A PHYLOGENETIC STUDY OF THE GENUS *CAMPYLOBACTER*

by

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(ABSTRACT)

The relationships of the fourteen (14) species of *Campylobacter* were derived by comparison of the partial 16S ribosomal RNA sequences determined using reverse transcriptase and oligonucleotide primers specific for regions in the 16S rRNA molecule. These species formed three distinct RNA sequence homology groups. We propose that the following species remain in the genus *Campylobacter* (homology group I): *C. coli*, *C. jejuni*, *C. laridis*, *C. fetus*, *C. hyointestinalis*, *C. concisus*, *C. mucosalis*, *C. sputorum* and the "catalase-negative to weakly-positive" (CNW) strains. *C. pylori*, "*C. cinaedi*" and "*C. fennelliae*" were not related to the true campylobacters at the genus level. However, they were related to each other and to *Wolinella succinogenes* at the genus level and constituted homology group II. These four species should be reclassified and placed within a single genus based on 16S rRNA sequence similarity. *C. cryaerophila* and *C. nitrofigilis* also exhibited a high level of RNA sequence homology with each other but not with any other species tested; they constituted homology group III and should be considered as being a single genus. A comparison of the 16S rRNA sequence data from the three homology groups with the same sequences from representatives of the three major

phylogenetic branches of the purple bacteria indicated that these genera form a single phylogenetic branch which is only distantly related to the purple bacteria.

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Table of Contents

List of Tables

List of Figures

INTRODUCTION

The genus Campylobacter contains gram-negative, microaerophilic vibrioid rods that are motile by a single polar flagellum at one or both ends of the cell. They neither ferment nor oxidize carbohydrates but obtain energy primarily by oxidizing amino acids and tricarboxylic acid cycle intermediates. The genus currently includes 14 species. Campylobacters can be found in various habitats including the reproductive tract, gastrointestinal tract and oral cavity of man or animals. One species is a commensal found in association with the roots of grasses in salt marshes.

Campylobacters have been recognized as important agents of veterinary disease for several decades. In recent years, however, the importance of these pathogens in human disease has become increasingly apparent. Campylobacter jejuni and Campylobacter coli are now considered the major causes of bacterial gastroenteritis in man, being isolated as frequently as Salmonella and Shigella combined. The isolation of Campylobacter pylori is highly correlated with incidence of human gastritis while several other species are known opportunistic pathogens in man.

The species currently placed within this genus show wide diversity with respect to physiology, biochemical characteristics, nutritional requirements and natural habitats. This diversity raises the question as to whether all of these species are sufficiently related to justify their classification within a single genus. Since campylobacters do not attack carbohydrates and are inert with regard to most of the traditional biochemical tests used for identification of bacteria, only a few tests are available for the identification and classification of

campylobacters. Consequently, the classification of campylobacters based on physiology and biochemical tests has a limited usefulness.

In order to determine relatedness of bacteria above the level of species, one must examine certain genes which accurately reflect the evolutionary distance between the organisms (i.e. genes which have a low rate of mutation with respect to the rest of the genome). Gene products can also be used in the analysis since these will reflect the conservation of the genes. Gene products used in this fashion are termed molecular chronometers. The 16S ribosomal ribonucleic acid (rRNA) molecule has become the standard molecular chronometer used in the classification of bacteria above the level of species. This is due to several features possessed by the 16S rRNA molecule: a universal distribution in bacteria, the importance of its cellular function which results in an extremely low tolerance of mutation, the ease of its isolation, the apparent lack of lateral transfer of rRNA genes between contemporaneous organisms, and a size (approx. 1500 bases) sufficient to accomodate a useful amount of genetic information.

The specific objectives of this research were:

1. To isolate 16S rRNA from the type strains representing all fourteen species of Campylobacter and determine the partial sequence of this RNA by a recently described rapid method of sequencing using reverse transcriptase and oligonucleotide primers that are specific for certain regions in the RNA molecule.

2. To use this sequence data base to estimate the relatedness exhibited by these species at the genus level of phylogenetic classification.

3. To use the relatedness values so obtained as a basis for deciding whether reclassification of various Campylobacter species should be done and, if so, what specific recommendations should be made in this regard.

LITERATURE REVIEW

Members of the genus Campylobacter are small, gram-negative curved rods that demonstrate a characteristic "corkscrew-like" motility. Carbohydrates are neither fermented nor oxidized. Tricarboxylic acid cycle intermediates and amino acids serve as carbon sources. Campylobacters are microaerophilic and grow best under an atmosphere of 3-6% O_2 and 5-10% CO_2 (Smibert 1984). One species is aerotolerant (Neill et al. 1985). Some species require hydrogen gas or formate as an electron donor, whereas other species which do not require H_2 may be stimulated by its presence (Smibert 1984). Some species can grow under anaerobic conditions in the presence of an alternate electron acceptor such as fumarate or nitrate, whereas other species are unable to grow anaerobically (Goodman and Hoffman 1983; McClung et al. 1985; Neill et al. 1985; Razi et al. 1981; Smibert 1984; Totten et al. 1985; Véron et al. 1981).

The current species of Campylobacter and their pathogenic significance are listed in Table 1, and the phenotypic characteristics which differentiate these species are listed in Table 2. The differential characteristics of the "catalase-negative to weakly-positive" (CNW) strains of Campylobacter can be found in previously published studies (Roop et al. 1984; Sandstedt et al. 1983). The Campylobacter species designations have been confirmed as distinct and separate species by previous DNA homology studies (Belland and Trust 1982; Benjamin et al. 1983; Harvey and Greenwood 1983; Hébert et al. 1983; Leaper and Owen 1982; Owen 1983; Owen and Leaper 1981; Roop et al. 1984, 1985; Ursing et al. 1983). The most comprehensive DNA homology study of this genus placed 118 strains of Campylobacter into 11 homology groups (Roop et al.

Table 1. Names and Clinical Significance of *Campylobacter* Species.

Species Name	Pathogenicity
CATALASE POSITIVE CAMPYLOBACTERS	
C. coli	Enteritis in humans (orally transmitted); normal intestinal flora of pigs and poultry
C. jejuni	Enteritis in humans; normal intestinal flora of cattle, sheep, dogs, cats, poultry, etc
C. fetus subsp. *fetus*	Sporadic abortion in cattle and sheep; blood infections in humans; orally transmitted
C. fetus subsp. *venerealis*	Sexually transmitted abortion in cattle
C. laridis	Intestines of seagulls, humans, dogs, horses; occasionally causes blood infections in humans
C. hyointestinalis	Proliferative ileitis in pigs; diarrhea in calves; occasionally causes blood infections in humans
C. sputorum biovar fecalis	Normal flora of sheep feces and bovine genitalia
C. pylori	Gastric and duodenal ulcers in humans
"*C. cinaedi*"	Proctitis in homosexual men
"*C. fennelliae*"	Proctitis in homosexual men
C. cryaerophila (sic)	Abortions in pigs, cattle, horses and sheep; occasionally causes blood infections in humans
C. nitrofigilis	Found in association with roots of salt marsh grasses
CATALASE NEGATIVE CAMPYLOBACTERS	
C. sputorum bicvar sputorum	Normal flora of the human mouth
C. sputorum biovar bubulus	Normal flora of bovine genitalia
C. mucosalis	Isolated from lesions of porcine intestinal adenomatosis
C. concisus	Isolated from humans with peridontal disease
"CNW Strains"	Feces of dogs and cats

TABLE 2. Differential characteristics of Campylobacter species[a]

Characteristics	C. fetus subsp. fetus	C. fetus subsp. veneralis	C. jejuni	C. coli	C. hyointestinalis	C. lanidis	C. cryaerophila	C. nitrofigilis	"C. cinaedi"	"C. fennelliae"	C. pylori	C. sputorum biovar sputorum	C. sputorum biovar bubulus	C. sputorum biovar fecalis	C. mucosalis	C. concisus
Type strain of species	ATCC 27374	ATCC 19438	ATCC 33560	ATCC 33559	ATCC 35217	ATCC 35221	NCTC 11885	ATCC 33309	ATCC 35683	ATCC 35684	NCTC 11637	ATCC 35980			NCTC 11000	ATCC 33237
Catalase	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-
Growth at:																
25°C	+	+	-	-	+	-	+	+	-	-	-	-	-	-	+	-
42°C	d	-	+	+	+	+	-	-	-	-	-			+	+	+
Growth in:																
1% glycine	+	-	+	+	+	+	-	-	+	+	d	+	+	+	+	+
1% oxgall												+	-	+	+	+
Minimal medium	d	d	-	+	-	-	d					-	d	d	-	-
1.5% NaCl plates	d	-	-	-	-	+	-					d	+	+	-	+
3.5% NaCl	-	-	-	-	-	-	-	+	-	-	-	-	d	+	-	-
Anaerobic growth in 0.1% TMAO	-	-	-	-	+	+	-					d	+	+	-	-
Sensitive to:																
nalidixic acid, 30 µg disk	-	-	+	+	-	-	d	+	+	+	-	d	d	-	d	-
cephalothin, 30 µg disk	+	+	-	d	+	-	-	+	+	+	+	+	+	+	+	-
Hippurate hydrolysis	-	-	+	-	-	-	-	-	-	-		-	-	-	-	-
H_2S production:																
SIM medium	-	-	-	-	-	-	-					d	+	+	+	+
TSI slants	-	-	-	+	+	-	-					d	+	+	+	+
Alkaline phosphatase	-	-	+	d	-	-	-					-	-	-	-	-
Aerobic growth	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+		+	-	-	+	+	+	+	+
Nitrite reduction	-	-	-	-	-	-	-		-	-	-	d	d	+	d	+
Nitrogenase activity	-		-	-				+								
H_2 or formate needed for microaerophilic growth; H_2 + fumarate, or formate + fumarate, needed for anaerobic growth												-	-	-	+	+
Fumarate, but not H_2 or formate, needed for anaerobic growth												+	+	+	-	-

[a] Symbols: see standard definitions.

1984, 1985). Since that study, three new species have been designated: Campylobacter pylori (Marshall et al. 1984), "Campylobacter cinaedi" and "Campylobacter fennelliae" (Totten et al. 1985). Although some DNA homology studies have indicated that certain species, such as C. coli, C. jejuni and C. laridis, are more related to each other than they are to other campylobacters (Belland and Trust 1982; Roop et al. 1984, 1985; Ursing et al. 1983), this type of analysis cannot answer the question as to whether the Campylobacter species are sufficiently related to warrant classification within a single genus. Measurement of the similarity of DNA between two organisms, expressed as a percent homology, is usually not useful in the classification of organisms above the level of species. This is because percent DNA homology reflects the similarity between the entire genomes of the two organisms. Since the bulk of a genome has a high tolerance for base mutations, DNA homology values are only meaningful when determining the relatedness between two organisms which have only recently diverged from one another (Johnson 1984; Schleifer and Stackebrandt 1983).

To accurately determine the relationships between more distantly related organisms, the similarity of certain genes which have a low rate of base mutation with respect to the bulk of the genome can be used to measure phylogenetic relationships above the level of species (Johnson 1984; Schleifer and Stackebrandt 1983). The products of these genes can be used for this measurement since they will accurately reflect this sequence conservation (De Wachter et al. 1985; Fitch and Margoliash 1967; Stackebrandt 1985; Woese 1985; Zuckerkandl and Pauling 1965). A gene product used in the fashion is termed a molecular chronometer.

The first molecular chronometer which was extensively used for phylogenetic measurement was cytochrome c. Although the use of this molecule was of great value in the confirmation of the eukaryotic evolutionary tree, it is not useful in the study of bacterial phylogeny for the following reasons. First, the "evolutionary clock" of cytochrome c is too fast to be accurate in bacterial classification. That is, the rates of base substitution and deletion occur too rapidly to allow measurement of the deep evolutionary divergences of bacteria. Secondly, cytochrome c does not remain functionally constant across the bacterial realm. Lastly, this molecule does not even occur in many bacterial groups, which results in an incomplete picture of bacterial evolution (Woese 1985).

As the ability to determine actual base sequences of nucleic acids was developed, attention turned to using the ribosomal ribonucleic acid (rRNA) molecules as chronometers. Some of the reasons for this are as follows: rRNAs are the key elements of the cellular translational apparatus and are therefore functionally and evolutionarily homologous in all organisms; the importance of their cellular function dictates a very low tolerance for base mutation and, therefore, they are moderately to highly conserved across kingdoms; rRNA constitutes a significant fraction of the cellular mass and is easily isolated; and rRNA can provide enough data to be statistically significant in phylogenetic analysis (Woese 1985). Additionally, there appears to be no lateral transfer of rRNA genes between contemporaneous organisms (Olsen et al. 1986). All procaryotic cells contain 5S, 16S and 23S rRNA. Their functionally and structurally similar counterparts in eucaryotes are 5S,

18S and 30S rRNA. The eukaryotes also commonly contain a fourth 5.8S species which is homologous to the 5' end of the bacterial 23S rRNA (Olsen et al. 1986). Since the sequencing methods of the early 1970's were not practical for the larger rRNAs, the 5S rRNA molecule was the initial choice to be used as a chronometer due to its small size (approx. 120 nucleotides). Although employed with some success (Hori and Osawa 1979; Küntzel 1982) the 5S rRNA is too small and too highly conserved to accurately estimate phylogenetic distance and branching order (De Wachter et al. 1985; Woese 1985). Following significant developments in methodology, the 16S rRNA molecule was found to be the ideal chronometer for the following reasons. First, the size of the 16S rRNA (approx. 1600 bases) is much larger than the 5S rRNA so that any "nonclocklike" base changes that may occur during evolution affect a relatively smaller portion of the molecule. Moreover, a larger sequence database is obtained. Secondly, the occurrence of base changes in the 16S molecule vary in frequency in different positions on the molecule. Some areas will change within a genus while other areas remain constant across kingdoms. This allows a wide range of phylogenetic distances to be analyzed (Olsen et al. 1986; Woese 1985).

Since 16S rRNA could not be used as chronometer due to the incompatibility of such a large molecule with the nucleotide sequencing procedures of the early 1970's, other analysis methods had to be developed. One of the most important techniques was RNase T_1 oligonucleotide cataloging. This procedure, developed by Carl Woese and his colleagues in the late 1970's, involves repeated enzymatic digestion of radioactively labeled 16S rRNA with RNases, each digestion being

followed by a two-dimensional electrophoresis. An autoradiograph of the sample is made and the exposed regions or spots (corresponding to oligonucleotides) are characterized for migration patterns which are dependent on particular base compositions and fragment lengths. The oligonucleotides are then put into a catalogue and it is these catalogues which are then compared from different organisms. The similarity between the catalogues is expressed as a binary association coefficient known as a S_{AB} value. Since these catalogues become part of a permanent record or database, they need to only be determined one time and stored in a computer. Later, these catalogues can be compared with new ones as they become available. Major discoveries in bacterial evolution were made possible by the application of this procedure. For example, the discovery of a third major line of evolutionary divergence, which occurred as early as the divergence of the procaryotes and the eucaryotes, radically altered the perception of evolution of life on primitive Earth. This third "kingdom", which is made up of a group of bacteria called the archaeobacteria, is no more related to the other "kingdom" of bacteria, now called the eubacteria, than the eubacteria are to the eucaryotes (Woese and Fox 1977). Another major discovery involved the elucidation of the origin of the membrane-bound organelles which are found within the eucaryotic cell. For several years it had been speculated that these organelles may have originated from symbiotic relationships between certain types of bacteria and primitive eucaryotes. This was based on such circumstantial evidence as the fact that chloroplasts of green plants contain the eubacterial 16S rRNA rather than the eucaryotic 18S rRNA. Chloroplasts and the cyanobacteria

also have similar photosynthetic membranes, pathways of carbon dioxide fixation and electron flow (Bonen and Doolittle 1975; Zablen et al. 1975). The definitive evidence came from the comparison of oligonucleotide catalogues created from chloroplast rRNA and cyanobacterial rRNA which revealed a significant evolutionary relationship between chloroplasts and certain cyanobacteria (Bonen and Doolittle 1975; Zablen et al. 1975). A similar relationship was demonstrated between mitochondria and certain purple nonsulfur eubacteria (Bonen et al. 1977; Küntzel 1982).

With the advent of DNA cloning and rapid nucleotide sequencing procedures, the cumbersome process of oligonucleotide cataloging is being used much less frequently. The use of DNA cloning in phylogenetic analysis involves locating the RNA cistrons within the genome, removing this region with restriction endonucleases and placing this DNA fragment within a suitable cloning vector, usually a plasmid. This insert within the plasmid is then subcloned into a vector especially designed for nucleotide sequencing using different sets of restriction enzymes to create a population of subclones whose inserts overlap, thus allowing the entire 16 rRNA sequence to be determined. This is a major advantage of the cloning procedure over cataloging, which yields only 25-30% of the total 16S rRNA sequence. Knowing the entire nucleotide sequence is important in functional analysis of rRNA which requires secondary structure determinations. However, it should be noted that determination of the entire sequence of 16S rRNA is not necessary in phylogenetic analysis because the partial sequence obtained reflects the evolutionary information contained within the entire sequence (Lane et

al. 1985). The major disadvantage of the cloning procedure occurs when a large number of organisms must be compared since each cistron from each organism must be successfully located, cloned and populations of overlapping subclones obtained. To avoid the cloning process, Lane et al. (1985) took advantage of the highly conserved regions of 16S rRNA and developed a nucleotide sequencing procedure which enabled researchers to determine the partial base sequence of 16S rRNA directly from the molecule itself. This is done by isolating and purifying the large rRNAs (16S and 23S) and hybridizing oligonucleotide primers which are made specifically complementary to the highly conserved regions of 16S rRNA. These primers provide the free 3' hydroxyl ends to reverse transcriptase, which can then initiate synthesis of a single strand of DNA that is complementary to the RNA. The DNA sequence can then be determined by the dideoxynucleotide chain termination method (Sanger 1981). Approximately 800 bases of sequence data can be obtained per organism in one week from cell pellet to autoradiograph (once the optimal conditions for the procedure have been determined for the organisms in question). Lane et al. (1985) also demonstrated that the analysis of the partial sequence data obtained by this method yields results which are statistically similar to the results obtained when the same analysis is performed using the entire sequences of the same 16S rRNAs.

