The isolation and identification of a bacterium from the Oconaluftee River, Great Smoky Mountains National Park

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12 December 2006

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Introduction

The purpose of this study was to isolate a single bacterial species from the Great Smoky Mountains National Park (GSMNP), to characterize its growth requirements and to identify it using its 16S rDNA sequence. A broader goal of this investigation was to contribute to the All Taxa Biodiversity Inventory (ATBI) of GSMNP.

The ATBI was formally conceived in 1997 with the goal of cataloging every species that inhabits GSMNP, an area encompassing over 2,000 square kilometers in western North Carolina and eastern Tennessee. The park is thought by many to represent the most diverse area, in terms of number of species present, in North America. Aside from a major survey of selected taxa in a small part of Costa Rica, no survey attempted by biologists to date even comes close to matching the enormity of this project (Sharkey, 2001). The ATBI group maintains a web page titled *Discover Life in America* (http://www.dlia.org/). A recent summary indicated that more than 4,400 species previously unknown in GSMNP had been cataloged. Of this number, more than 600 were entirely unknown to biologists prior to the beginning of the inventory. The work continues and biologists estimate that the total number of species (excluding microbes) within the park's boundaries may exceed 100,000.

Three primary goals of the ATBI inventory have been established. The first two involve naming and cataloging each species as well as establishing biogeographical data (i.e. what and where). A further goal is to understand each organism's role in the complex ecosystem that is the Smoky Mountains (Sharkey, 2001). Even the most seasoned naturalist may become so caught up in learning about the park's birds, arthropods and flora that microbial diversity may be

forgotten or given only a passing thought. Yet, the numbers of microbes added to the inventory continues to grow.

The first secrets of the microbial world were revealed to the Dutchman Antoni van Leeuwenhoek in the 1670s. His writings (as quoted by Bauman, 2004 p. 3) preserved his enthusiasm and awe.

> …the motion of these animalcules in the water was so swift, and so various, upwards, downwards, and round about, that 'twas wonderful to see…the number of these animals in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom…

Since that time we have come to understand some of the many things that microbes do. It seems that there are microbes to fill every conceivable biological role. Some are autotrophs and therefore represent producers in complex food webs. Others are consumers or decomposers. Many are involved in keeping various biogeochemical cycles continuing through time (Ward, 2002). Microorganisms are part of complex symbiotic relationships with many higher organisms. They help synthesize vitamins, provide food and keep the organisms healthy (Bauman, 2004). Most of the time their work goes unnoticed. However, humans have harnessed the powers of microbes to treat sewage and other wastes (Ward, 2002). We have recognized and taken advantage of their metabolic activities to help us produce foods like cheese and soy sauce, useful chemicals such as acetone and various drugs, animal foods such as silage and countless other products that are a part of our daily lives (Bauman, 2004). Microbes cause a number of diseases of plants and animals as well (Ward, 2002). For humans the list is exhaustive and

includes leprosy, anthrax, aspergillosis, rubella, tetanus, malaria, legionellosis, giardiasis and cholera to name a few (Bauman, 2004).

Leeuwenhoek was ecstatic about his discovery of microbes. In time, however, other researchers would become even more astonished by the sheer numbers and kinds of these previously unseen organisms in the biosphere. Entire kingdoms of life were added when so many newly observed life forms did not obviously fit the traditional "plant or animal" mindset of earlier times. The diversity of bacteria, in particular, is still poorly understood. One thing that most researchers agree on is that the total number of bacteria in the world, at least in a practical sense, may be unknowable (Curtis, Sloan & Scannell, 2002; Schloss & Handelsman, 2004). Some estimates place the number within the magnitude of 10^7 to 10^9 individual species (Schloss & Handelsman, 2004). Some have suggested that we may increase the number of biological kingdoms to more than 50 as we learn more about bacteria (Bauman, 2004). Fewer than 10,000 individual species have been named to date (Overman & van Gemerden, 2000). As noted above, humans have already found countless practical applications in our miniscule knowledge of bacteria. Knowing more would increase our understanding of medicine, the environment, food production, chemistry and symbiosis. And of course there is value in simply knowing more for the sake of knowledge. Ward (2002) also suggested that we may better understand how our own activities may impact microbial communities.

