel, and C. Matthies. 2002. Ecological consequences of the phylogenetic and physiological diversities of acetogens. Antonie Van Leeuwenhoek. 81: 203-213.

Esiobu, N., Armenta, L., and Ike, J. 2002. Antibiotic resistance in soil and water environments. Int. J. Environ. Health Res. 12, 133-144.

Kusel, K., Dorsch, T., Acker, G., Stackebrandt, E. and Drake, H. L. 2000. *Clostridium scatologenes* strain SL1 isolated as an acetogenic bacterium from acidic sediments. Int. J. Syst. Evol. Microbiol. 50: 537-546.

Levy, S. B. 2002. Factors impacting on the problem of antibiotic resistance. J. Antimicrob. Chemother. 49, 25-30.

Mills, A. L. 1997. Metal requirements and tolerance. In: Hurst CJ, Knudsen GR, McInerney MJ, Stetzenbach LD, Walter MV (eds) Manual of Environmental Microbiology. American Society of Microbiology, DC, pp. 349-357.

Mingardon, F., Perret, S., Belaich, A., Tardif, C., Belaich, J-P., and Fierobe, H-P. 2005. Heterologous production, assembly, and secretion of a minicellulosome by *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. 71: 1215-1222.

Murray, W. D., Wemyss, K. B., and Khan, A. W. 1983. Increased ethanol production and tolerance by a pyruvate-negative mutant of *Clostridium saccharolyticum*. Eur. J. Appl. Microbiol. Biotechnol. 18: 71-74.

Ohkuma, M. and Kudo, T. 1996. Phylogenetic Diversity of the intestinal bacteria community in the termite *Reticulitermes speratus*. Appl. Environ. Microbiol. 62: 461-468.

Stackebrandt, E. and Hippe, H. 2001. Taxonomy and systematics,. In *Clostridia*, pp. 19-48. Edited by H. Bahl & P. Dürre, New York City, NY: Wiley-VCH.

Wheelis, M. L., Kandler, O., and Woese, C. R. 1992. On the nature of global classification. Proc. Natl. Acad. Sci. U.S.A. 89: 2930-2934.