The question of the practicality of a phylogenetically based system of classification for prokaryotes is often raised. Until the work of Woese and colleagues, phylogenetic classification of organisms above the species level was basically speculative. Bacteria were

classified primarily on phenotypic characteristics and morphology. Those that resembled each other and had similar metabolisms, growth requirements, etc. were placed within single families and in some cases, single genera. For example, the gram-negative photosynthetic bacteria were grouped together because the capacity for photosynthesis was thought to be a phylogenetically important phenotype. Those photosynthetic bacteria which could reduce particular compounds and those which could not reduce these compounds were further classified as separate groups. Phylogenetic classification, based on the use of nonhomologous phenotypes and characteristics of differing complexities, is not possible (Fox et al. 1980; Stackebrandt 1987; Woese 1985). It has subsequently been found that the photosynthetic bacteria are scattered throughout several branches of the bacterial phylogenetic tree established from rRNA cistron similarity studies (Fox et al. 1980; Stackebrandt 1985; Stackebrandt and Woese 1984; Woese 1985, 1986). Since this type of tree is based on genetic relationships which remain constant, the classification scheme itself will remain stable. However, what good is this type of classification to the microbiologist if the only criterion for this scheme is the similarity of the ribosomal RNA? Other than being stable, a classification scheme for procaryotes should be practical. The laboratory microbiologist faced with having to identify a particular bacterium needs relatively simple, reliable and, in some cases rapid, phenotypic tests which can be used to identify this organism. The acknowledgement of true phylogenetic relationships, however, should not be sacrificed for the sake of practicality. Unifying phenotypic characteristics must be found which accurately

reflect the evolutionary relationships elucidated from rRNA similarity analyses. Although, a few of the higher taxa can be successfully described phenotypically as well as phylogenetically (Schlesner and Stackebrandt 1986; De Ley et al. 1986), phenotypic descriptions of the majority of the higher taxa which correlate with the phylogenetic hierarchy must wait until new insights are available (Stackebrandt 1987).

The classification scheme of higher organisms which now exists and has been relatively stable since its inception is based on evolutionary divergence proposed through analysis of an accurate fossil record of these organisms. These fossilized skeletons can be analyzed in many respects such as complexity, age, habitat, etc. to determine its relationship to other organisms. For example, the arrangement and shape of teeth present in an animal skull can tell the anthropologist whether this animal was a carnivore or a herbivore. Although fossils of primitive bacteria do exist, these cannot be used in the same manner as the fossils of the higher organisms. One cannot determine whether the fossilized bacterium was auxotrophic or chemotrophic, whether or not it fermented glucose, or analyze its nucleic acids to determine its relationship to other bacteria. One can only say that this fossilized bacterium morphologically resembles or does not resemble one that is known to exist today. Therefore, the phylogenetic analysis of the procaryotes needed to form the base of a stable classification scheme, must involve the analysis of the molecular chronometers, such as 16S rRNA, which exist in the present day bacteria. This can be accomplished with the knowledge that mutations in this molecule occur at specific

frequencies, that these frequencies will remain constant over extremely long periods of time, and that the mutational rate of the 16S rRNA molecule runs isosynchronously in all known lines of descent (Stackebrandt 1987). By determining the number of base changes which have occurred at a single position over time, one can accurately approximate the evolutionary distance between the divergence of two organisms. The appropriate branch in the evolutionary tree to which these organisms belong can be determined by studies of the similarity between the 16S rRNA sequences of these organisms and those known to be in certain branches.

Many of the procaryotes which have been placed within one of the various branches in the phylogenetic tree have also been classified on the basis of oligonucleotide signature sequences. These are short sequences (approx. 6-15 bases in length) which are generated from the oligonucleotide cataloging procedure and occur at specific positions on the 16S rRNA, depending, of course, on the size of the molecule, which can vary from organism to organism. Using signature sequences as a basis for classification, the eubacteria can be divided into 10 major "phyla". These are: the gram-positive bacteria, the purple bacteria and their relatives (including the mitochondria), the spirochetes and their relatives, the bacteroides, the flavobacteria and cytophagas, the cyanobacteria and chloroplasts, the green sulfur bacteria, the chloroflexus group, the sulfate reducing bacteria, the radioresistant micrococci and the planctomyces-pasteuria group. The gram-positive bacteria can be further divided into the low mol% guanine plus cytosine (G + C) and the high mol% G + C branches. The purple bacteria are

divided into three main branches: the alpha, beta and gamma branches. The archaeobacteria fall into two main branches: the methanogens, extreme halophiles and the genus Thermoplasma comprising one branch and the sulfur dependent archaeobacteria ("thermoacidophiles") comprising the other (Fox et al. 1980; Stackebrandt and Woese 1984; Woese 1985, 1987; Woese et al. 1985).

Romaniuk et al. (1987) have performed the only rRNA sequence analysis of the genus Campylobacter to date. Using the nucleotide sequencing procedure described by Lane et al. (1985), this group derived partial 16S rRNA sequences from Campylobacter jejuni, C. coli, C. laridis, C. fetus subsp. fetus, C. sputorum biovar sputorum and C. pylori. These sequences were then compared to several others which had been previously published. Their results show that C. pylori is not related to the other campylobacters tested at the genus level and is, in fact, more closely related to Wolinella succinogenes than it is to the other Campylobacter species. The remaining five Campylobacter species were found to be related to each other at the genus level and, collectively, this group was found to be related to, but phylogenetically distinct from W. succinogenes and a Thiovulum species (a marine sulfide-dependent bacterium). Based on signature sequences available from these data, this study also concluded that the campylobacters comprise a previously undefined phylogenetic branch. Although this study clarifies the relationship of C. pylori to some Campylobacter species, it does not answer the question as to the relationship of C. pylori to the remaining species, or whether these species show a degree of relatedness to each other sufficient to warrant

classification within a single genus. For example, do the aerotolerant species Campylobacter cryaerophila and the salt-requiring, nitrogen-fixing species Campylobacter nitrofigilis belong within a genus containing microaerophilic, non-salt requiring, non-nitrogen-fixing bacteria? A study of the partial 16S rRNA sequences from strains representing all of the species now classified as campylobacters is necessary to decide whether these species belong together in a single genus, or whether they should be placed into other existing genera and/or new genera.

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The Genus Campylobacter: a Phylogenetic Study

Abstract

The phylogenetic relationships of all species in the genus Campylobacter, Wolinella succinogenes and other gram-negative bacteria were determined by comparison of partial 16S ribosomal RNA sequences. The results of this study indicate that the genus Campylobacter is composed of three separate rRNA sequence homology groups. Homology group I contains the true Campylobacter species: Campylobacter coli, C. jejuni, C. laridis, C. fetus, C. hyointestinalis, C. concisus, C. mucosalis, C. sputorum and the "catalase-negative to weakly-positive" (CNW) strains. "C. cinaedi", "C. fennelliae", C. pylori and Wolinella succinogenes constitute homology group II. Homology group III contains C. cryaerophila and C. nitrofigilis. We consider the three homology groups to represent separate genera but, at present, phenotypic characteristics needed to differentiate them are not available. The three homology groups were only distantly related to representatives of the alpha, beta and gamma branches of the purple bacteria, indicating that these bacteria do not belong to any previously defined branch of this "phylum."

Introduction

Campylobacters have been recognized as important agents of veterinary disease for many years. The type strain of this genus, Campylobacter fetus subsp. fetus, is the cause of orally transmitted sporadic abortions in cattle and sheep, and Campylobacter fetus subsp. venerealis is the cause of sexually transmitted abortion in cattle (Smibert 1984). Other species such as Campylobacter hyointestinalis and Campylobacter mucosalis are also animal pathogens (Gebhart et al. 1985; Lawson and Roland 1974; Smibert 1984). In recent years, however, the importance of campylobacters as agents of human disease has become increasingly apparent. Campylobacter jejuni and Campylobacter coli are now considered major causes of bacterial gastroenteritis in humans, and several species of Campylobacter which were previously thought to be strictly animal pathogens have been identified as opportunistic human pathogens (Butzler and Skirrow 1979; Fennell et al. 1987; Karmali and Fleming 1979; Rettig 1979; Simor and Wilcox 1987). Since 1984, three new species of human pathogens have been described: Campylobacter pylori, which is a probable causative agent of gastric and duodenal ulcers (Marshall et al. 1984), and "Campylobacter cinaedi" and "Campylobacter fennelliae", which have been identified as causes of proctitis in homosexual men (Fennell et al. 1984; Totten et al. 1985).

The classification of the genus Campylobacter has always been somewhat difficult. Because these organisms do not catabolize carbohydrates and are inert with regard to most traditional biochemical tests used for the identification of bacteria, only a small number of

tests are available for the identification and classification of campylobacters (Roop et al. 1984, 1985). Consequently, phylogenetic classification of campylobacters based on biochemical tests has a limited usefulness. This lack of significant numbers of useful phenotypes has resulted in a poor definition of the genus Campylobacter. In turn, this had allowed the inclusion of several species into this genus which do not conform to its current definition. These include: C. pylori which differs markedly from other campylobacters in its morphological characteristics, fatty acid and protein compositions (Goodwin et al. 1985; Megraud et al. 1985); Campylobacter cryaerophila which is aerotolerant (Neill et al. 1985); and Campylobacter nitrofigilis, a NaCl-requiring, nitrogen-fixing bacterium associated with the roots of salt marsh grasses (McClung et al. 1983).

Although DNA homology studies have shown that the current Campylobacter species are distinct from one another (Belland and Trust 1982; Benjamin et al. 1983; Harvey and Greenwood 1983; Hébert et al. 1983; Leaper and Owen 1982; Owen 1983; Owen and Leaper 1981; Roop et al. 1984, 1985; Ursing et al. 1983), they have not answered the question as to whether these species are sufficiently related to justify classification within a single genus. The comparison of 16S rRNA sequences for phylogenetic analysis has proven to be a powerful tool for the accurate classification of microorganisms above the level of species (Fox et al. 1980; Olsen et al. 1986; Stackebrandt 1985; Stackebrandt and Woese 1984; Woese 1985, 1987). The recent development of a technique which facilitates the rapid generation of partial 16S rRNA sequences has allowed researchers to accurately classify newly described

microorganisms (Lane et al. 1985). The application of this technique using five representative species of Campylobacter and C. pylori has already revealed that C. pylori is not related at the genus level to C. jejuni, C. coli, C. fetus subsp. fetus, C. laridis or C. sputorum biovar sputorum (Romaniuk et al. 1987). However, the relatedness of C. pylori to the remaining Campylobacter species and the relatedness of these species to one another and to other campylobacters, has not been determined. We report here the results of comparisons of partial 16S rRNA sequences from all fourteen species of Campylobacter and their relationships to one another and other gram-negative bacterial species.

Materials and Methods

Bacterial strains and growth of stock cultures. The Campylobacter strains used in this study are listed in Table 1. The identity of each strain was confirmed using differential biochemical tests (Roop et al. 1984, 1985) or by comparison of rRNA sequence data with those sequences which had been previously published (Romaniuk et al. 1987). C. coli, C. jejuni, C. fetus subsp. fetus, C. fetus subsp. venerealis, C. hyointestinalis, C. laridis, C. sputorum biovar sputorum and CNW strain CG-1 were cultured under an air atmosphere at 37°C in Brucella semisolid medium (Brucella broth [Gibco] containing 0.15% agar; BSS). C. cryaerophila and C. nitrofigilis were cultured in BSS medium under an air atmosphere at 30°C. The medium for C. nitrofigilis was supplemented with 1.0% NaCl. C. mucosalis, C. concisus, C. pylori, "C. cinaedi" and "C. fennelliae" were cultured in BSS medium at 37°C under an atmosphere of 6% O_2, 10% CO_2, 15% H_2 and 69% N_2. The medium for C. mucosalis and C. concisus was supplemented with 0.3% fumaric acid.

Isolation of RNA. Biphasic culture systems employing Roux bottles were used for the growth of all strains as described by Roop et al. (1984, 1985). Bulk cellular rRNA was isolated by a modification of a previously described procedure (Kirby 1965; Johnson 1981). Lysates of cells disrupted with a French pressure cell were extracted with phenol-cresol solution. 16S rRNA was selectively precipitated from the soluble RNA and DNA by the addition of cold (-20°C) 7.5 M ammonium acetate. The rRNA was stored in 1 x SSC-1.0 mM HEPES/1.0% SDS at -20°C.

Table 1. Strains of **Campylobacter** species used in this study.

Species	Strain
CATALASE POSITIVE CAMPYLOBACTERS	
C. coli	ATCC[a] 33559[T]
C. jejuni	ATCC 33560[T]
C. fetus	
subsp. *fetus*	ATCC 27374[T]
subsp. *venerealis*	ATCC 19438[T]
C. hyointestinalis	80-4577-4 (ATCC 35217; proposed type strain[b])
C. laridis	NCTC[c] 11352[T]
C. cryaerophila	NCTC 11885[T]
C. nitrofigilis	ATCC 33309[T]
"*C. cinaedi*"	ATCC 35683 (proposed type strain[d])
"*C. fennelliae*"	ATCC 35684 (proposed type strain[d])
C. pylori	NCTC 11637[T]
CATALASE NEGATIVE CAMPYLOBACTERS	
C. mucosalis	NCTC 11000[T]
C. concisus	VPI 13086[T]
C. sputorum biovar sputorum	VPI S-17 (proposed neotype strain[e])
CNW[f] Strain	CG-1 (reference strain[g])

[a] American Type Culture Collection, Rockville, Md., U. S. A.
[b] Gebhart et al. 1985.
[c] National Collection of Type Cultures, London, England.
[d] Totten et al. 1985.
[e] Roop et al. 1985.
[f] CNW = "catalase-negative or weakly-positive"
[g] Gebhart et al. 1984.

Synthesis and Purification of Oligonucleotide Primers. Primers complementary to conserved regions of the 16S rRNA molecule were prepared by means of a DNA synthesizer (Model 381A, Applied Biosystems, Foster City, CA). The five primers used in this study were complementary to the following regions of E. coli 16S rRNA (5' to 3'): 321-340, 519-536, 907-926, 1220-1239 and 1388-1407. Crude oligonucleotide preparations were purified by thin-layer chromatography (Brian Reid, personal communication), diluted to a concentration of 0.1 mg/ml with TE buffer (10 mM Tris-base and 0.1 mM EDTA, pH 8.0), and stored at -20°C.

Preparation of RNA for Sequencing. The SDS present in the rRNA storage solution was removed prior to base sequencing by extraction with phenol/chloroform/salts solution followed by extraction with secondary butanol to remove any residual phenol. The rRNA was precipitated in two volumes of cold (-20°C) 95% ethanol and dissolved in TE buffer to a concentration of 2.0 mg RNA/ml.

Determination of RNA Base Sequence. Partial 16S rRNA sequences were determined by a procedure based on Lane et al. (1985). Modifications in this procedure were made to enhance the resolution of base sequence ambiguities. Microfuge tubes (Sarstedt, 0.5 ml) served as reaction vessels throughout the procedure. Oligonucleotide primers were hybridized to the 16S rRNA template in a reaction mixture consisting of 1.0 µl of RNA (2.0 mg/ml), 2.0 µl of primer (0.1 mg/ml), 1.0 µl of 5X hybridization buffer (250 mM Tris-HCl, 500 mM KCl, pH 8.5) and 1.0 µl of H_2O. The reaction mixtures were heated at 90°C for 2 min, allowed to cool to 60°C over a period of 30 min, and then placed on ice. To this

mixture was added: 5.0 µl of 5X reverse transcriptase buffer (250 mM Tris-HCl, 250 mM KCl, 50 mM dithiothrietol, 50 mM $MgCl_2$, pH 8.3), 2.0 µl of ^{35}S-dATP (10 µCi/µl), 1.0 µl of AMV-reverse transcriptase (Promega Biotec, 9 U/µl) and 2.0 µl of H_2O. Three microliters of this mixture was added to each of four reaction vessels containing 2.0 µl of a solution composed of 10 µM dATP, 250 µM dCTP, dGTP, dTTP and one of the following: 1.25 µM ddATP, 5.0 µM ddCTP, 7.5 µM ddGTP or 10 µM ddTTP. Reaction mixtures were incubated at 48°C for 20 min. One microliter of chase mix (1.0 mM dNTP, 9 U AMV-RTase, 10 mM Tris-HCl, pH 8.3) was added and the mixtures were incubated an additional 15 min. Reactions were stopped by adding 6.0 µl of stop mix (86% formamide, 10 mM EDTA, 0.08% xylene cyanol, 0.08% bromophenol blue). cDNA was denatured from the RNA template by heating each sample at 90°C for 2 min immediately prior to loading the sample on the sequencing gel. Sequencing gels used were 8% polyacrylamide/8 M urea buffer gradient gels and were 0.4 mm thick and 40 cm in length.