As noted above, bacterial diversity is clearly vast. Since Leeuwenhoek's initial writings and diagrams first appeared to the public, microbiologists have set out to describe this diversity. Traditionally, most data pertaining to bacterial diversity were reasoned from various growth-based lab techniques. In other words, biologists would subject the organism of interest to various environmental conditions in the lab, then describe and classify them based on these

similarities and on morphology. For example, some bacteria are able to grow only in the absence of oxygen gas. Others are extremely salt tolerant. Some carry out complex fermentation reactions and some are able to utilize exotic growth conditions to their biological advantage (Bauman, 2004). While this sort of study yields valuable information about microbes, it has (according to some) little value in establishing phylogenetic relationships in such a vast group of life forms as bacteria (Olsen, et al., 1994; Ward, 2002).

From an historical perspective, many point to the work of Carl Woese in the 1970s as being a defining moment in the understanding of bacterial diversity. His efforts centered on molecular biology of the gene (Ward, 2002; Bauman, 2004). This use of genetics itself was not revolutionary; it dates at least as far back as the 1950s and 1960s (Olsen, et al., 1994; Bauman, 2004). Yet, Woese focused on the previously neglected ribosomal DNA (rDNA) sequences of microbes. Prokaryotic ribosomes differ from those of eukaryotes with regard to their sedimentation rates (measured in Svedberg units [S]). They are often called 70S ribosomes based on this difference. Further, these 70S ribosomes are composed of two subunits. One of these subunits contains a single 16S rRNA strand, ultimately coded for by the rDNA genes (Bauman, 2004). These genes are recognized as being both universal and highly conservative. In other words, the genes tend to vary little from one organism to another. Variations, when present, are significant in that they allow possible phylogenetic relationships to be deduced (Weisburg, et al., 1991; Ward, 2002).

It would seem, then, that knowing the various growth and metabolic requirements of bacteria will continue to be important in a practical sense. As noted above, these things can help us understand an organism's role in its environment or help us to manage a disease causing microbe. Yet, compared with the number of species, the fact remains that bacteria (as a whole)

tend to be incredibly nondescript in terms of their superficial morphologies and growth requirements (Olsen, et al., 1994; Bauman, 2004). It is for this reason that most modern microbiologists have turned to the molecular study of rDNA as being a more reliable indicator of relationships among these organisms (Weisburg, et al. 1991; Olsen, et al., 1994; Ward, 2002).

Methods and Materials

Collection and Isolation of Organism

A water sample was collected from the Oconaluftee River in the Great Smoky Mountains National Park in Swain County, North Carolina, near the Oconaluftee Indian Village (GPS coordinates: N35.30.71; W83.18.15) in late August, 2006, following a heavy rain. Using aseptic technique, a sterile 50 mL centrifuge tube was used to collect ca. 40 mL of running surface water at ca. 1 m from the stream bank. The culture tube was packed in ice and then placed in refrigeration until five days from start (DFS). At five DFS the water sample was subjected to standard dilution to extinction and spread plating using R2A medium. The plates were incubated at room temperature until 12 DFS (S. O'Connell, personal communication, September 5, 2006). At this time, a sample from a single colony was isolated by streak plating onto R2A medium. This colony was maintained and observed for isolation by means of further streak plating at 14, 19, 21 and 26 DFS.

The isolated colony was prepared for storage by way of inoculation onto a slant tube containing R2A medium at 28 DFS. This culture was stored at 4 °C and was used for all subsequent characterization of the isolate, except as noted below. Further, two R2A plates were prepared for long term storage of the organism. One was stored at -20 °C, and used for DNA extraction; the other was stored at -70 °C.

Characterization of Organism

All morphological and metabolic laboratory tests were replicated three times. Colonies were observed and characterized macroscopically. Samples of the organism were examined and measured microscopically by way of negative staining with nigrosin dye and by Gram staining with known positive and negative controls (Hudson & Sherwood, 1997; Bauman, 2004). Environmental parameters of the organism were explored by way of plating onto R2A medium. As needed, the basic medium was amended with NaCl to test for salt tolerance. To determine pH ranges, the medium was amended with sodium hydroxide or sulfuric acid. It should be noted that for the pH 3 plates, the growth medium was altered due to instability of the R2A in that condition. Ten percent tryptic soy broth agar, with gellan gum, was used in this case. Range of temperatures was tested by way of incubating cultures at various temperatures. Oxygen tolerance was checked by way of inoculation of the isolate into molten R2A medium with sodium thioglycollate and reazurin added (Hudson & Sherwood, 1997; Bauman, 2004). All cultures were incubated for one week. Data for neutral pH, 0% NaCl concentration and 20% O_2 were obtained during streak plating of the isolate described above (see Table 1 for data). Further, various metabolic characters of the isolate were tested by way of the Enterotube® II system (Holmes, 1989). The test for indole was not performed. Finally, a test for catalase (Cappuccino & Sherman, 2005) was performed on a subsequently prepared three day old streak plate of the isolate. Also of note was a principal components analysis (PCA), performed to compare the isolate with others collected on the same day in terms of morphology and growth parameters.

DNA Extraction and PCR

DNA was extracted from the isolate using the Ultra Clean Microbial DNA Isolation Kit (Mo Bio Inc., Solana Beach, CA). Partial gene fragments of the 16S rDNA operon were targeted

using PCR for sequencing and identification purposes. The total reaction consisted of 50 µL with the following ingredients: 0.05% IgePal (Sigma-Aldrich, St. Louis, MO), nuclease free water (Promega Corporation, Madison, WI), 1X PCR buffer, 1.5mM $MgCl_2$, 0.20mM dNTP's, and 2.5 U Taq DNA polymerase (Eppendorf Corporation, Westbury, NY), 0.25 µM each primer (bacterial –specific primers 341F and 097R) and 1 µL DNA template.

A touchdown PCR approach was employed, consisting of 5 min of initial denaturation at 94 °C, then 30 cycles of denaturation at 94 °C for 1 min, annealing for 1 min, and extension for 3 min at 72 °C. The annealing temperature in the first two rounds was 65 °C, followed by one round each at 1 °C lower than the previous round, and finally eighteen rounds at 55 °C. A final extension for 7 min at 72 °C was employed and then amplicons were stored at 4 °C until they could be sequenced.

Sequencing and Analysis of 16S rDNA operon

The Big Dye Terminator, Version 3.0 Cycle sequencing kit, as well as the 3130 automated Sequencer (Applied Biosystems, Inc., Foster City, CA) was used for sequencing of the gene. Analysis was provided by use of the Ribosomal Database Project II (RDP II) website (Maidak et al., 2001) and the Basic Local Alignment Search Tool (BLAST) website (Altschul et al., 1997). The top five sequence matches in each database were noted during the analysis.

Results

The isolated organism was characterized macroscopically as forming white, flat, opaque, shiny, circular, mucilaginous colonies, with entire margins; that averaged greater than 7 mm in diameter. Microscopically, organisms from fresh (less than one week old) colonies were characterized as Gram positive rods (see Discussion for clarifications), ca. 2 micrometers wide and 5-7 micrometers in length. Organisms from a one week old, and a 20 day old, steak plate

demonstrated significant changes in their microscopic morphology. Few rod-shaped cells were present on these plates. A significant number of irregularly shaped masses and coiling filaments were found. Most exceeded 7 micrometers in length. They displayed neither overt branching patterns nor obvious reproductive spores or spore-bearing structures.

Environmental parameters (Table 1) were unremarkable except for the organism's ability to tolerate anaerobic conditions and its wide pH tolerance. Profuse growth was observed at all ranges tested from pH 3 to 9. Based on the Enterotube ® II test, the isolate was found to ferment glucose to acid and gas. Further, tests for fermentation of lactose, arabinose and sorbitol were positive; as was the test for hydrolization of urea. All other tests performed via the Enterotube ® II were negative. A delayed but robust reaction to a 3% solution of hydrogen peroxide was noted during the catalase tests.