Comparison of RNA sequences. Sequence homologies were calculated by using methods previously described (McCarroll et al. 1983; Olsen et al. 1986). Positions of unknown base composition and ambiguous regions of alignment were eliminated prior to homology calculations. Alignment gaps were assigned one half the value of a nucleotide base in the same position. Similarity coefficients were calculated and a dendrogram constructed by the Numerical Taxonomy System (NTSYS) analysis program at Virginia Polytechnic Institute and State University.

Results

The partial 16S rRNA sequences determined from the 14 Campylobacter species are aligned in Figure 1 with the known sequences from representatives of the three major branches of the purple bacteria (Agrobacterium tumefaciens, alpha branch; Pseudomonas testosteroni, beta branch; and Escherichia coli, gamma branch [Yang et al. 1985]) and Wolinella succinogenes (provided by C. R. Woese, Department of Genetics and Development, University of Illinois, Urbana). The 14 Campylobacter species could be assigned to three distinct rRNA sequence homology groups. The three groups are especially apparent when the positions of constant base sequence, which are of little phylogenetic value (Weisburg et al. 1985), are removed from consideration (lower portion of Table 2). Organisms that are closely related continue to exhibit high rRNA sequence homology. The relationships among the various species are represented graphically in Figure 2.

Group I contains nine species: C. coli, C. jejuni, C. laridis, CNW strain CG-1, C. fetus (both subspecies), C. hyointestinalis, C. concisus, C. mucosalis, and C. sputorum. These species have interspecies homology values ranging from 83.2 to 96.8%. Group I could be further divided into two subgroups. Subgroup 1 includes C. coli, C. jejuni, C. laridis, and CNW strain CG-1, which have interspecies homology values ≥92.0%. Subgroup 2 includes C. fetus, C. hyointestinalis, C. concisus, C. mucosalis, and C. sputorum, which have interspecies homology values ≥88.0%.

Primers A and B:

C. coli	UCGGUGUAGG	AUGAGACUnU	AUAGUAUCAG	CUAGUUGGUA	AGGUAAUGGC	UAACCAAGGC	UnUGACGCUU	AACUGGUCUG	AGAGGAUGAU	CAGUCACACU	GGAACUGAGA
C. jejuni	.....	..n.....A.	.....	..n.....	.....	.....	.n.....	.....	.....	.....	.....
C. laridis	.....	.C.....n.	.....	.....UGG	.....	A...GG....	.nG.....	.....	.....	.....	.....
CNW strain CG-1	.....	.nU.....n.	.....	..n.....	.....A...	.....	.n.....n	....n.....	.....	.....	.....
C. fetus fetus	.....	.....nC	..U.....	..n.G.....	.....	.....	.C.....A.	.....	.....	.....	.....
C. fetus venerealis	.....	.....n.	..U.....	..n.....	.....	.....	.nG.....A.	.....	.....	.....	.....
C. hyointestinalis	.....	.....n.	..U.....	.....	.....	....A.....	.A.....A.	.....	.....	.....	.....
C. concisus	.....	..n..G..n.	..U.....	.....G	.....	....n.....	.n.....A.	.....	.....	.....	.....
C. mucosalis	....CA....	.....G..nC	..U.....	.....	.....	.U.....	.n.....A.	.....	.....	.....G..	.....
C. sputorum	...C.A.G..	.....G..n.	..U.....	.....	.....	.....	.n.....	.....	.....	.....	.....
C. pylori	...C.AAGA.	..n..CU.n.	G.CC.....	..U.....	.....	....U.....	.A.....GG.	.U.C..C.Cn	.....G...A	.G.A.....	.....
"C. cinaedi"	...C.AA...	..C..nn.n.	..CC.....	..U...n...	....n.....	.....	.n.....GGn	...-..C...	.....G.n.A	.G.A.....	.....
"C. fennelliae"	C..C.AA...	..Un.U..A.	G.CC.....	..U.....G	.....	.C.....	.n.....GG.	.U.C..C...	.....G...A	.G.A....n.	.....
C. cryaerophila	.U...AAGA.	..U..C..n.	..U.....	U....n..nG	G.....	nU.....A.	AA....A.A.	....n..U..	.....n..	.....	.....
C. nitrofigilis	.G.C.C....	..UG.C..n.	CCG.....	.....n...G	....C.....	.C..A.....	AA....A.C.	....n..U..	.....	.....	.....
W. succinogenes	-..C.A.G..	..C..C..A.	G.CC.....	..U.....G	.....	.C.....	.A.....GG.	.U.C..C...	.....G....	.G.A.....	.....
E. coli	.U.CCUAC..	...U.C.CAG	..G.G..U..	.....A...G	G.....C...	.C...U....	GAC..UC.C.	.G.....	C	...C.....	.....
P. testosteroni	GU.C.ACUA.	.GCG.CUGA.	GGCAG..U..	G.....G	G.....A...	.U.....C.	.GC..UCUG.	.G.....	.....C..C	...C.....	..G.....
A. tumefaciens	....G...U.	.....C.CGC	G.U.G..U..	.....G	G.....A...	CU.....	GAC..UC.A.	.G.....	.....	...C....U.	..G.....

C. coli	CACGGUCCAG	ACUC-UUCGG	GAGGCGGCAG	UAGGGAAUAU	UGCGCAAUGG	GGGAAACCCn	GACGCAGCAA	CGCCGCGUGG	AGGAUAACAC	UUUUAGGAGC	GUAAACUnCn
C. jejuni	.....	....-.....	.....	.....	.....	.....n	.....	.....	.....G....	....C.....	.....C.n
C. laridis	.....	....-.A...	....Gn....	.....	.....	.....n	.....	.....	.....	.n..C.....	.....CGn
CNW strain CG-1	.....	n...C-n...	.....C....	.....	.....C....	.....n	.....	.....	.....n....	....C.....	.....n.n
C. fetus fetus	.....	....-.n...	.....U..U.	.....	...U.Un...	.....n	..A..U..U.	.....	.....G..U.	....C.....	.....n.n
C. fetus venerealis	.....	n...-.n...	.....A....	.....	...U.An...	.....n	..A.....	.....	.....G....	....C.....	.....n.n
C. hyointestinalis	.....	....-.n...	.....AC...	.....	...Un.....	.....n	..A...n...	.....n....	.....n....	....C.....	.....n.n
C. concisus	.....	n...-GA...	.....	.....	...U..n...	.....n	..A.....	.....	.n...G....	....C.....	.....n.n
C. mucosalis	.....	....-.....	.....	.....	.....	.....n	..A.....G	...A.....	.....	....C.....	.....n.n
C. sputorum	.....	....-.A...	.....A....	.....	...U.....	.....U	..A...n...	.....	.....G....	....C.....	.....C.n
C. pylori	.....	....-.A...	.....	.....	.....n.C..	.....n	..A.....G	.....	.....G.AGG	...A....UU	.....n.U
"C. cinaedi"	.....	....-.A...	.....n....	.....	...U.....	.....n	n.A.....	.....	.....AGG	Unnn....UU	.....nnn
"C. fennelliae"	.....	....-.A...	.....n.	.....	...n..n...	.....n	..A.....	.....	.....G.AGG	UnnA....UU	.....n.n
C. cryaerophila	.....n.	C...GGG...	.....A....	.G.....	...A....n.	AC....GU.U	C.U....nn.	.....	.....n....	A...C..U..	..n....n.U
C. nitrofigilis	.....	....-..n..	.....A...n	.G.....	...A.....	AC....GU.U	n.U.....	.....	.....G....	A...C..U..	.....n.n
W. succinogenes	.....	....C.A...	.....A....	.....	...U.....	.C....G..U	..A.....a.	.....	.....G.AGG	.C..C...UU	.....C.U
E. coli	.....	....C.A...	.....A....	.G.....	...A.....	.C.C..G..U	..U.....C.	U.....U	.U..AG.AGG	CC..C..GUU	.....A.U
P. testosteroni	.....C....	....C.A...	.....A....	.G.....U.	..GA.....	.C....G..U	..UC.....	U.....C	.....G.AGG	CCC.C..GUG	.....G.U
A. tumefaciens	.....C...A	....C.A...	.....A....	.G.....	..GA.....	.C.C..G..U	..UC....C.	U.....A	GU...G.AGG	CC.....GUU	.....GCU.U

			Primer C:								
C. coli	UUUCUUAGGG	AAGAA	AAAUCCGUAC	AGAUCACCAA	GAAUACCCAU	UGCGAAGGCG	AUCUGCUnGA	ACUnAACUnA	CGCGAAU-GC	GUnAAAGCGU	GGGGAGCAAA
C. jejuni	.....n....	.....	.....G	.U.....	.....	.....	.....G..	...C....G.	...U..G-..	.CG.....	.....
C. laridis	.....	.....	.....G	.U.....	.....	.....G.	.....G..	...n....G.	...U..G-..	.CG.....	.....C....
CNW strain CG-1	.....nU...	.....	.....G	.U.....	.....	.....	.....G..	...n....G.	.....G-..	.nG.....	.....
C. fetus fetus	...G.....	.....	.....G	.....G	.....	.....G.	.....G..	...n....G.	...Un..-..	..G.....	.....
C. fetus venerealis	...G.....	.....	.....G	.....G	.....	.....Gn	.....G..	...n....n.	...U...-..	..n.....	.....
C. hyointestinalis	...G.....	.....	.....G	.....G	.....	.....G.	.....G..	...n....G.	...U...-..	..n.....	.....
C. concisus	...G.A....	.....	.....G	.....G	.....	.....	.....G..	...C....G.	...U...-..	..n.....	.....
C. mucosalis	...G.....	.....	.....G	.....G	.....	.....	.....G..	...C....G.	...U..G-..	..n.....	.....
C. sputorum	n.....U...	.....	.....G	.U....AG.G	.....UU.n	C	.....A..	...U....G.	.A.U...-..	..n.....	.....
C. pylori	...G.A..A.	....n	.....G	.....AG.G	.....U...	.....	.C.....G..	..AUU...G.	...UG..U.U	.Cn.....	.....
"C. cinaedi"	n.GG.A..A.	....-	.....G	.....AG.G	.....	.....	.C.....G..	..AUU...G.	...UG..-..	..n.....	.....
"C. fennelliae"	...G.A..A.	....-	.....G	.....AG.G	....G.U..n	.....	.C..n..n..	..AUU...G.	...UC..-..	.CG.....	.....
C. cryaerophila	n..A.AU.A.	....U	.....G	.....GA.G	.....G..	.....	....A..G..	..AUU.U.G.	n..UG.G-nU	.CGn...n..	.....G.
C. nitrofigilis	...A.AUA..	....U	.....G	.U....GA.G	.....G..	.....	....A..G..	..AUn.U.G.	...UG.G-A.	.CG.....	.....
W. succinogenes	.....A..A.	...--	.....G	.....GAG.G	.....U...	....a...cg	.C.....G..	..AUU...G.	...UG.u-.U	.CGa...cu.	.....
E. coli	..CAGCG...	.G...	....G....G	.....UGG.G	.....GG.	G.....	GC.CC..G..	CGAAG...G.	...UC.G-.U	.CG.....UG	.....
P. testosteroni	...G.AC..A	.C...	....G....G	.U..GCGG..	...C...G..	G.....A	...CC..G.G	C..GC...G.	...UC..-..	ACG.....	.....
A. tumefaciens	..CACCG.A.	....U	....U....G	.U..UCGG.G	...CG..AG.	G.....	GCU.A..G.U	C.AUU...G.	...UG.G-.U	.CG.....	.....

C. coli	CAGGAUUAGA	UACCCUnGUA	GUCCACGCCC	UAAACGAUGU	AUACUAGUUG	UUGCUCUGCU	A-GUUAGGGC	AGUAAUGCAC	-UAACGGAUU	AA	
C. jejuni	.....	.....n...	.....	.....	.C.....	...GGG....	.-..C.U.U.	.....	G.....C...	..	
C. laridis	.....	.....n...	.....	.n.....	..G..U....	...GGG....	.-....UnU.	.....	G.....C...	..	
CNW strain CG-1	.....C.	.....n...	.....G....	.nn.....	..G..n...n	..UGGG.n..	n-..n.UCU.	.....-	G.....C...	..	
C. fetus fetus	.....	.....n...	.....	.....	.....	.....G....	.-..n.C...	n.....	-.....	..	
C. fetus venerealis	.....GG...	.....n...	.....	.....	.....	.....G....	.-..n.CC..	n.....	-.....	..	
C. hyointestinalis	.....	.....n...	.....	n.....	..n..n....	.....G....	.-....C...	.....	-.....	..	
C. concisus	.....	.....n...	.....	n.....	..C...n....	.....AA...	n-..nUU...	.....	-.....	..	
C. mucosalis	.....	.....n...	..GG.....	.....	.....	.....AA...	.-..nUU...	.....	-.....	..	
C. sputorum	.....	.....n..n	.....n....	.n.....	..n..n....	.....n....	.-..C.U...	.....C...	-.....	..	
C. pylori	G.....	.....n...	.....	.....G	..n..n....	...GAGG...	.n..CUnUC.	.....	C.....C...	..	
"C. cinaedi"	.....	.....n...	.....	.n.....A	..n..n....	....C.....	.-..A....n	n.....-	G.n...C...	..	
"C. fennelliae"	.....n....	.G....n..G	.....	.n.....A	..n..n....	....C..n..	C-..n....n	n.....C.	-.....C...	..	
C. cryaerophila	.....	.....n...	.....n....	.....	.C...n....	...UGAGA..	n-.AnCUU..	.....G	U....AC...	..	
C. nitrofigilis	.....	.....n...	.....	.n.....	.C...n....	...UGAG...	n-.ACCUn..	.....G	U....AC...	..	
W. succinogenes	.....	.....G..a	.....	.....A	..G.....	....C.....	U-..n....c	.....G	U....AC...	CU	
E. coli	.....	.....G...	.....G	.....	CG...U.GA.	G.UG.GCC..	U-.AGGC.UG	GC.UCC.G.G	C.....CG..	..	
P. testosteroni	.....	.....G...	.....GC...	.....	CA...G....	...GGUC-U.	.-AC.GACU.	.....C.A.G	C.....CG.G	..	
A. tumefaciens	.....	.....G...	.....G	.....A	..GU...CC.	.C.GG.A.-.	.-UACU.UU.	G..GGC...G	C.....C...	..	

Primers D and E:

C. coli	GACAGG-UGC	UGCACGGCUG	UCGUCAGCUC	GUGUCGUGAG	AUGUUGGGUU	AAGUCCCGCA	ACGAGCGCAA	CCCACGUnUU	UAGUUGCUAA	CGGUUCG-GC	CGAGCnCUCU
C. jejuni	......-...	....	....	....	....	...n......	....	.......n..	....	.......-..	.....n....
C. laridis	......-...	....	....	....	........C.	..C......n	....	.......n..	....	.AC....-.G	U....A....
CNW strain CG-1	......G...	....	....	....	....	....	....	...Un..CC.	....	..A....-.U	.....A....
C. fetus fetus	......-...	....	....	....	....	...n......	....	.......n..	....	.A.....-..	Un...A....
C. fetus venerealis	......-...	....	....	....	....	...n......	....	.......n..	....	.A.....-..	Un...A....
C. hyointestinalis	......-...	....	....	....	....	...n......	....	......nn..	....	.A.....-..	UC...C....
C. concisus	......-...	....	....	....	....	...n......	....	.......CA.	....	.A.....-..	Un...n....
C. mucosalis	......-...	....	....	....	....	...n......	....	.......n..	....	.....A.-..	.....n....
C. sputorum	......-...	..n.......	....	....G.....	....	....	........G.	......nn..	....	.A..AA.-..	An...A.A.n
C. pylori	A.....-...	....	....	....	....	...n......	........U.	....nC.nC.	....	.A.G..AU..	Un..AA....
"C. cinaedi"	A.....-...	.........n	....	....n.....	....	....	....	....n..CC.	n....A...G	.A...nAA..	.n...A....
"C. fennelliae"	A.....-...	....	....	....	....	..nn......	....	...Un..CC.	n.....U..G	.A.....-..	nn...n....
C. cryaerophila	U.....-...	....	....	....	....	....	....	...Un..n..	....	.A.....-..	Un..AA....
C. nitrofigilis	U.....-...	.........n	....U.....	....	....	....	.......nG.	...Un..n..	....	.A.....-..	Un.nAA....
W. succinogenes	aa....-...	....	....	....	....	....	....	...U...CAC	....	.......-..	.....A....
E. coli	......-...	....U.....	....	....U....A	....	....	....	...UUA.CC.	.U.....C.G	....C..-..	..G.AA...A
P. testosteroni	C.....-...	....U.....	....	....	....	....	....	...UU.CCA.	.........-	.AU.CA.-U-	U....A....
A. tumefaciens	A.....-...	....U.....	....	....	....	....	....	...U..CCC.	.......C.G	.AU..A.-U-	U.G..A....