From the rDNA sequence, several matches were found. Both RDPII and BLAST reported *Rhizobium sp.* matches with a greater than 98% similarity. In both cases, the top matches were associated with plant centered habitats. A detailed list of the top five matches for both databases may be found in Table 2.

Figure 1 displays a PCA analysis plot for all organisms isolated during this study. One overt variance among the data points may be observed along the first coordinate (Factor 1). This component represents reaction to Gram Stain. The author's isolate (identified as "Eddie") is situated within a cluster of Gram negative isolates within this aspect of the plot. With regard to other trends, the author's isolate is positioned within a cluster of organisms including Tom, Ben, Joe, Misty and Britt. They all share two negative test results in common: presence of ornithine decarboxylase and lysine decarboxylase. Four of these isolates: Tom, Eddie, Ben and Joe form a smaller cluster that all had negative test results for the enzyme phenylalanine deaminase. The

isolates Ben and Eddie further shared a positive result for lactose fermentation which may account for their being grouped in a smaller sub cluster. In summary then, the isolate identified as "Eddie" clusters with a group of organisms that are unable to metabolize certain amino acids (Holmes, 1989). Comments concerning the phylum for the isolate, *Proteobacteria*, are included in the Appendix.

Discussion

As previously noted, the isolate identified as "Eddie" had significant rDNA sequence matches with organisms of the genus *Rhizobium* (Table 2). One of the top matches was recovered from the leguminous (Wofford, 1989) tree *Robinia pseudoacacia*. Members of the genus *Rhizobium* are very commonly associated with such organisms in an endosymbiotic relationship involving nitrogen fixation (Madigan, Martinko & Parker, 2003; Bauman, 2004). The major incongruity between this match lies in the Gram stain reaction obtained in lab. It is an almost sure bet that the Gram tests were faulty since the rDNA sequence matched in excess of at least 98% with a known Gram negative genus. With regard to microscopic morphology, the concept of pleomorphy is clearly in accord with characteristics of the genus. Pleomorphic organisms have a variable cell shape (Bauman, 2004). Recall that the isolate displayed rods in younger colonies and a highly variable shape in older colonies. The cells in the younger colonies were longer by about 2 μm than expected (Holt, et al., 1994). As the already nutrient poor culture aged, one would expect to observe pleomorphy within species in the genus *Rhizobium*. Further, from a macroscopic standpoint, colony morphology was congruent with expected characteristics (Holt, et al., 1994; Ulrich & Zaspel, 2000) with the sole exception of elevation. Convex colonies are consistent with the genus (Holt, et al., 1994) but flat colonies were observed. Gas fermentation was noted but not expected. Further, published descriptions of

Rhizobium indicate that amino acids may be widely utilized by organisms in the genus. Yet, tests performed for particular amino acid specific enzymes (see Results) were negative. Growth at extreme pH levels (Table 1) was noted. Optimal pH for the genus ranges between 6 and 7. Finally the organism grew under conditions of low oxygen in the lab and showed growth in aerobic conditions, both of which are expected (Holt, et al., 1994).

The isolation of the organism from this study has some potential benefit to the ATBI being conducted in GSMNP. The class *Alphaproteobacteria*, of which the isolate is a member (Brenner, Krieg & Staley, 2005a) has been sparsely represented in the inventory of microbes since its onset. About two years ago, an isolate with an rDNA sequence matching *R. tropici* with nearly 100% similarity was recovered. Other genera in the class that have been found include *Agrobacterium* and *Sphingomonas* (Curtis, et al., 2005). It is unlikely that the current isolate in question represents a species new to science. It could, however, represent a species or variety new to the GSMNP inventory. Since the genus is usually found in endosymbiotic associations with plant roots (Bauman, 2004) one may expect that it is not easily recovered by the type of sampling described in the methods section, above. Recovery of *Rhizobium* from water samples would seem particularly unusual but certainly not improbable, especially considering the heavy rains that had fallen near the time of the organism's collection. Therefore, any documentation of the genus in GSMNP could only represent a plus toward the inventory effort. Even if no species new to GSMNP has been isolated by the author, this study provides confirmation of the work of previous researchers and could, possibly, provide some indirect indication of richness of the genus within the park boundaries.

Madigan, et al. (2003) noted that different species and varieties of *Rhizobium* tend to form cross inoculations most efficiently with different species of leguminous plants. These

findings have already led to studies involving cultivars of some species of the genus for commercial purposes (Bauman, 2004; Madigan, et al., 2003). In other words, if research demonstrates that a particular species or variety of *Rhizobium* tends to do especially well in establishing endosymbiotic associations with a particular crop plant, there may be a commercial application (Bauman, 2004). From the standpoint of the science of microbiology as a whole, the isolate recovered in this study could have some similar future use.

In reflecting about the soundness of the current study, several things are of note. First, as previously mentioned, the diversity of the GSMNP is poorly documented, especially in terms of its microbial life forms. Any effort to understand this complex habitat is worthwhile. It may be beneficial to future researchers in the park to specifically target collection efforts toward bacteria associated with leguminous plants. For example, root nodules of species known to form endosymbiotic associations with proteobacteria could be harvested and macerated. Fluids from these preparations could then be plated for isolation of colony forming units. This may increase the amount of diversity that has been documented for root endosymbionts in the study to date.

One of the most problematic outcomes of the current study is, of course, the Gram stain reaction for the isolate. Three replications of the Gram stain procedure (Hudson & Sherwood, 1997) were completed on the isolate and compared against known Gram positive and Gram negative controls. All three replications produced results that were opposite of the result expected. The Gram stain is one of the most basic and useful tests for identification of microbes. Some species display a variable reaction to the stain but all known *Rhizobium* are Gram negative (Madigan, 2003; Bauman, 2004; Brenner et al., 2005a). To reduce the effect of operator error, it may have been useful to have other members of the research team perform the Gram stain procedure on one another's' isolates. To assess any potential problems with reagents used in the

Gram procedure, it may have been helpful to repeat the test with chemicals from multiple commercial sources.

Another area of note for improvement involves documenting the microscopic morphology of the isolate. As previously noted, the observations made generally fell within expected ranges reported in published literature. Yet, since *Rhizobium* routinely is pleomorphic, it may have been beneficial to complete negative staining procedures over the course of time to more clearly document any changes in cell shape. Subsequent observations of the isolate that revealed changes in morphology were initiated purely on a whim but did lead to important findings about pleomorphy in the isolate.

Rhizobium species are known to be motile. They achieve this motility by means of peritrichous, polar- or sub polar flagella. Fimbriae have also been observed (Holt, et al., 1994). No flagella were seen at 1000X with light microscopy. Madigan, et al. (2003) noted that flagella are highly useful in classification of bacteria. Flagellar patterns are particularly useful in distinguishing among some species of *Rhizobium* (Holt, et al., 1994). One flagellum may be as thin as 20 nm and, therefore, rarely visible under light microscopy. Use of Leifson flagellar stain is recommended as one easy method to observe these structures (Madigan, et al., 2003). It may be beneficial in the future to include flagellar staining in the standard morphological studies of the isolates described in the methods section. Electron microscopy tends to reveal flagella at higher magnifications as well (Madigan, et al., 2003).

Culturing the isolate on a highly enriched agar may help to provide even more data on morphology. For example, some species of *Rhizobium* require water soluble vitamins, particularly biotin, in order to grow. Two other B vitamins, pantothenate and thiamine, can help to distinguish among *R. galegage, R. meliloti*, *R. loti and R. leguminosarum* when added to the

culture medium. Also, *R. galegage* displays a susceptibility to the bacteriophage identified as "phage gal 1/R" whereas the other previously mentioned species do not (Holt, et al., 1994).

Whether new to science or not, the isolate obtained from GSMNP and described in this study represents one of perhaps hundreds of millions of bacterial species in the world. It is clear that microbiologists have much work to do even if they establish the goal of knowing just a fraction of this number. There is no doubt that the ATBI has already contributed much to our understanding of microbes on a local, and even a global, scale. As the work continues, new species will doubtless become known to biologists.