C. coli	AAAUAGACUG	CCUUCG-UAA	GGAn-GAGGA	AGGUGUGGAC	GACGUCAAGU	CAUCAUGGCC	CUUAUGCCCA	GGGCGACACA	CGUGCUACAA	UGGCAUAUAC	AAUGAGACGC
C. jejuni	....	......-...	...n-.....	....	....	....	....	....	....	....	....
C. laridis	....	......-...	..nn-.....	.......n..	....	....	....	....	....	....	....
CNW strain CG-1	..GG......	.n....-...	..nn-.....	....	....	....	.......U..	....	....	....	....
C. fetus fetus	....	......-C..	..nn-.....	....	....	....	....	....	....	....	.......U..
C. fetus venerealis	....	......-C..	..nn-.....	.......n..	....	....	....	....	....	....	C......U..
C. hyointestinalis	....	......-...	..nn-.....	....	....	....	....	....	....	....	....
C. concisus	..UG......	......-...	..nG-.....	....	....	....	......U..G	....	....	....	.......A..
C. mucosalis	....n.....	.......-C..	..nn-.....	....	....	....	......U..G	....	....	....	....
C. sputorum	n..C......	......-...	...n-.....	....	....	....	......U..G	....	....	....	...A......
C. pylori	..GG.U....	...C..-...	...A-.....	.....G....	....	....	....C...n.	....U.....	....	...GG.GC..	..A....A..
"C. cinaedi"	..GG......	......-...	...G-.....	.....A....	.....n....	....	........G.	....	....	...GGCGC.n	..A...GA..
"C. fennelliae"	..GG......	....n.CA..	...G-.....	.....A....	....	....	....C.....	....U.....	....	...GG.GC..	..A....A..
C. cryaerophila	n..C......	...A..-C..	.U.G-.....	.....A....	......C...	....	...GC.U...	....U.....	....	...G......	C.A...C...
C. nitrofigilis	..CG......	......-C..	.U.G-.....	.....A....	....	....	...UC.A...	....U.....	....	...GG.....	C.A...C...
W. succinogenes	.GUG......	......-C..	...G-.....	.....A....	....	....	....C.ncc.	....U.....	....	...UG.G...	..A....A..
E. coli	..GG......	..AGU.A...	ACUG-.....	.....G...U	....	....	....C.A...	....U.....	....	....GC....	..A....A..
P. testosteroni	..UGG.....	..GGU.AC..	ACCG-.....	.....G...U	....	.C........	.....AGGUG	....U.....	...CA.....	....UGG...	..A.G.UU..
A. tumefaciens	..GGG.....	..GGU.A...	.CCGA.....	.....G...U	....	.C........	....C.GG.U	....U.....	....	...UGGUG..	.G..G.CA..

C. coli	AAUACCGCGA	GGUnGAGCGA	AUCU-nUAAA	A-UAUGUCCC	AGUUCGGAUn	GUUCUCUGCA	ACUCGAGAGC	AUGAAGCCGG	AAUCGCUnGU	AAUCGUAGAU	CAGCCAUGC
C. jejuni	.....	...n....A.	....-n....	.-.....	.....n	.....	.....	.....	.....n..	.....	.....
C. laridis	.....C.	n..n....A.	....-n....	.-.....	.....n	.....	.....	.....	.....n..	.....	.....
CNW strain CG-1	.....	...n....A.	....-n.n..	.-.....	.....n	.....	.....	.....	.....A..	.....C.A..	.....U
C. fetus fetus	....U.....	.A.G...nn.	....-n....	.-.....	.....U	.GAG.....	...n..CUC.	.....	.....n..	.....	.....
C. fetus venerealis	....U.....	.A.G...nn.	....-A....	.-.....	.....U	.GAG.....	...n..CUC.	.....	.....n..	.....	.....
C. hyointestinalis	....U.....	.A.n....AC	....-n.n..	.-.....	.....n	.....	.....	.....	.....n..	.....	.....
C. concisus	....U.....	.A.n....n.	....-n....	.-.....	.....U	.GAG.....	...n..CUC.	.....	.....n..	.....	.....
C. mucosalis	....U.....	.A.n...n..	....-n....	.-.....	.....n...U	.GAG.....	.....CUC.	.....	.....n..	.....	.....
C. sputorum	....U.....	.A.n....A.	....-n.n..	.-.....	.....U	.GAG.....	.....CUC.	.....	.....n..	.....	.....
C. pylori	.....U....	A..G....C.	....-UC...	.-C.CC..U.	.....A	..AGG.....	.....CCUn.	.....Un.	.....n..	.....C.A..	.....U
"C. cinaedi"	.....	...n....A.	.A..-nn...	.-CG.C....	.....U	..nG.....	C.....CUn.	..n.....n.	.....n..	.....n...	....n...U
"C. fennelliae"	....U.....	.A.G....A.	....-CC...	.-CGCC..U.	.....U	..nG.....	.....CUn.	..A....Un.	.....n..	n....C.A..	....A...U
C. cryaerophila	...G.G.U..	C..G....C.	....UA.n..	.-...C....	.....U	..AG.....C	...n..CUn.	.....UU..	.....A..	.....	UC..U....
C. nitrofigilis	G....G.U..	C..n.....	....-UC...	.-.GCC....	.....U	..nG.....C	.....CUn.	.....UU..	.....Cn..	.....	.C..U....
W. succinogenes	.....	...G....A.	....-U....	.-C.CA..U.	.....U	..AG.....	.....CUA.	.....U..	.....A..	.....	....A....
E. coli	G.CCU.....	.AGCA....G	.C..-CAU..	.G.GC...GU	...C.....U	.GAG..GU..	.....CUC.	.....U...	.....A..	.....G...	...-A....
P. testosteroni	C.AC.....	..GG....U.	...C-CAU..	.GCCA...GU	...C.....C	.CAG.....	.....CU..	G.....U...	.....A..	.....G...	...-A...U
A. tumefaciens	G.G..A....	U..C....U.	....-CC...	.-GCCA..U.	.....U	.CA.....	.....U..	.....UU..	.....A..	.....C....	...-.....

Figure 1. Alignment of partial 16S rRNA sequences (5' to 3' based on E. coli sequence) from fourteen species of Campylobacter, Wolinella succinogenes, Escherichia coli, Pseudomonas testosteroni and Agrobacterium tumefaciens. Dots represent bases identical to the base in that position in the C. coli sequence. Hyphens represent alignment gaps. Small-case letters represent ambiguous bases and "n" represents unidentified bases. All oligonucleotide primers were synthesized complementary to conserved regions of the 16S rRNA sequence of E. coli. Primer A is complementary to positions 321-340. Primer B is complementary to positions 519-536. Primer C is complementary to positions 907-926. Primer D is complementary to positions 1220-1239. Primer E is complementary to positions 1388-1407.

Table 2. Homology of *Campylobacter* 16S rRNA sequences compared with other procaryotic species

Species (reference)	% Homology[a]																		
	C. coli	*C. jejuni*	*C. laridis*	*C. fetus* subsp. *fetus*	*C. fetus* subsp. *venerealis*	*C. hyointestinalis*	*C. concisus*	*C. mucosalis*	*C. sputorum*	CNW CG-1	*C. cinaedi*	*C. fennelliae*	*C. pylori*	*W. succinogenes*	*C. cryaerophila*	*C. nitrofigilis*	*E. coli*	*P. testosteroni*	*A. tumefaciens*
C. coli		98.1	96.8	96.5	96.4	97.7	96.4	95.7	94.7	96.7	90.6	88.1	87.4	88.7	87.4	88.2	78.5	78.1	78.1
C. jejuni	96.8		97.7	96.0	95.8	97.3	95.8	95.1	94.8	97.5	90.0	87.6	86.7	88.5	88.1	88.8	79.2	79.7	79.0
C. laridis	93.6	96.0		94.5	94.6	95.8	95.0	93.4	93.2	96.6	89.0	86.8	85.2	87.5	86.5	87.3	77.6	78.5	78.4
C. fetus subsp. *fetus*	92.4	90.4	88.0		98.5	98.0	98.0	97.7	95.5	94.2	90.4	89.0	86.6	88.9	87.7	87.7	79.4	78.9	78.1
C. fetus subsp. *venerealis*	92.0	90.0	88.4	96.4		97.9	97.9	97.7	95.5	94.4	90.3	88.7	86.3	88.9	87.9	87.5	79.4	79.1	78.1
C. hyointestinalis	95.2	93.6	91.6	95.2	94.8		98.0	97.2	95.5	95.9	90.4	88.9	86.8	88.5	87.9	87.9	79.0	78.3	78.5
C. concisus	90.8	89.6	87.6	94.4	94.0	94.0		97.4	96.2	93.9	90.8	89.7	87.4	89.2	88.2	88.4	79.9	79.8	79.1
C. mucosalis	89.6	87.6	84.4	93.6	92.8	92.4	92.8		94.9	92.9	89.4	87.9	86.4	87.9	87.7	87.1	79.4	78.1	77.6
C. sputorum	88.4	87.6	85.6	90.0	89.6	89.6	89.2	88.0		92.6	89.6	88.2	87.3	88.7	87.1	87.5	78.8	78.6	77.0
CNW strain CG-1	92.4	94.0	92.0	86.4	86.0	88.8	86.0	83.2	83.6		89.0	87.4	86.7	87.2	86.8	87.2	78.5	79.0	79.0
"*C. cinaedi*"	77.5	76.1	74.5	78.0	77.2	77.6	77.6	74.8	75.9	74.9		95.2	92.5	92.4	84.8	85.4	79.2	78.7	79.2
"*C. fennelliae*"	72.1	70.1	69.3	73.7	73.3	73.3	74.1	71.7	72.1	71.3	88.8		93.4	94.2	84.1	85.7	79.4	78.7	79.6
C. pylori	69.0	69.1	67.1	69.0	68.2	69.8	71.0	68.6	69.8	68.3	82.5	85.7		91.5	83.7	83.7	77.8	77.6	78.3
W. succinogenes	73.5	72.4	70.0	72.8	72.3	72.3	74.4	71.5	72.8	70.0	82.4	86.8	80.1		86.2	87.2	79.4	79.1	80.1
C. cryaerophila	69.5	70.9	68.4	71.3	72.1	71.3	72.5	71.3	69.7	68.5	63.7	62.2	62.3	67.6		94.2	78.6	77.8	78.3
C. nitrofigilis	71.3	72.1	69.7	70.9	71.7	70.5	72.1	69.3	69.7	69.7	65.3	63.8	61.1	68.8	86.9		79.5	78.0	78.5
E. coli	48.6	49.5	47.9	50.6	50.2	49.8	52.2	49.8	49.0	49.0	50.6	49.9	47.3	51.7	49.5	51.5		81.9	82.4
P. testosteroni	48.0	50.5	49.7	49.2	49.2	49.2	51.6	48.0	48.8	51.7	50.8	49.3	47.9	50.7	47.3	46.9	57.0		81.4
A. tumefaciens	48.0	49.3	50.1	47.6	46.8	48.0	50.0	47.2	45.2	50.8	49.6	50.1	48.9	52.3	49.1	48.9	58.2	56.0	

[a] Values in the upper portion of the table are percent similarities of the aligned sequences after removal of all base positions of unknown composition and all ambiguous sequence alignments. Alignment gaps were assigned half the value of a nucleotide in the same position. Values in the lower portion of the table were determined in a similar fashion, except that all positions of constant base composition were also eliminated from consideration.

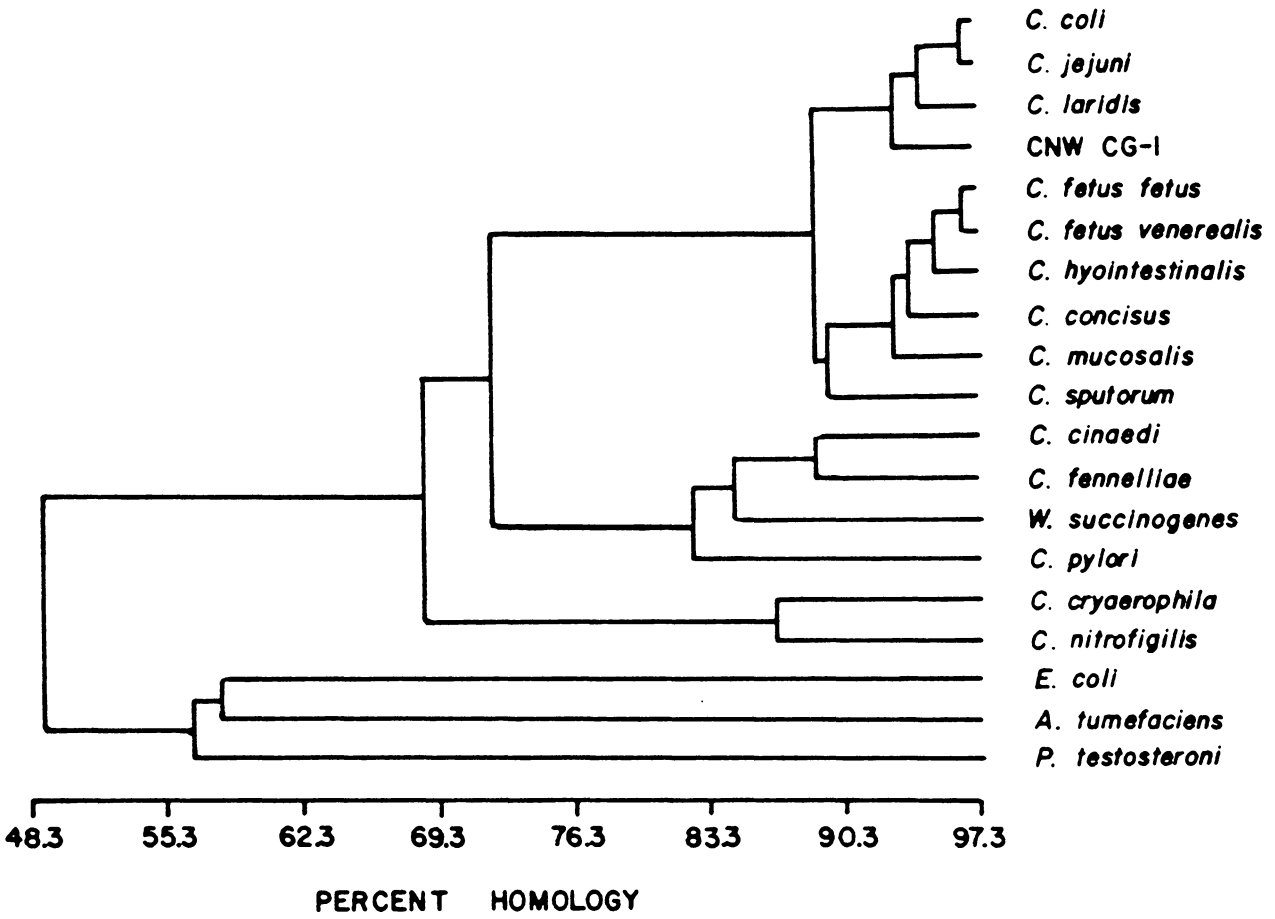

Figure 2. Phylogenetic relationships of the Campylobacter species, Wolinella succinogenes and representatives of the major branches of the purple phototrophic bacteria. The horizontal scale is the percent 16S rRNA sequence homology, calculated by the Numerical Taxonomy System (NTSYS) analysis program, using the values found in the lower portion of Table 2.

Group II contains "C. cinaedi", "C. fennelliae", and C. pylori and had interpecies homology values ranging from 82.8 to 88.8%. Group II also includes Wolinella succinogenes, which had a level of homology of 80.1 to 86.8% with the three Campylobacter species.

Group III contains C. cryaerophila and C. nitrofigilis, which have an interspecies homology value of 86.9%.

The homology level between groups I and II is 72.4%. Groups I and II are related to Group III at a level of 68.8%.

The level of 16S rRNA sequence homology between the three major branches of the purple phototrophic bacteria was determined by comparing the known sequences from representatives of each branch. A. tumefaciens and P. testosteroni were related at a level of 56.0%, A. tumefaciens and E. coli were related at a level of 58.2%, and E. coli and P. testosteroni were related at a level of 57.0%. Groups I, II and III exhibited an overall rRNA sequence homology of 49.3% with the above three representative species.

Discussion

The intergroup rRNA sequence homology values among Groups I, II and III indicate that these groups should not continue to be included within a single genus. However, the interspecies homology values within each group are high enough to warrant consideration of each group as a separate genus. Group I contains C. fetus, the type species of the genus Campylobacter; consequently, we propose that only Group I should represent the genus Campylobacter.

A review of general phenotypic characteristics which are shared by the species in homology group I is as follows: gram-negative vibrioid cells 0.2 to 0.5 μm wide by 1.0 to 5.0 μm long; motility occurs by a single, unsheathed, polar flagellum at one or both ends of the cell; oxidase-positive; reduce nitrate to nitrite; do not oxidize or ferment carbohydrates; require organic sources of nitrogen; occur in the reproductive organs, intestinal tract, or oral cavity of humans and animals. The mol% guanine + cytosine (G + C) of their DNA is 30-39%. Other characteristics vary considerably among the species, even among the species within each of the two subgroups in Group I.

The finding that one subgroup of Group I contains C. coli, C. jejuni, and C. laridis is consistent with previous data from DNA reassocation experiments: these three species were more closely related to one another by DNA reassociation than to any other Campylobacter species (Belland and Trust 1982; Owen and Leaper 1981; Roop et al. 1984; Ursing et al. 1983). Our findings indicate that CNW strain CG-1, which represents a separate species by DNA homology (Roop et al. 1985), also

belongs to this subgroup. A comparison of the phenotypic properties of the four species indicates that they all can grow at 42°C (Benjamin et al. 1983; Hébert et al. 1982; Roop et al. 1984, 1985; Smibert 1984). These species have the ability to use hydrogen as an electron donor when growing microaerophilically, but cannot grow anaerobically with fumarate as an electron acceptor. C. laridis can grow anaerobically using trimethylamine N-oxide (TMAO) (Roop et al. 1984, 1985). They are all catalase-positive except CNW strains, which may give a negative, weakly positive, or delayed reaction depending on the cultural conditions (Sandstedt et al. 1983).

The second subgroup within Group 1 includes C. fetus and C. hyointestinalis; this is consistent with previous data from DNA reassociation experiments that indicated that these two species were related (Roop et al. 1985). Our findings indicate that C. concisus, C. mucosalis, and C. sputorum also belong to this subgroup.

A comparison of phenotypes exhibited by these species is as follows: all the species except C. fetus can grow at 42°C; the catalase reaction varies, even within a single species (C. sputorum biovar fecalis is catalase-positive whereas C. sputorum biovars sputorum and bubulus are negative); and all species have the ability to grow anaerobically with H_2 as the electron donor and fumarate as the electron acceptor. C. concisus and C. mucosalis require H_2 or formate as the electron donor for microaerophilic growth, and H_2 or formate as the electron donor plus fumarate as the electron acceptor for anaerobic growth. C. sputorum requires only fumarate for anaerobic growth. C. fetus and C. hyointestinalis require both H_2 and fumarate for anaerobic

growth. All the organisms except C. fetus subspecies venerealis can grow in the presence of 1% glycine, and all except C. concisus are susceptible to cephalothin (Roop et al. 1984, 1985).

Group II includes C. pylori, "C. cinaedi", "C. fennelliae" and W. succinogenes. Romaniuk et al. (1987) previously reported that C. pylori should be excluded from the genus Campylobacter, i.e, from Group I; our results confirm this and also indicate that the percent rRNA sequence homology between C. pylori and W. succinogenes is 80.1% and, as a group, W. succinogenes, "C. cinaedi" and "C. fennelliae" are related to C. pylori at a level of 82.8%. Because the error in the RNA sequencing procedure used in this study is approximately 1.0% (Lane et al. 1985), it seems unreasonable to exclude any of these species from Group II.

It is difficult to identify phenotypic features that could be used to define Group II. Although all the species are asaccharolytic, C. pylori differs from "C. cinaedi", "C. fennelliae" in regard to morphology and in the type of infection. C. pylori has a cellular dimensions of 0.5 μm wide by 3.0 μm long and is associated with antral gastritis, whereas "C. cinaedi" and "C. fennelliae" have cellular dimensions of 0.3 μm wide by 1.5 μm long and are associated with diarrhea in homosexual males. However, all three species grow poorly under microaerobic conditions in the absence of H_2 (Megraud et al. 1985; this study, data not shown).

Because W. succinogenes is the type species of the genus Wolinella, the genus represented by Group II would be called Wolinella. Tanner et al. (1981) created the genus Wolinella and placed two species within this genus, one of these being W. succinogenes (formerly Vibrio

succinogenes). Tanner et al. described this species as an anaerobe capable of growth in 5% O_2 but not in air enriched with CO_2. This characterization of W. succinogenes as being an anaerobe is contradicted by evidence from earlier work conducted on this organism. In 1961, Wolin et al. first isolated this species from the bovine rumen and subsequently named it Vibrio succinogenes. It is clearly shown in that study that this species is capable of using O_2 as a terminal electron acceptor under microaerobic conditions (approx. 2% O_2) but not under atmospheric levels of O_2. This finding, along with further confirmatory evidence (Jacobs and Wolin 1963), indicate that W. succinogenes is a microaerophile and not an anaerobe as previously suggested. Due to this evidence, we feel that the genus Wolinella should be redefined to include the microaerophilic species: "C. cinaedi", "C. fennelliae" and C. pylori. Additionally, the definition of W. succinogenes should be emended to describe this species as a microaerophile capable of anaerobic growth when alternate electron acceptors are supplied (e.g. fumarate or nitrate), and not as a strict anaerobe stated by the current definition (Tanner and Socransky 1984).

Group III consists of C. cryaerophila and C. nitrofigilis, which are related by a rRNA sequence homology value of 86.9%. A phenotypic definition of the genus represented by these two species is elusive. C. cryaerophila is an aerobe that causes abortion in pigs and other animals and occasionally causes blood infections in humans, whereas C. nitrofigilis is a microaerophilic, NaCl-requiring nitrogen-fixer associated with the roots of marsh grasses. However, both species have optimal growth temperatures below 37°C, and they can grow at

temperatures as low a 6°C (McClung et al. 1983; Neill et al. 1985). Moreover, it is interesting that 80% of the C. nitrofigilis strains characterized by McClung et al. (1983) exhibited urease activity, and 75% of the strains could grow in 1% bile. We feel that such features might be indicative of a bacterium which has an animal host, and this may suggest that the habitat of this species might not be, or have been, limited only to marsh grass roots.

The difficulty in arriving at suitable, mutually exclusive phenotypic definitions of the genera represented by Groups I, II, and III can be attributed to the scarcity of phenotypic characterization data for campylobacters and also to the fact that not all the organisms have been compared by the same tests and methods. Now that the relationships among the campylobacters have been delineated, it is to be hoped that unique phenoytpic features of each genus may be discovered.

It is not yet clear just where campylobacters belong among the eubacteria. The fact that Groups I, II and III were related to one another at a level of 68% strongly suggests that the three groups belong together in the same phylogenetic branch. Romaniuk et al. (1987) indicated that campylobacters appeared to belong to a previously undefined phylogenetic branch, based on signature sequence analysis and on comparison of sequence data with partial sequences from representatives of 8 of the 10 major phylogenetic branches of the eubacteria. Our data indicate that Groups I, II and III have an overall RNA sequence similarity of 49.3% to representatives of the alpha, beta, and gamma phylogenetic branches of the purple bacteria. This is a depth of divergence that is even greater than that between the three branches

themselves, and is consistent with the conclusion reached by Romaniuk et al.

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Appendix A
Alignment of 16S rRNA Sequences

The following is the alignment of partial 16S rRNA sequences of fifteen strains of Campylobacter and four other gram-negative bacterial species (Agrobacterium tumefaciens, Pseudomonas testosteroni, Escherichia coli and Wolinella succinogenes). Numbers to the left represent strain designations for each species or subspecies. The strain designation for W. succinogenes is not known. Numbers across the top are nucleotide positions based on the published sequence of E. coli. Small-case letters represent ambiguous bases, "n" represents unknown base compositions, and "-" represents a gap in that sequence.

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          218        228        238        248        258        267
           |          |          |          |          |          |
30105      UCGGGGUAUGAUGAGCCCGCGUUGGAUUAGCUAGUUGGUGGGGUAAAGGC
11996      GUGCUACUAGAGCGGCUGAUGGCAGAUUAGGUAGUUGGUGGGGUAAAGGC
K-12       UUGCCUACGGAUGUGCCCAGAUGGGAUUAGCUAGUAGGUGGGGUAACGGC
WS         -CGCUAUGGGAUCAGCCUAUGUCCUAUCAGCUUGUUGGUGAGGUAAUGGC
33559      UCGGUGUAGGAUGAGACUnUAUAGUAUCAGCUAGUUGGUAAGGUAAUGGC
33560      UCGGUGUAGGAUnAGACUAUAUAGUAUCAGCUnGUUGGUAAGGUAAUGGC
11352      UCGGUGUAGGACGAGACUnUAUAGUAUCAGCUAGUUGUGGAGGUAAUGGC
27374      UCGGUGUAGGAUGAGACUnCAUUGUAUCAGCUnGGUGGUAAGGUAAUGGC
19438      UCGGUGUAGGAUGAGACUnUAUUGUAUCAGCUnGUUGGUAAGGUAAUGGC
35217      UCGGUGUAGGAUGAGACUnUAUUGUAUCAGCUAGUUGGUAAGGUAAUGGC
S-17       UCGCUAUGGGAUGAGGCUnUAUUGUAUCAGCUAGUUGGUAAGGUAAUGGC
CG-1       UCGGUGUAGGAnUAGACUnUAUAGUAUCAGCUnGUUGGUAAGGUAAAGGC
13086      UCGGUGUAGGAUnAGGCUnUAUUGUAUCAGCUAGUUGGUGAGGUAAUGGC
11637      UCGCUAAGAGAUnAGCUUnUGUCCUAUCAGCUUGUUGGUAAGGUAAUGGC
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35683     UCGCUAAAGGAUCAGnnUnUAUCCUAUCAGCUUGUUnGUAAGGUnAUGGC
35684     CCGCUAAAGGAUUnGUCUAUGUCCUAUCAGCUUGUUGGUGAGGUAAUGGC
11885     UUGGUAAGAGAUUAGCCUnUAUUGUAUCAGUUAGUnGGnGGGGUAAUGGC
33309     UGGCUCUAGGAUUGGCCUnUCCGGUAUCAGCUAGUnGGUGAGGUCAUGGC
11000     UCGGCAUAGGAUGAGGCUnCAUUGUAUCAGCUAGUUGGUAAGGUAAUGGC
        268       278       288       298       308       318
          ¦         ¦         ¦         ¦         ¦         ¦
30105     CUACCAAGGCGACGAUCCAUAGCUGGUCUGAGAGGAUGAUCAGCCACAUUG
11996     UUACCAAGCCUGCGAUCUGUAGCUGGUCUGAGAGGACGACCAGCCACACUG
K-12      UCACCUAGGCGACGAUCCCUAGCUGGUCUGAGAGGAUGACCAGCCACACUG
WS        UCACCAAGGCUAUGACGGGUAUCCGGCCUGAGAGGGUGAUCGGACACACUG
33559     UAACCAAGGCUnUGACGCUUAACUGGUCUGAGAGGAUGAUCAGUCACACUG
33560     UAACCAAGGCUnUGACGCUUAACUGGUCUGAGAGGAUGAUCAGUCACACUG
11352     AAACGGAGGCUnGGACGCUUAACUGGUCUGAGAGGAUGAUCAGUCACACUG
27374     UAACCAAGGCUCUGACGCAUAACUGGUCUGAGAGGAUGAUCAGUCACACUG
19438     UAACCAAGGCUnGGACGCAUAACUGGUCUGAGAGGAUGAUCAGUCACACUG
35217     UAACAAAGGCUAUGACGCAUAACUGGUCUGAGAGGAUGAUCAGUCACACUG
S-17      UAACCAAGGCUnUGACGCUUAACUGGUCUGAGAGGAUGAUCAGUCACACUG
CG-1      UAACCAAGGCUnUGACGCUnAACUnGUCUGAGAGGAUGAUCAGUCACACUG
13086     UAACnAAGGCUnUGACGCAUAACUGGUCUGAGAGGAUGAUCAGUCACACUG
11637     UAACUAAGGCUAUGACGGGUAUCCGGCCCnAGAGGGUGAACGGACACACUG
35683     UAACCAAGGCUnUGACGGGnAAC-GGCCUGAGAGGGUnAACGGACACACUG
35684     UCACCAAGGCUnUGACGGGUAUCCGGCCUGAGAGGGUGAACGGACACAnUG
11885     nUACCAAGACAAUGACACAUAACUnGUUUGAGAGGAUnAUCAGUCACACUG
33309     UCACAAAGGCAAUGACACCUAACUnGUUUGAGAGGAUGAUCAGUCACACUG
11000     UUACCAAGGCUnUGACGCAUAACUGGUCUGAGAGGAUGAUCAGUCACGCUG
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          319        329        339        349        359        369
           |          |          |          |          |          |
30105     GGACUGAGACACGGCCCAAACUCCUACGGGAGGCAGCAGUGGGGAAUAUUG
11996     GGACUGAGACACGGCCCAGACUCCUACGGGAGGCAGCAGUGGGGAAUUUUG
K-12      GAACUGAGACACGGUCCAGACUCCUACGGGAGGCAGCAGUGGGGAAUAUUG
WS        GAACUGAGACACGGUCCAGACUCCUACGGGAGGCAGCAGUAGGGAAUAUUG
33559     GAACUGAGACACGGUCCAGACUC-UUCGGGAGGCGGCAGUAGGGAAUAUUG
33560     GAACUGAGACACGGUCCAGACUC-UUCGGGAGGCGGCAGUAGGGAAUAUUG
11352     GAACUGAGACACGGUCCAGACUC-UACGGGAGGGnGCAGUAGGGAAUAUUG
27374     GAACUGAGACACGGUCCAGACUC-UnCGGGAGGCUGCUGUAGGGAAUAUUG
19438     GAACUGAGACACGGUCCAGnCUC-UnCGGGAGGCAGCAGUAGGGAAUAUUG
35217     GAACUGAGACACGGUCCAGACUC-UnCGGGAGGCACCAGUAGGGAAUAUUG
S-17      GAACUGAGACACGGUCCAGACUC-UACGGGAGGCAGCAGUAGGGAAUAUUG
CG-1      GAACUGAGACACGGUCCAGnCUCC-nCGGGAGGCCGCAGUAGGGAAUAUUG
13086     GAACUGAGACACGGUCCAGnCUC-GACGGGAGGCGGCAGUAGGGAAUAUUG
11637     GAACUGAGACACGGUCCAGACUC-UACGGGAGGCGGCAGUAGGGAAUAUUG
35683     GAACUGAGACACGGUCCAGACUC-UACGGGAGGCnGCAGUAGGGAAUAUUG
35684     GAACUGAGACACGGUCCAGACUC-UACGGGAGGCGGCnGUAGGGAAUAUUG
11885     GAACUGAGACACGGUCCnGCCUCGGGCGGGAGGCAGCAGUGGGGAAUAUUG
33309     GAACUGAGACACGGUCCAGACUC-UUnGGGAGGCAGCAnUGGGGAAUAUUG
11000     GAACUGAGACACGGUCCAGACUC-UUCGGGAGGCGGCAGUAGGGAAUAUUG

          370        380        390        400        410        420
           |          |          |          |          |          |
30105     GACAAUGGGCGCAAGCCUGAUCCAGCCAUGCCGCGUGAGUGAUGAAGGCCU
11996     GACAAUGGGCGAAAGCCUGAUCCAGCAAUGCCGCGUGCAGGAUGAAGGCCC
K-12      CACAAUGGGCGCAAGCCUGAUGCAGCCAUGCCGCGUGUAUGAAGAAGGCCU
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WS       CUCAAUGGGCGAAAGCCUGAAGCAGCaACGCCGCGUGGAGGAUGAAGGUCU
33559    CGCAAUGGGGGAAACCCnGACGCAGCAACGCCGCGUGGAGGAUAACACUUU
33560    CGCAAUGGGGGAAACCCnGACGCAGCAACGCCGCGUGGAGGAUGACACUUU
11352    CGCAAUGGGGGAAACCCnGACGCAGCAACGCCGCGUGGAGGAUAACACUnU
27374    CUCUnUGGGGGAAACCCnGAAGCUGCUACGCCGCGUGGAGGAUGACUCUUU
19438    CUCAnUGGGGGAAACCCnGAAGCAGCAACGCCGCGUGGAGGAUGACACUUU
35217    CUnAAUGGGGGAAACCCnGAAGCAnCAACGCCGnGUGGAGGAUnACACUUU
S-17     CUCAAUGGGGGAAACCCUGAAGCAnCAACGCCGCGUGGAGGAUGACACUUU
CG-1     CGCCAUGGGGGAAACCCnGACGCAGCAACGCCGCGUGGAGGAUnACACUUU
13086    CUCAnUGGGGGAAACCCnGAAGCAGCAACGCCGCGUGGAnGAUGACACUUU
11637    CGCnACGGGGGAAACCCnGAAGCAGCAGCGCCGCGUGGAGGAUGAAGGUUU
35683    CUCAAUGGGGGAAACCCnnAAGCAGCAACGCCGCGUGGAGGAUAAAGGUnn
35684    CnCAnUGGGGGAAACCCnGAAGCAGCAACGCCGCGUGGAGGAUGAAGGUnn
11885    CACAAUnGACGAAAGUCUCAUGCAGnnACGCCGCGUGGAGGAUnACACAUU
33309    CACAAUGGACGAAAGUCUnAUGCAGCAACGCCGCGUGGAGGAUGACACAUU
11000    CGCAAUGGGGGAAACCCnGAAGCAGCAGCGCAGCGUGGAGGAUAACACUUU
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                                                  694          707
         421        431        441        451    457          704
          |          |          |          |      ||           |   |
30105    UAGGGUUGUAAAGCUCUUUCACCGGAGAAGAUAA---AAAUUCGUAGAUAU
11996    UCGGGUGGUAAACUGCUUUUGUACGGAACGAAAAGCCAAAUGCGUAGAUAU
K-12     UCGGGUUGUAAACUACUUUCAGCGGGGAGGAAGGGAGAAAUGCGUAGAGAU
WS       UCGGAUUGUAAACUCCUUUUCUAAGAGAAG-------AAAUCCGUAGAGAU
33559    UAGGAGCGUAAACUnCnUUUCUUAGGGAAGAAUUCUAAAAUCCGUACAGAU
33560    UCGGAGCGUAAACUCCnUUUCUnAGGGAAGAAUUCUAAAAUCCGUAGAUAU
11352    UCGGAGCGUAAACUCGnUUUCUUAGGGAAGAAUUCUAAAAUCCGUAGAUAU
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27374    UCGGAGCGUAAACUnCnUUUGUUAGGGAAGAACCUUGAAAUCCGUAGAGAU
19438    UCGGAGCGUAAACUnCnUUUGUUAGGGAAGAACCAUCAAAUCCGUAGAGAU
35217    UCGGAGCGUAAACUnCnUUUGUUAGGGAAGAACCAUGAAAUCCGUAGAGAU
S-17     UCGGAGCGUAAACUCCnnUUCUUUGGGAAGAAUUAAUAAAUCCGUAGAUAU
CG-1     UCGGAGCGUAAACUnCnUUUCUnUGGGAAGAAUUCUCAAAUCCGUAGAUAU
13086    UCGGAGCGUAAACUnCnUUUGUAAGGGAAGAAUAAUAAAAUCCGUAGAGAU
11637    AAGGAUUGUAAACUnCUUUUGUAAGAGAAGAnAAUAAAAAUCCGUAGAGAU
35683    nAGGAUUGUAAACUnnnnUGGUAAGAGAAGA-UUAUAAAAUCCGUAGAGAU
35684    AAGGAUUGUAAACUnCnUUUGUAAGAGAAGA-UUAUAAAAUCCGUAGAGAU
11885    UCGGUGCGUnAACUnCUnUUAUAUGAGAAGAUAAUAAAAAUCCGUAGAGAU
33309    UCGGUGCGUAAACUnCnUUUAUAUAGGAAGAUAAUGAAAAUCCGUAGAUAU
11000    UCGGAGCGUAAACUnCnUUUGUUAGGGAAGAACCAUGAAAUCCGUAGAGAU