Appendix: Description of Phylum

The bulk of evidence concerning the morphology and growth habits of the isolate collected from GSMNP suggests that it would be placed in the phylum or division known as *Proteobacteria*. This group represents the most physiologically diverse and numerically largest of all known bacteria (Gupta, 2000). A brief consideration of the etymology of the phylum name can provide some interesting details of the group's characteristics. The phylum name was derived from two Greek terms: *proteus* and *baktron.* Proteus was an early Greek god of the sea that was thought to be able to assume a wide variety of shapes. Not only does this prefix often denote "first," it may also be used to mean versatility. The Greek root *baktron*, meaning rod, has been widely used to refer to any number of prokaryotic organisms, regardless of their cell shape. Members of the phylum *Proteobacteria* may display a number of shapes including rods, helices, filaments and spheres (Brenner, Krieg & Staley, 2005b). Many species may change their shapes due to differing growth conditions (Holt, et al., 1994). Therefore, the term proteobacteria may not be an altogether descriptive moniker for the morphology of the various members of the

division. However, *Bergey's Manual of Systematic Bacteriology, 2nd edition* uses *Proteobacteria* as the name of this diverse phylum (Brenner, et al., 2005a; 2005b).

The various growth and metabolic habits of the members of phylum *Proteobacteria* were arranged into 12 phenotypes (Boone & Castenholz, 2001) that help to emphasize how truly diverse the various species making up the phylum are. On thing that all species share in common is a negative reaction to Gram stain (Madigan, et al., 2003; Bauman, 2004.). Apart from that, the twelve phenotypes are summarized below (Boone & Castenholz, 2001).

- Sheathed bacteria that often display filamentous growth. Some have flagella; others are nonmotile.
- Nonmotile, or rarely motile, curved cells that act as saprobes.
- Symbiotic species, including intracellular parasites, of vertebrates and invertebrates.
- Phototrophic species containing bacteriochlorophyll.
- Chemolithic but nonphototrophic species that include nitrifiers, sulfur oxidizers and hydrogen oxidizers.
- Non-fruiting (non reproductive spore forming) cells that move by gliding.
- Cells that produce fruiting bodies such as sporangia and move by gliding.
- Organisms displaying budding and/or appendaged growth.
- Aerobic and microaerophilic rods and cocci.
- Aerobic and microaerophilic helices and vibroids.
- Facultatively anaerobic rods.
- Anaerobic cells with straight, curved or helical shapes.

There are further items of note with regard to the taxonomy of the phylum *Proteobacteria*. The name was proposed at the class level in the late 1980s but was elevated to the rank of phylum by 2001. There has been considerable revision and expansion of the phylum within the last five years (Brenner, et al., 2005b). One of the most significant shifts has been to establish five classes within the division, each named for a letter of the Greek alphabet (Bauman, 2004). A short summary of these classes is shown below.

Alphaproteobacteria

Seven orders make up this class (Brenner et al., 2005a). A variety of shapes are represented including rods, spheres, spirals and others. Pleomorphy is common and there is a wide range of nutritional habits in the group (Bauman, 2004). Genera such as *Nitrobacter*, *Rhizobium*, and *Azospirillum* are important in the nitrogen cycle. The latter two are nitrogen fixers that can utilize nitrogen gas and convert it to nitrate and/or ammonia (Madigan, et al., 2003). *Nitrobacter* has the ability to oxidize ammonia to nitrate. *Acetobacter* species have been used in industry due to their ability to make acetic acid (Bauman, 2004). Some pathogens of humans, plants and other animals also occur in the group. Crown gall tumors are initiated by *Agrobacterium tumefaciens* (Madigan, et al., 2003). Intracellular parasites from the genera *Ehrlichia* and *Rickettsia* are responsible for a number of often deadly infections in humans (Bauman, 2004). *Brucella suis* is significant in that it may lead to spontaneous abortions in swine. *B. melitensis* may have the same effect in goats, sheep and cattle (Tsolis, 2002).