         708       718       728       738       748       758
          |         |         |         |         |         |

30105    UCGGAGGAACGCCAGUGGCGAAGGCGGCUUACUGGUCCAUUACUGACGCUG
11996    GCGGAAGAACACCGAUGGCGAAGGCAAUCCCCUGGGCCUGCACUGACGCUC
K-12     CUGGAGGAAUACCGGUGGCGAAGGCGGCCCCCUGGACGAAGACUGACGCUC
WS       CGAGAGGAAUACUCAUUGCGaAGGcgACCUGCUGGAACAUUACUGACGCUG
33559    CACCAAGAAUACCCAUUGCGAAGGCGAUCUGCUnGAACUnAACUnACGCGA
33560    CACCAAGAAUACCCAUUGCGAAGGCGAUCUGCUGGAACUCAACUGACGCUA
11352    CACCAAGAAUACCCAUUGCGAAGGGGAUCUGCUGGAACUnAACUGACGCUA
27374    CACCAGGAAUACCCAUUGCGAAGGGGAUCUGCUGGAACUnAACUGACGCUn
19438    CACCAGGAAUACCCAUUGCGAAGGCnAUCUGCUGGAACUnAACUnACGCUA
35217    CACCAGGAAUACCCAUUGCGAAGGGGAUCUGCUGGAACUnAACUGACGCUA
S-17     CAAGAGGAAUACUUAnUGCGAAGGCCAUCUGCUAGAACUUAACUGACACUA
CG-1     CACCAAGAAUACCCAUUGCGAAGGCGAUCUGCUGGAACUnAACUGACGCGA
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13086     CACCAGGAAUACCCAUUGCGAAGGCGAUCUGCUGGAACUCAACUGACGCUA
11637     CAAGAGGAAUACUCAUUGCGAAGGCGACCUGCUGGAACAUUACUGACGCUG
35683     CAAGAGGAAUACCCAUUGCGAAGGCGACCUGCUGGAACAUUACUGACGCUG
35684     CAAGAGGAAUGCUCAnUGCGAAGGCGACCUnCUnGAACAUUACUGACGCUC
11885     CAGAAGGAAUACCGAUUGCGAAGGCGAUCUACUGGAACAUUAUUGAnGCUG
33309     CAGAAGGAAUACCGAUUGCGAAGGCGAUCUACUGGAACAUnAUUGACGCUG
11000     CACCAGGAAUACCCAUUGCGAAGGCGAUCUGCUGGAACUCAACUGACGCUA
        759         769         779         789         799         809
          ¦           ¦           ¦           ¦           ¦           ¦
30105    AG-GUGCGAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUGGUAGUCCACG
11996    AU-GCACGAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUGGUAGUCCAGC
K-12     AG-GUGCGAAAGCUGGGGGAGCAAACAGGAUUAGAUACCCUGGUAGUCCACG
WS       Au-GUGCGaAAGcuUGGGGAGCAAACAGGAUUAGAUACCCUGGUaGUCCACG
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33560    AG-GCGCGAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUnGUAGUCCACG
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27374    AU-GCGUGAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUnGUAGUCCACG
19438    AU-GCGUnAAAGCGUGGGGAGCAAACAGGAGGAGAUACCCUnGUAGUCCACG
35217    AU-GCGUnAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUnGUAGUCCACG
S-17     AU-GCGUnAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUnGUnGUCCAnG
CG-1     AG-GCGnGAAAGCGUGGGGAGCAAACAGGAUUACAUACCCUnGUAGUCCAGG
13086    AU-GCGUnAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUnGUAGUCCACG
11637    AUUGUGCnAAAGCGUGGGGAGCAAAGAGGAUUAGAUACCCUnGUAGUCCACG
35683    AU-GCGUnAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUnGUAGUCCACG
35684    AU-GCGCGAAAGCGUGGGGAGCAAACAGGAnUAGAUGCCCUnGUGGUCCACG
11885    AG-nUGCGnAAGnGUGGGGAGCAGACAGGAUUAGAUACCCUnGUAGUCCAnG
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33309    AG-ACGCGAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUnGUAGUCCACG
11000    AG-GCGUnAAAGCGUGGGGAGCAAACAGGAUUAGAUACCCUnGUAGUGGACG
         810        820        830        840        850        860
         |          |          |          |          |          |
30105    CCGUAAACGAUGAAUGUUAGCCGUCGGGCAG-UA-UACUGUUCGGUGGCGCA
11996    CCCUAAACGAUGUCAACUGGUUGUUGGGUC-UUA-ACUGACUCAGUAACGAA
K-12     CCGUAAACGAUGUCGACUUGGAGGUUGUGCCCUU-GAGGCGUGGCUUCCGGA
WS       CCCUAAACGAUGAAUGCUAGUUGUUGCCCUGCUU-GUnAGGGcAGUAAUGCA
33559    CCCUAAACGAUGUAUACUAGUUGUUGCUCUGCUA-GUUAGGGCAGUAAUGCA
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19438    CCCUAAACGAUGUAUACUAGUUGUUGCUGUGCUA-GUnACCGCnGUAAUGCA
35217    CCCnAAACGAUGUAUnCUnGUUGUUGCUGUGCUA-GUUACGGCAGUAAUGCA
S-17     CCCUnAACGAUGUAUnCUnGUUGUUGCUnUGCUA-GUCAUGGCAGUAAUCCA
CG-1     CCCUnnACGAUGUAUGCUnGUUnUUUGGGUnCUn-GUnAUCUCAGUAAUGCA
13086    CCCUnAACGAUGUAUCCUnGUUGUUGCUAAGCUn-GUnUUGGCAGUAAUGCA
11637    CCCUAAACGAUGGAUnCUnGUUGUUGGAGGGCUAnGUCUnUCCAGUAAUGCA
35683    CCCUnAACGAUGAAUnCUnGUUGUUGCCCUGCUA-GUAAGGGnnGUAAUGCA
35684    CCCUnAACGAUGAAUnCUnGUUGUUGCCCUnCUC-GUnAGGGnnGUAAUGCC
11885    CCCUAAACGAUGUACACUnGUUGUUGUGAGACUn-GAnCUUGCAGUAAUGCA
33309    CCCUnAACGAUGUACACUnGUUGUUGUGAGGCUn-GACCUnGCAGUAAUGCA
11000    CCCUAAACGAUGUAUACUAGUUGUUGCUAAGCUA-GUnUUGGCAGUAAUGCA
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                  1033
    861           873       1040          1050        1060       1070
      ¦            ¦¦         ¦             ¦           ¦          ¦
30105 GCUAACGCAUUAACUGG--CCCCAG-AACAGGUGCUGCAUGGCUGUCGUCAGCU
11996 GCUAACGCGUGAAAGAA--CCUGCA-CACAGGUGCUGCAUGGCUGUCGUCAGCU
K-12  GCUAACGCGUUAAGGAA--CCGUGA-GACAGGUGCUGCAUGGCUGUCGUCAGCU
WS    GUUAACACAUUCUGGAG--CUUGAa-aaCAGGUGCUGCACGGCUGUCGUCAGCU
33559 C-UAACGGAUUAAAGAA--CUUAnA-GACAGGUGCUGCACGGCUGUCGUCAGCU
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19438 C-UAACGGAUUAAAGAAA-GUU-GA-GACAGGUGCUGCACGGCUGUCGUCAGCU
35217 C-UAACGGAUUAAAGAAA-UUA-GU-GACAGGUGCUGCACGGCUGUCGUCAGCU
S-17  C-UAACGGAUUAAAAAU-GUUU-nA-GACAGGUGCUGnACGGCUGUCGUCAGCU
CG-1  -GUAACGCAUUAAAGAA-UGUU-GAGACAGGGUGCUGCACGGCUGUCGUCAGCU
13086 C-UAACGGAUUAAAGAAAUGUU-AA-GACAGGUGCUGCACGGCUGUCGUCAGCU
11637 CCUAACGCAUUAAAGAC--CUU-GAAAACAGGUGCUGCACGGCUGUCGUCAGCU
35683 -GUnACGCAUUAAAGAG--CUU-AAAAACAGGUGCUGCACGGCUnUCGUCAGCU
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33309 GUUAACACAUUAAAGAAA-CUUACn-UACAGGUGCUGCACGGCUnUCGUUAGCU
11000 C-UAACGGAUUAAAGAA-UGUU-GA-GACAGGUGCUGCACGGCUGUCGUCAGCU
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11996    CGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCGCAACCCUUGCCAU
K-12     CGUGUUGUGAAAUGUUGGGUUAAGUCCCGCAACGAGCGCAACCCUUAUCCU
WS       CGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCGCAACCCUCGUCAC
33559    CGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCGCAACCCACGUnUU
33560    CGUGUCGUGAGAUGUUGGGUUAAGnCCCGCAACGAGCGCAACCCACGUnUU
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27374    CGUGUCGUGAGAUGUUGGGUUAAGnCCCGCAACGAGCGCAACCCACGUnUU
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35217    CGUGUCGUGAGAUGUUGGGUUAAGnCCCGCAACGAGCGCAACCCACGnnUU
S-17     CGUGUGGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCGCGACCCACGnnUU
CG-1     CGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCGCAACCCUnGUCCU
13086    CGUGUCGUGAGAUGUUGGGUUAAGnCCCGCAACGAGCGCAACCCACGUCAU
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35683    CGUGUnGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCGCAACCCAnGUCCU
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11885    CGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCGCAACCCUnGUnUU
33309    CGUGUCGUGAGAUGUUGGGUUAAGUCCCnGAACGAGCGCAACCCUnGUnUU
11000    CGUGUCGUGAGAUGUUGGGUUAAGnCCCGCAACGAGCGCAACCCACGUnUU
     1122       1132       1142       1152       1162       1172
        ¦          ¦          ¦          ¦          ¦          ¦
30105   UAGUUGCCAGCAUUUAG-U-UGGGCACUCUAAGGGGACUGCCGGUGAUAAGC
11996   UAGUUGCUA-CAUUCAG-U-UGAGCACUCUAAUGGGACUGCCGGUGACAAAC
K-12    UUGUUGCCAGCGGUCCG-GCCGGGAACUCAAAGGAGACUGCCAGUGAUAAAC
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WS      UAGUUGCUAACGGUUCG-GCCGAGCACUCUAGUGAGACUGCCUUCG-CAAGG
33559   UAGUUGCUAACGGUUCG-GCCGAGCnCUCUAAAAUAGACUGCCUUCG-UAAGG
33560   UAGUUGCUAACGGUUCG-GCCGAGCnCUCUAAAAUAGACUGCCUUCG-UAAGG
11352   UAGUUGCUAACACUUCG-GGUGAGCACUCUAAAAUAGACUGCCUUCG-UAAGG
27374   UAGUUGCUAACAGUUCG-GCUnAGCACUCUAAAAUAGACUGCCUUCG-CAAGG
19438   UAGUUGCUAACAGUUCG-GCUnAGCACUCUAAAAUAGACUGCCUUCG-CAAGG
35217   UAGUUGCUAACAGUUCG-GCUCAGCCCUCUAAAAUAGACUGCCUUCG-UAAGG
S-17    UAGUUGCUAACAGUAAG-GCAnAGCACACnnAACAGACUGCCUUCG-UAAGG
CG-1    UAGUUGCUAACGAUUCG-GUCGAGCACUCUAAGGAGACUGCnUUCG-UAAGG
13086   UAGUUGCUAACAGUUCG-GCUnAGCnCUCUAAUGAGACUGCCUUCG-UAAGG
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     1173       1183       1193       1203       1213       1223
        |          |          |          |          |          |

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K-12    UG-GAGGAAGGUGGGGAUGACGUCAAGUCAUCAUGGCCCUUACGACCAGGGC
WS      AG-GAGGAAGGUGAGGACGACGUCAAGUCAUCAUGGCCCUUACGnccAGGGC
33559   An-GAGGAAGGUGUGGACGACGUCAAGUCAUCAUGGCCCUUAUGCCCAGGGC
33560   An-GAGGAAGGUGUGGACGACGUCAAGUCAUCAUGGCCCUUAUGCCCAGGGC
11352   nn-GAGGAAGGUGUGnACGACGUCAAGUCAUCAUGGCCCUUAUGCCCAGGGC
27374   nn-GAGGAAGGUGUGGACGACGUCAAGUCAUCAUGGCCCUUAUGCCCAGGGC
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35217    nn-GAGGAAGGUGUGGACGACGUCAAGUCAUCAUGGCCCUUAUGCCCAGGGC
S-17     An-GAGGAAGGUGUGGACGACGUCAAGUCAUCAUGGCCCUUAUGUCCGGGGC
CG-1     nn-GAGGAAGGUGUGGACGACGUCAAGUCAUCAUGGCCCUUAUGCCUAGGGC
13086    nG-GAGGAAGGUGUGGACGACGUCAAGUCAUCAUGGCCCUUAUGUCCGGGGC
11637    AA-GAGGAAGGUGGGGACGACGUCAAGUCAUCAUGGCCCUUACGCCnAGGGC
35683    AG-GAGGAAGGUGAGGACGACGUnAAGUCAUCAUGGCCCUUAUGCCGAGGGC
35684    AG-GAGGAAGGUGAGGACGACGUCAAGUCAUCAUGGCCCUUACGCCCAGGGC
11885    AG-GAGGAAGGUGAGGACGACGUCCAGUCAUCAUGGCCCUUGCGUCCAGGGC
33309    AG-GAGGAAGGUGAGGACGACGUCAAGUCAUCAUGGCCCUUUCGACCAGGGC
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         1224      1234      1244      1254      1264      1274
          |         |         |         |         |         |

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11996     UACACACGUCAUACAAUGGCUGGUACAAAGGGUUGCCAACCCGCGAGGGGG
K-12      UACACACGUGCUACAAUGGCGCAUACAAAGAGAAGCGACCUCGCGAGAGCA
WS        UACACACGUGCUACAAUGGUGUGUACAAAGAGAAGCAAUACCGCGAGGUGG
33559     GACACACGUGCUACAAUGGCAUAUACAAUGAGACGCAAUACCGCGAGGUnG
33560     GACACACGUGCUACAAUGGCAUAUACAAUGAGACGCAAUACCGCGAGGUnG
11352     GACACACGUGCUACAAUGGCAUAUACAAUGAGACGCAAUACCGCCAnGUnG
27374     GACACACGUGCUACAAUGGCAUAUACAAUGAGAUGCAAUAUCGCGAGAUGG
19438     GACACACGUGCUACAAUGGCAUAUACCAUGAGAUGCAAUAUCGCGAGAUGG
35217     GACACACGUGCUACAAUGGCAUAUACAAUGAGACGCAAUAUCGCGAGAUnG
S-17      GACACACGUGCUACAAUGGCAUAUACAAUAAGACGCAAUAUCGCGAGAUnG
CG-1      GACACACGUGCUACAAUGGCAUAUACAAUGAGACGCAAUACCGCGAGGUnG
13086     GACACACGUGCUACAAUGGCAUAUACAAUGAGAAGCAAUAUCGCGAGAUnG
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11637     UACACACGUGCUACAAUGGGGUGCACAAAGAGAAGCAAUACUGCGAAGUGG
35683     GACACACGUGCUACAAUGGGGCGCAnAAAGAGGAGCAAUACCGCGAGGUnG
35684     UACACACGUGCUACAAUGGGGUGCACAAAGAGAAGCAAUAUCGCGAGAUGG
11885     UACACACGUGCUACAAUGGGAUAUACCAAGAGCCGCAAUGCGGUGACGUGG
33309     UACACACGUGCUACAAUGGGGUAUACCAAGAGCCGCGAUACGGUGACGUnG
11000     GACACACGUGCUACAAUGGCAUAUACAAUGAGACGCAAUAUCGCGAGAUnG
     1275       1285       1295       1305       1315       1325
        ¦          ¦          ¦          ¦          ¦          ¦
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11996    AGCUAAUCC-CAUAAAGCCAGUCGUAGUCCGGAUCGCAGUCUGCAACUCGAC
K-12     AGCGGACCU-CAUAAAGUGCGUCGUAGUCCGGAUUGGAGUCGUCAACUCGAC
WS       AGCAAAUCU-UUAAAA-CACAUCUCAGUUCGGAUUGUAGUCUGCAACUCGAC
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CG-1     AGCAAAUCU-nUnAAA-UAUGUCCCAGUUCGGAUnGUUCUCUGCAACUCGAG
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11885    AGCCAAUCUUAUnAAA-UAUCUCCCAGUUCGGAUUGUAGUCUGCCACUnGAC
33309    AGCGAAUCU-UCAAAA-UGCCUCCCAGUUCGGAUUGUnGUCUGCCACUCGAC
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11000	AGnGAAUCU-nUAAAA-UAUGUCCCAGUUCnGAUUGGAGUCUGCAACUCGAC	
	1326 1336 1346 1356 1366	# of
	¦ ¦ ¦ ¦ ¦	bases
30105	UGCAUGAAGUUGGAAUCGCUAGUAAUCGCAGAUCAG-CAUGC	749
11996	UGCGUGAAGUCGGAAUCGCUAGUAAUCGUGGAUCAG-AAUGU	752
K-12	UCCAUGAAGUCGGAAUCGCUAGUAAUCGUGGAUCAG-AAUGC	754
WS	UACAUGAAGCUGGAAUCGCUAGUAAUCGUAGAUCAGCAAUGC	745
33559	AGCAUGAAGCCGGAAUCGCUnGUAAUCGUAGAUCAGCCAUGC	751
33560	AGCAUGAAGCCGGAAUCGCUnGUAAUCGUAGAUCAGCCAUGC	752
11352	AGCAUGAAGCCGGAAUCGCUnGUAAUCGUAGAUCAGCCAUGC	752
27374	UCCAUGAAGCCGGAAUCGCUnGUAAUCGUAGAUCAGCCAUGC	751
19438	UCCAUGAAGCCGGAAUCGCUnGUAAUCGUAGAUCAGCCAUGC	751
35217	AGCAUGAAGCCGGAAUCGCUnGUAAUCGUAGAUCAGCCAUGC	751
S-17	UCCAUGAAGCCGGAAUCGCUnGUAAUCGUAGAUCAGCCAUGC	751
CG-1	AGCAUGAAGCCGGAAUCGCUAGUAAUCGCAAAUCAGCCAUGU	751
13086	UCCAUGAAGCCGGAAUCGCUnGUAAUCGUAGAUCAGCCAUGC	752
11637	UnCAUGAAGCUnGAAUCGCUnGUAAUCGCAAAUCAGCCAUGU	755
35683	UnCAUnAAGCCnGAAUCGCUnGUAAUCGUnGAUCAGCnAUGU	750
35684	UnCAUAAAGCUnGAAUCGCUnGUnAUCGCAAAUCAGCAAUGU	751
11885	UnCAUGAAGUUGGAAUCGCUAGUAAUCGUAGAUUCGCUAUGC	754
33309	UnCAUGAAGUUGGAAUCGCCnGUAAUCGUAGAUCCGCUAUGC	752
11000	UCCAUGAAGCCGGAAUCGCUnGUAAUCGUAGAUCAGCCAUGC	751

Appendix B
Materials and Methods

Culture media (Roop, Ph.D. dissertation, 1985):

1. Brucella broth and agar.
2. Brucella semisolid agar (BSS). Brucella broth supplemented with 0.15% agar.
3. BSSF: Brucella semisolid agar supplemented with 0.3% fumaric acid and adjusted to pH 7.0 using KOH.
4. BSSS: Brucella semisolid agar supplemented with 1.0% NaCl.
5. FBP broth: (George et al. 1978) Brucella broth supplemented with 0.025% sodium bisulfite, 0.05% ferrous sulfate ($7H_2O$) and 0.05% sodium pyruvate (for use in Roux bottles).
6. FBP agar: (George et al. 1978) Brucella broth supplemented with 0.025% each of sodium bisulfite, ferrous sulfate ($7H_2O$) and sodium pyruvate and 2.5% agar (for use in Roux bottles).
7. F and F broth: Brucella broth supplemented with 0.3% fumaric acid and 0.2% sodium formate and adjusted to pH 7.0 using KOH (for use in Roux bottles).
8. F and F agar: F and F broth supplemented with 2.5% agar (for use in Roux bottles).
9. BF broth: Brucella broth supplemented with 0.3% fumaric acid and adjusted to pH 7.0 using KOH (for use in Roux bottles).
10. BF agar: BF broth supplemented with 2.5% agar (for use in Roux bottles).
11. BS broth: Brucella broth supplemented with 1.0% NaCl (for use in Roux bottles).

12. BS agar: BS broth supplemented with 2.5% agar (for use in Roux bottles).

Maintenance and preservation of stock cultures

The incubation conditions and media used for the maintenance of all stock cultures and for the RNA isolation are presented in Table 1.

Type strains of each species and subspecies used in this study were preserved in liquid nitrogen. Strains were grown in the appropriate semisolid medium for 24-96 hours and transferred to four Brucella agar slants per strain, each supplemented according to growth requirements. These slants were incubated for 24-96 hours in anaerobe jars containing 6% O_2, 10% CO_2 and 84% N_2 for all strains except C. mucosalis, C. concisus, C. pylori, "C. fennelliae" and "C. cinaedi" which were incubated in the same atmospheric content used for the growth of these stock cultures (see above). Growth from the slants was washed off with sterile nutrient broth containing 10% sterile dimethyl sulfoxide (DMSO). Approximately 0.5 ml of this mixture was placed into sterile, cotton-stoppered glass cryules (Wheaton 200), sealed and placed in liquid nitrogen.

Table 1. Media and culture conditions used for the growth of Campylobacter cultures.

Species	Stock culture medium[a]	RNA isolation medium[a]	Incubation temperature (°C)	Atmosphere
C. coli	BSS	FBP	37	AIR
C. jejuni	BSS	FBP	37	AIR
C. fetus fetus	BSS	BF	37	AIR
C. fetus venerealis	BSS	BF	37	AIR
C. hyointestinalis	BSS	BF	37	AIR
C. laridis	BSS	BF	37	AIR
C. concisus	BSSF	BF	37	ADJUSTED[b]
C. mucosalis	BSSF	BF	37	ADJUSTED
C. sputorum	BSS	BF	37	AIR
CNW strain CG-1	BSS	FBP	37	AIR
C. pylori	BSS	UB[c]	37	ADJUSTED
"C. cinaedi"	BSS	UB	37	ADJUSTED
"C. fennelliae"	BSS	UB	37	ADJUSTED
C. cryaerophila	BSS	UB	30	AIR
C. nitrofigilis	BSSS	BS	30	AIR

[a] see culture media section of appendix b for explanation of abbreviations

[b] ADJUSTED = 6% O_2, 10% CO_2, 15% H_2, and 69% N_2

[c] UB = unsupplemented Brucella medium

Growth of cultures for RNA isolation

Biphasic culturing systems employing Roux bottles were used for the growth of all Campylobacter cultures used for RNA isolation (Roop et al. 1984, 1985). Roux bottles (Corning 1290) were prepared according to Roop (Ph.D. dissertation 1985). Each bottle was typically inoculated with the growth from two 48- to 96-hr-old semisolid cultures. Cultures grown in an air atmosphere were inoculated into five Roux bottles per organism. Three bottles per organism were used for those cultures which required incubation in an atmosphere supplemented with hydrogen gas and carbon dioxide. This was due to the space limitation of the incubator. Originally, an attempt was made to adjust the atmosphere of each bottle individually. However, it was found that stoppers which sealed each bottle could not maintain a vacuum and therefore could not hold the adjusted atmosphere. Therefore, an anaerobic incubator (Thelco, Precision Scientific Co.) was used for these organisms since it had the capacity for atmospheric adjustment and could hold this atmosphere for several days.

C. jejuni, C. coli and CG-1 grew in an air atmosphere in Roux bottles containing FBP medium (George et al. 1978, Roop et al. 1984) at 37°C. The same medium was initially used for the growth of C. fetus (both subspecies), however, only scant growth occurred. BF medium was subsequently used and resulted in good growth of these organisms.

The broth overlays from each bottle containing a specific culture were pooled aseptically into a sterile, RNase-treated 250-ml plastic centrifuge bottle for RNA isolation. Each Roux bottle was checked for purity by phase contrast microscopy immediately prior to pooling.

The reference strain E. coli K-12 was grown by inoculating 250 ml of Trypticase-soy broth with the growth from a 24-hr-old T-soy agar slant. The culture was incubated at 37°C for 24 hrs with intermittant shaking. This culture was also checked for purity by phase contrast microscopy prior to RNA isolation.

Buffers and reagents used for RNA isolation

The following reagents and buffers were used in the isolation of RNA from all species in this study. Due to the presence of RNases, all solutions were made using autoclaved water treated with diethyl pyrocarbonate to inactivate this enzyme. Additionally, all glassware used in the preparation and storage of the solutions were baked in a sterilizing oven at approximately 375°F for 12 hrs. All plasticware was autoclaved for 45 min in a solution of detergent (Sparkleen, Fisher Scientific), rinsed under hot tap water and autoclaved empty for 30 min. Latex gloves were worn throughout the preparation of these solutions.

Water: Distilled water was placed in a baked 4 liter Erlenmeyer flask along with a baked teflon-coated stirring bar. To this was added diethyl pyrocarbonate to a concentration of 0.2%. This solution was stirred on a magnetic stir plate for 4 hours followed by autoclaving for 45 min. This RNase-free water was then used for the preparation of all buffers and reagents.

Extraction buffer: 0.25 M sucrose (85.6 g/l), 0.2 M NaCl (11.7 g/l), 0.1 M Tris/HCl (15.8 g/l) and 10 mM magnesium acetate (2.15 g/l). This solution was adjusted to pH 9.0 using KOH and stored at 4°C in a 1 liter screw-capped bottle. Immediately prior to use, a portion of the stock

buffer was removed, heated in a boiling water bath, allowed to cool to room temperature, followed by the addition of 370 µl/l of 2-mercaptoethanol.

Phenol-cresol mixture: 550 ml water saturated phenol, 70 ml m-cresol and 0.5 g 8-hydroxyquinoline. Sterile RNase-free water was added to the phenol in a separatory funnel, shaken and allowed to separate into two layers. The lower phenol layer was drained off, mixed again with fresh treated water and allowed to separate again. This was repeated until no more water was taken up by the phenol. The reagents were mixed and stored in a 2 liter glass-stoppered reagent bottle at 4°C in the dark.

7.5 M ammonium acetate: 578 g ammonium acetate per liter. Added to autoclaved RNase-free water and allowed to dissolve. The solution was stored at -20°C in a 1-liter glass-stoppered reagent bottle.

Standard saline citrate (SSC) buffer: The stock solution of this buffer was made at a 20 fold concentration (20 x SSC) consisting of 3.0 M NaCl (175.32 g/l) and 0.3 M trisodium citrate (77.42 g/l). These reagents were dissolved in untreated distilled water and adjusted to pH 7.0 with KOH. Diethyl pyrocarbonate was added to a concentration of 0.2% and autoclaved for 30 min. This solution was stored at 4°C. Only autoclaved RNase-free water was used for the working dilution of 1 x SSC.

Sodium dodecylsulfate (SDS): 200 g SDS per liter (20% SDS wt/vol). The SDS was dissolved in autoclaved RNase-free water and stored at room temperature in a 1-liter screw-capped bottle.

95% ethanol: Stored at -20°C in a 1-liter screw-capped bottle.

HEPES buffer: 100 mM HEPES stock solution (4.76 g/200 ml). Dissolved in autoclaved RNase-free water and stored at 4°C in a 150-ml dilution bottle.

1 x SSC-1 mM HEPES buffer: 40 ml 20 x SSC, 8.0 ml 100 mM HEPES, 752 ml sterile RNase-free water. Stored at 4°C in 1-liter screw-capped bottle.

0.5 M EDTA: 190.1 g tetrasodium EDTA per liter of sterile RNase-free water. The pH was adjusted to 8.0 with KOH and stored at 4°C in a 1-liter screw-capped bottle.

RNA isolation procedure

16S rRNA was isolated by a variation of the procedure previously described (Kirby 1965; Johnson 1981). The cells were harvested by centrifugation in RNase-free (see buffers and reagents for RNA isolation) 250-ml polypropylene centrifuge bottles at 7250 x g for 20 min at approximately 4°C. The supernatant was poured off and the cell pellet resuspended in 15 ml of extraction buffer which had been pre-reduced by placement in a boiling water bath for 15 min followed by the addition of 2-mercaptoethanol. The cell suspension was added to a baked, 50-ml screw-capped test tube and placed on ice. Diethyl pyrocarbonate (25 µl) was added to the cell suspension immediately prior to lysing of the cell suspension. Diethyl pyrocarbonate serves to inactivate RNases.

The cell suspension was lysed by passage through a French pressure cell (Carver Laboratory Press, Model C) at approximately 12,000 psi into a 125-ml glass-stoppered Erlenmeyer flask containing: 10 ml of phenol-cresol/8-hydroxyquinoline, 7.0 ml of extraction buffer, 1.5 ml 20% of

SDS, 1.2 ml of 0.5 M EDTA and 25 µl of diethyl pyrocarbonate. The flask was constantly swirled as the lysate entered the flask to keep the contents thoroughly mixed. The flasks were then shaken for 20 min on a wrist action shaker to allow denaturation of the cellular proteins. The lysate was then placed in a RNase-free 50-ml polypropylene centrifuge tube and centrifuged at 17,400 x g for 10 min. During the centrifugation, the 125- ml flask was washed out under hot water and allowed to drain. It is important here that all flasks and centrifuge tubes are labeled to insure that cultures do not get mixed. The centrifuge tube was carefully removed from the rotor so as not to disturb the layers that have formed. A sterile, RNase-free 10-ml pipet was used to remove the upper aqueous phase which contains the nucleic acid. To do this without disturbing the lower phenol layer and the denatured protein which lies at the interface, the pipet was inverted, being careful not to touch the mouth end of the pipet even while wearing gloves. The upper aqueous phase was transferred back into the Erlenmeyer flask from which it was taken and 10 ml of phenol-cresol/8-hydroxyquinoline was added. The flask was again shaken for 20 min, centrifuged and the aqueous phase removed. This was repeated until no substantial protein layer was present. Normally, three phenol extractions were required for a RNA preparation to be free of protein.

The aqueous phase from the final phenol extraction was placed in a RNase-free 250-ml polypropylene centrifuge bottle. Two volumes of 95% ethanol (-20°C) was added, gently mixed and placed at -20°C for 60 min. The bottle was centrifuged at 7250 x g for 15 min, the ethanol carefully decanted and the bottle placed back in the centrifuge in the original

orientation and centrifuged again at 1380 x g for 5 min. Any remaining ethanol was removed with a sterile Pasteur pipet. At this point the pellet contains both RNA (primarily ribosomal and some transfer RNA) and DNA. The pellet was resuspended in 20 ml of 1X SSC-1 mM HEPES buffer and transferred to a RNase-free 50-ml polypropylene centrifuge tube. 10 ml of 7.5 M ammonium acetate was added, mixed well and placed at -20°C for 30 min. The ammonium acetate will selectively precipitate the large ribosomal RNA (23S and 16S) while leaving the small rRNA, tRNA and DNA in solution. Occasionally, the pellet may contain some 5S rRNA. This usually results only when a substantial yield of RNA has been recovered. Since the sequencing procedure is specific for 16S rRNA, the presence of these other rRNA species is of no consequence. The tube was centrifuged at 17,400 x g for 10 min, the supernatant decanted, and the pellet allowed to dry thoroughly. This pellet was resuspended in 10 ml 1X SSC-1 mM HEPES buffer and to this was added 20 ml of cold (-20°C) 95% ethanol. This solution was gently mixed and placed at -20°C for 60 min. The tube was centrifuged at 17,400 rpm for 15 min and the ethanol carefully decanted.

After the resulting pellet had dried (usually by being placed in a 37°C incubator for 10 min), it was dissolved in 5.0 ml of 1X SSC-1 mM HEPES buffer and transferred to a 10-ml RNase-free screw-capped test tube. Electrophoretic grade 20% SDS was added to a final concentration of 0.5%. Addition of the SDS at this point is critical for the stable storage of the RNA preparation. The SDS will protect the RNA from RNase contamination which may result upon the frequent handling the tubes and the withdrawing of samples.

Determination of concentration and purity of RNA preparations and detection of intact 16S ribosomal RNA

The concentration of RNA in the preparations was determined using a Bausch and Lomb Spectronic 2000 recording spectrophotometer. Concentrations were determined by making a 1:50 dilution of the RNA sample in 1X SSC-1 mM HEPES buffer in a 3.0-ml quartz cuvette and measuring the absorbance at 260 nm. The following equation was used to calculate RNA concentration:

mg RNA/ml = OD260 nm x dilution factor/ 23

The purity of the RNA sample was determined by scanning the RNA sample in the dilution buffer from 350 nm to 200 nm and looking for a peak at 260 nm. Any peak at 280 nm or any skewing of the peak at 260 nm towards the upper part of the scale was suggestive of protein contamination. However, the RNA isolation procedure used in this study consistently gave samples in which there was no detectable or minimal presence of protein.