Betaproteobacteria

This class is also divided into seven orders (Brenner, et al., 2005a). Morphology includes cocci, spirals and other forms. Typically, species in this class thrive in habitats with low nutrient availability. Genera in this class have additional involvement in the nitrogen cycle.

Nitrosomonas, for example oxidizes nitrite to nitrate (Bauman, 2004). Until very recent times it has been widely assumed that only certain *Alphaproteobacteria* could form root nodules in legumes (Chen, et al., 2003). At least two genera of *Betaproteobacteria*, *Burkholderia* and *Ralstonia*, have been shown to have species or varieties capable of doing so (Moulin, et al, 2001; Chen, et al., 2003). Human diseases are also caused by species in this class. *Neisseria gonorrhoeae* causes gonorrhea and other infections while *Spirillum minus* (vectored by rats) may cause an acute illness in humans (Bauman, 2004). *Zoogloea ramigera* has found commercial use in the waste treatment industry (Madigan, et al., 2003). A few species are known to be endosymbioants of certain hemipterans of class insecta (Thao, Gullan & Baumann, 2002).

Gammaproteobacteria

The *Gammaproteobacteria* are divided into 14 orders (Brenner et al., 2005a). Practically every known common bacterial shape is represented in this class. Once again, the nitrogen cycle has important representation in this group. Species of *Azomonas* and *Azotobacter* are not known to associate with plant roots, but are nitrogen fixers (see above) that inhabit soil (Bauman, 2004). An unusual group, often known as purple sulfur bacteria, is found within this class as well. They carry out photosynthesis but do not liberate oxygen gas as a byproduct since they utilize hydrogen sulfide as an electron source. Some forms are extreme halophiles. Examples of purple sulfur bacteria include *Ectothiorhodospira* (Madigan, et al., 2003). In terms of human pathogens, *Pseudomonas* may cause urinary tract and ear infections while *Legionella pneumophilia* could cause an often deadly lung infection known as Legionnaires' disease (Bauman, 2004). Some species of *Gammaproteobacteria* actually live in a meta-endosymbiotic relationship with endosymbionts of some insects (Thao, et al., 2002).

Deltaproteobacteria

Eight orders comprise this class (Brenner, et al., 2005a). At least three genera in this group, *Desulfovibrio* among them, are important to the sulfur cycle. This genus reduces sulfate to hydrogen sulfide (Madigan, et al., 2003). *Bdellovibrio* invades and kills other species of Gram negative bacteria during its life cycle for purposes of reproduction (Bauman, 2004). Three species are known (Madigan, et al., 2003). Several other genera, often collectively called the myxobacteria, have a life cycle that resembles that of slime molds. During poor environmental conditions, individual bacterial cells may glide together to form aggregations. Dormant vegetative spores may form on sporangia at this stage. During favorable conditions these spores may become active (Boone & Castenholz, 2001; Bauman, 2004).

Epsilonproteobacteria

The final recognized class consists of Proteobacteria contains only one order, *Camphylobacterales* (Brenner, et al., 2005a). Shapes include spirals, vibrios and rods (Bauman, 2004). Two of the eight genera in the group are widely known as human pathogens. *Helicobacter pylori* was identified in the early 1980s from human intestinal tissue samples. Since that time, *H. pylori* has been strongly implicated in development of gastric ulcers and other gastrointestinal conditions (Madigan, et al., 2003). *Campylobacter* species commonly infect a wide variety of domesticated animals (Bauman, 2004). *C. fetus* often causes spontaneous abortion in farm animals (Madigan, et al., 2003). *C. jejuni* is part of the native intestinal flora of poultry. Humans who acquire the microbe usually have acute symptoms that include pain and diarrhea. The disease may be particularly troublesome for infants (Bauman, 2004; Madigan, et al., 2003).