It was necessary in this study to not only isolate rRNA from each species of Campylobacter, but also to isolate it intact (i.e. not degraded or fragmented during the isolation procedure). To determine whether or not the rRNA was intact, the sample was loaded into a 2% agarose gel and electrophoresed to reveal either distinct migrating bands of 23S and 16S rRNA indicating intact RNA, or smears of RNA indicating degradation. A 2% agarose gel was prepared by adding 0.5 g of agarose to 25 ml of TBE buffer (0.089 M Tris-base, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) containing ethidium bromide at a

concentration of 0.5 mg/liter. The solution was heated to dissolve the agarose and allowed to cool to approximately 50°C before pouring into a horizontal gel electrophoresis unit (Hoefer Scientific, Model HA 33). Approximately 2.0 µg of a sample RNA was mixed with a tracking dye (0.25% bromophenol blue and 25% glycerol) and 1X SSC-1 mM HEPES buffer to a final volume of 15 µl and loaded into the gel. The running buffer for the electrophoresis was the same as the buffer used in making the agarose gel. The samples were electrophoresed at a voltage of 135-145 V for approximately one hour or until the tracking dye was three quarters of the way through the gel. The rRNA within the gel was visualized using an ultraviolet light source.

Preparation of RNA for sequencing procedure

The SDS present in the RNA storage buffer had to be removed prior to the sequencing procedure. This was accomplished by the following procedure:

Approximately 50 µg of RNA was placed in a sterile 0.5 ml Eppendorf centrifuge tube and brought up to 100 µl total volume using RNase-free (see buffers and reagents for RNA isolation) TE buffer (10 mM Tris-base and 0.1 mM EDTA, pH 8.0). To this solution was added 50 µl of phenol-chloroform and the tube was gently vortexed and centrifuged at approx. 12,000 rpm using a microcentrifuge (Eppendorf Microfuge). The upper aqueous phase was carefully removed using a micropipettor and placed in another sterile Eppendorf tube. The phenol extraction was repeated and the aqueous phase again removed to a sterile Eppendorf tube. The solution was now free of SDS; however, it may now contain

some residual phenol. This phenol was removed by the addition of 50 µl of secondary butanol, vortexed and separated by centrifugation at 12,000 rpm. The upper secondary butanol layer was carefully removed using a micropipettor and discarded. The secondary butanol extractions were repeated usually two times which results in an aqueous volume of approx. 50 µl. In addition to removing the residual phenol, the secondary butanol facilitates concentration of the RNA since it also takes up water from the aqueous phase. Five microliters of 3.5 M sodium acetate was added and mixed; this resulted in a sufficiently high salt concentration to allow ethanol precipitation of the RNA. Cold (-20°C) 95% ethanol (110 µl) was added and the tube gently mixed and placed at -20°C for at least 1 hour. After precipitation, the tube was centrifuged at 12,000 rpm for 10 min and the ethanol carefully decanted. The tubes were placed back in the rotor and centrifuged again at 12,000 rpm for a few seconds and the remaining ethanol was removed using a micropipettor. After the pellet had dried thoroughly, the RNA was dissolved in 25 µl of TE buffer to give a final concentration of 2.0 mg/ml.

Buffers and reagents for the RNA sequencing procedure

The following reagents and buffers were used in the procedure used to determine the sequence of the rRNA:

1.0 M KCl: 14.9 g of KCL dissolved in 200 ml of sterile RNase-free water (see buffers and reagents for RNA isolation). This solution was autoclaved for 20 min and stored in screw-capped bottle at -20°C.

1.0 M Tris-HCl: 31.52 g of Tris-HCl in 200 ml of sterile RNase-free water, autoclaved for 20 min and stored in screw-capped bottle at -20°C.

1.0 M $MgCl_2$: 20.33 g of $MgCl_2(6H_2O)$ in 100 ml of sterile RNase-free water, autoclaved for 20 min and stored in screw-capped bottle at -20°C.

1.0 M dithiothrietol: 15.49 g of dithiothrietol in 100 ml of sterile RNase-free water. This solution was sterilized by passage through an Acrodisc filter (0.20 μm pore size; Gelman Sciences) and stored in a screw-capped bottle at -20°C.

5X hybridization buffer: 250 mM Tris-HCl and 500 mM KCl, pH 8.5. This solution was made by placing: 5.0 ml of 1 M KCl, 2.5 ml of 1 M Tris-HCl, pH 8.0 and 2.5 ml of sterile RNase-free water in a RNase-free 50-ml polypropylene centrifuge tube and adjusted to pH 8.5. This solution was then divided into 50 μl aliquots in sterile 0.5-ml Eppendorf tubes and stored at -20°C.

5X reverse transcriptase buffer: 250 mM Tris-HCl, 250 mM KCl, 50 mM $MgCl_2$, 50 mM dithiothrietol, pH 8.3. This solution was made by placing: 50 μl of 1 M dithiothrietol, 250 μl of 0.2 M $MgCl_2$, 250 μl of 1 M KCl, 250 μl of 1 M Tris-HCl, pH 8.3 and 200 μl of sterile RNase-free water in a sterile 1.5-ml Eppendorf tube. 100 μl aliquots were then placed into sterile 0.5-ml Eppendorf tubes and stored at -20°C.

Deoxynucleotide and dideoxynucleotide solutions: Each of the original stock solutions of deoxynucleotides was 18 mM and each of the dideoxynucleotide stock solutions was 10 mM. Aliquots of the these were taken and diluted to various working concentrations using sterile RNase-free water. Dilutions of these stock solutions were necessary since the final concentration of the solutions was in the micromolar range.

Deoxynucleotide mix: 10 µM dATP, 250 µM dCTP, dGTP, dTTP. This solution was made by mixing: 5.0 µl of 1.0 mM dATP, 6.94 µl each of 18 mM dCTP, dGTP and dTTP, and 474.2 µl of sterile RNase-free water in a sterile 1.5-ml Eppendorf tube.

Dideoxynucleotide mixes: Each solution was made in a separate sterile 0.5-ml Eppendorf tube in a final volume of 100 µl. The final concentrations for these solutions were: 1.25 µM ddATP, 5.0 µM ddCTP, 7.5 µM ddGTP and 10.0 µM ddTTP.

The working nucleotide mix was made by mixing 100 µl of deoxynucleotide mix with 100 µl of one of the four dideoxynucleotide mixes for a final volume of 200 µl and stored at -20°C.

4X chase mix: 4.0 mM dNTP's and 40 mM Tris-HCl, pH 8.3. This solution was made by mixing 22.2 µl of each stock solution of deoxynucleotide, 4.0 µl of 1 M Tris-HCl (pH 8.3) and 7.2 µl of sterile RNase-free water in a sterile 0.5-ml Eppendorf tube and stored at -20°C.

Stop mix: 86% formamide, 10 mM EDTA, 0.08% xylene cyanol and 0.08% bromophenol blue. This stock solution had a final volume of 30.0 ml and was stored in a 50-ml screw-capped test tube at 4°C. A 3.0 ml aliquot was placed in a 5-ml screw-capped test tube and stored at room temperature.

Buffers and reagents for the preparation of the sequencing gel

The following buffers and reagents were used in the preparation of the polyacrylamide sequencing gels. In this study all gels were 8% polyacrylamide/ 8 M urea buffer gradient gels.

10X TBE buffer: 108 g of Tris-base, 55 g of boric acid and 9.3 g of EDTA (free acid). These reagents are brought up to 1 liter using distilled water and stored in a 1-liter screw-capped bottle at room temperature.

40% acrylamide stock solution: 38 g of acrylamide and 2 g of bisacrylamide. These reagents were brought up to 100 ml. A mixed-bed resin was added and stirred. The solution was filtered to remove all particulate matter and stored in a 1-liter screw-capped bottle in the dark at 4°C.

Top sequencing gel: 93.6 g of urea, 29.3 ml of 40% acrylamide stock and 9.75 ml of 10 x TBE buffer. Boiling distilled water was added to bring the volume to 195 ml. The solution was stored in the dark at 4°C.

Bottom sequencing gel: 21.6 g of urea, 4.5 g of sucrose, 6.75 ml of 40% acrylamide stock solution, 11.25 ml of 10 x TBE buffer and 0.3 ml of bromophenol blue (0.01 g/ml). Boiling distilled water was added to bring the volume to 45 ml. The solution was stored in the dark at 4°C.

Preparation of the polyacrylamide sequencing gel

It is important when preparing a sequencing gel that the glass plates into which the liquid gel is poured are extremely clean. If any dust, fingerprints, etc. are present on the suface of the glass, bubbles will form between the plates. The larger of the two glass plates was cleaned by wiping down the surface which will contact the gel with 95% ethanol using Kimwipes. This was usually repeated three times. Next, the plate was cleaned with Windex. This provides a good surfactant on the glass surface which makes pouring of the gel proceed more smoothly.

The smaller of the two plates was also cleaned with ethanol three times. This plate was then siliconized on the surface which contacts the gel. Silicon on the surface enables this plate to be removed without tearing of the gel. Any laboratory glassware siliconizing agent will work. Prosil was used in this study by making a 1:100 dilution of the stock solution with distilled water. This solution was then wiped over the plate, making sure that the entire surface was covered. The excess solution was then wiped off and the plate allowed to dry. 0.4 mm spacers were placed on the first plate and the second siliconized plate placed on top of the first plate with the treated side facing down. Next, the two plates were taped together, making sure that the two lower corners were securely taped, since this was where leakage of the gel usually occurred.

The gel itself consists of two separate gels: a top gel and a bottom gel. The bottom gel has a higher buffer concentration than the top. This serves to retard the movement of small DNA fragments as they reach the lower regions of the gel, which results in an arithmetic rather than a logarithmic separation of the DNA fragments, allowing a larger number of bases to be visualized on a single gel. Sixty-five milliliters of the top gel solution was removed and placed in a 125-ml side-arm Erlenmeyer flask; to this was added 284 µl of 10% ammonium persulfate. The solution was then degassed by aspiration for about 1 min. Removal of oxygen is essential since polymerization is inhibited by oxygen. Fifteen milliliters of the bottom gel was placed in a 50-ml side-arm Erlenmeyer flask; to this was added 60 µl of 10% ammonium persulfate, and the solution was degassed. TEMED (32.5 µl) was added to

the top gel and 14 µl added to the bottom gel. This initiates the polymerization of the acrylamide and bisacrylamide; thus one must work quickly from this point. Using an automatic pipettor, 12-13 ml of the top gel was drawn into a 25-ml pipet followed by all 15 ml of the bottom gel. The two gels were slightly mixed in the pipet by drawing up an air bubble. This solution was then slowly displaced in between the glass plates. Once this gel had been fully added to the plates, the remaining space between the plates was filled with the top gel that was left over. Before the gel polymerized the comb which forms the well was inserted into the top of the gel and the plates clamped together. After approximately 45 min, the comb and the tape were removed and the plates clamped on the sequencing apparatus (IBI, Model STS 45). 1X TBE buffer was placed into the top and bottom wells of the sequencing apparatus and the "shark tooth" comb placed in the top of the gel.

RNA sequencing procedure

This sequencing procedure is based on a protocol previously described (Lane et al. 1985). Some modifications were made to enhance base ambiguities due primarily to the secondary structure encountered in the 16S rRNA molecule. It was found that the salt concentration in the reaction buffers was critical to success. Upon testing of various salt concentrations, those recommended by Lane et al. were found to be the most effective. Increasing the temperature of the elongation reaction from 37°C to 48°C increased the number of readable bases. This is thought to be due to denaturation of secondary structure allowing the reverse transcriptase to read through those regions. Allowing the

hybridization reactions to cool slowly and only to 60°C significantly decrease the background on the gels by presumably eliminating the nonspecific binding of the primers to other areas of the 16S rRNA molecule and possibly to the 23S rRNA molecule which is also present. It was also found that the Avian Myeloblastosis Virus reverse transcriptase was superior to the cloned Moloney Murine Leukemia Virus reverse transcriptase under the conditions used in this study.

All buffers, reagents, etc. were pre-treated to inactivate RNases (see buffers and reagents for RNA isolation). All pipet tips were bought already packaged and pre-sterilized. Eppendorf tubes were removed directly from their package and autoclaved for 30 min in baked beakers. Eppendorf tubes (0.5-ml) were used as reaction vessels throughout the procedure. All reagents and buffers were stored at -20°C unless otherwise stated and all reactants were kept on ice during the procedure.

Oligonucleotide primer was hybridized to the RNA template under the following conditions: 1.0 µl of RNA template (2.0 mg/ml), 2.0 µl of primer (0.1 mg/ml), 1.0 µl of 5X hybridization buffer and 1.0 µl of H_2O for a total volume of 5.0 µl. All tubes were incubated at 90°C for 2 min in a heating block filled with water. After 2 min, the entire heating block was removed and placed in a 60°C incubator for 15 min. The tubes were then placed in Eppendorf centrifuge racks and allowed to incubate an additional 15 min at 60°C. Upon completion of the hybridization, all reactions were immediately placed on ice. During this time, 2.0 µl of the appropriate nucleotide mixture (see buffers and reagents for the RNA sequencing procedure) was placed in one of the four

labeled tubes that were used per hybridization reaction. These tubes were then placed at 4°C until ready for use. To the hybridization tubes were added: 5.0 µl of 5X reverse transcriptase buffer, 2.0 µl of ^{35}S-dATP (10 µCi/µl), 2.0 µl of H_2O and 1.0 µl of AMV-RTase (9 U/µl) for a total volume of 15.0 µl. These reaction tubes were briefly centrifuged to mix the reactants and 3.0 µl of these mixtures was added to each of the four reaction tubes per hybridization which now contain the nucleotide mixture. The tubes were briefly centrifuged to mix the reactants and placed in a 48°C water bath for 20 min. During the incubation, the chase mix was prepared by diluting the 4X chase stock solution with sterile RNase-free water (see buffers and reagents for RNA isolation) to a volume which would accommodate 1.0 µl of chase per reaction (# of reactions = # of hybridizations x 4). Included in the chase volume was 9 units of reverse transcriptase. At the end of 20 min, each reaction then received 1.0 µl of chase, the reactions were centrifuged briefly and placed back in the same water bath for an additional 15 min. To stop the reactions, each tube received 6.0 µl of "stop mix". This mix includes formamide and EDTA which completely inhibits the activity of the reverse transcriptase. Additionally, the tracking dyes, xylene cyanol and bromophenol blue, are present in the "stop mix".

Immediately prior to loading 2.0 µl of each reaction on the sequencing gel, all tubes were heated at 90°C for 2 min. This is to denature the cDNA from the RNA template. The gel must also be pre-warmed to approximately 55 to 60°C at this step to prevent any renaturation of the DNA in the wells or in the gel (the gel also

contains 8 M urea which serves to prevent renaturation of the DNA). Normally the gels were warmed for about 30 min at 65-70 watts (approx. 2000 volts). The voltage needed to warm the gel to the proper temperature depended on the ambient temperature of the laboratory. If, for some reason, the gel had not been pre-warmed, reactions were kept on ice until the gel was at the proper temperature for loading.

Electrophoresis of the DNA ladders proceeded at 2100-2200 volts for approx. 2 hours. The electrophoresis was stopped when the bromophenol blue dye front reached the 45-cm mark. Next, the DNA fragments were "fixed" in the gel. This was done by removing the siliconized plate and placing the gel and remaining plate in a solution of 10% glacial acetic acid and 10% methanol in distilled water (total volume approx. 1500 ml) for 20 min. This solution also served to remove the urea from the gel which, if left in, would make the gel brittle upon drying. The solution was then carefully siphoned off and the gel removed from the glass plate by transfer to filter paper. The filter paper was then placed within a drying apparatus and allowed to dry for 25-30 min (Drying is necessary to allow the signal from the radionuclide to expose the x-ray film). The dried gel and filter paper was placed inside a cassette and allowed to expose 48 hours to 2 weeks depending on the level of incorporation of the ^{35}S-dATP as measured by a Geiger counter.

Comparison of RNA Sequences

All of the Campylobacter RNA sequences were manually aligned using a word processing computer program. This was done by initially aligning

the oligonucleotide hybridization sites since these primers bind to highly conserved regions. Once these regions were found the remaining sequences were aligned and gaps assigned where necessary. After successful alignment, all bases which were not represented in the shortest sequence (excluding internal gaps) were removed. This left approximately 750 bases of nucleotide sequence per organism. Next, base positions of unknown composition were deleted from all 19 sequences as were all ambiguous alignments. Homology values were then calculated by the following formula (McCarroll et al. 1983):

$$\frac{\#\text{ of bases common to each sequence}}{\text{total }\#\text{ of bases in both sequences} + (0.5)\text{total }\#\text{ of gaps in both sequences}}$$

Next, all positions which contained bases common to all 19 sequences were removed, leaving approximately 250 bases per organism. Homology values were again calculated according to the same formula.

The set of homology values which was calculated upon removal of common bases were put into the Numerical Taxonomy System (NTSYS) analysis program. This program calculated similarity coefficients based on the homology matrix, constructed a dendrogram based on these coefficients, and calculated how well the dendrogram fits the data in the matrix, expressed as a correlation coefficient.

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