Summary of Appendix

Within just the last five years, the phylum *Proteobacteria* has undergone considerable expansion and revision. In 2001, 1300 validly named species were reported among 348 genera (Boone & Castenholz, 2001). By 2003, this number had increased significantly to 2279 species within 521 genera. While all classes have undergone change, with orders being shifted among them and new genera being added, the *Deltaproteobacteria* in particular has been extensively modified (Brenner, et al., 2005a). A great deal of revision in the phylum was due to molecular studies of proteins that identified important signature sequences at the species level or at higher taxonomic levels. This work has strongly suggested that the *Proteobacteria* share common ancestry with all other phyla of eubacteria (Gupta, 2000). A further change in thinking is more directly related to the phylum *Proteobacteria* itself. In the past, *Proteobacteria* have been thought of as representing a monophyletic taxon or clade (Brenner, et al., 2005a). In other words, all members of the phylum were believed to be descended from a common ancestor contained within the phylum. The previously described work initiated by Woese in the 1970s has called this assumption into question. The current view is that five separate lines of descent are represented in the phylum. This assertion is based largely on PCA plotting of various members of the phylum involving their 16S rRNA sequences (Brenner, et al., 2005a).

In summary, the *Proteobacteria* represent an extremely varied and complex assemblage of organisms. It is very likely that this phylum will continue to undergo taxonomic revision as new information comes to the attention of microbiologists. As with bacterial taxonomy in general, rRNA analysis methods will probably have a key role in contributing to understanding the mind-boggling diversity of this phylum. Our understanding of the world changes with changing perspective. In the 1600s, Leeuwenhoek had his own understanding of life turned upside down as he peered through his microscope. That dissonance spread throughout the

scientific community. The work of Carl Woese has provided modern biologists with a no less thrilling new perspective with which to try to understand our world.

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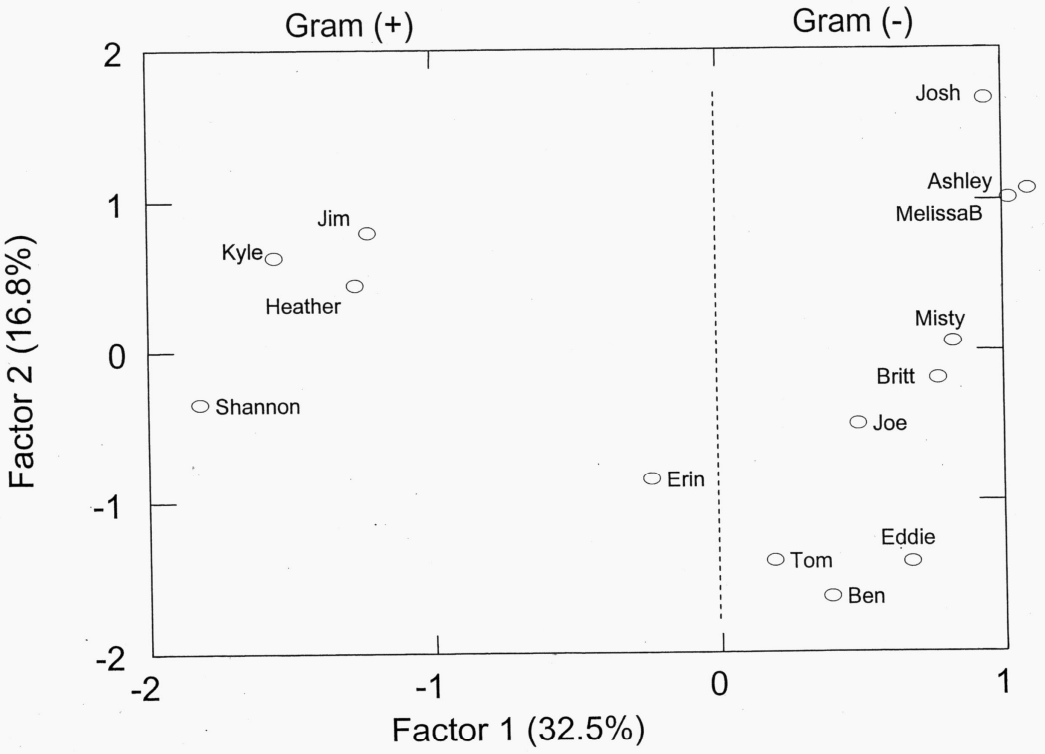

Figure 1. Principal components analysis (PCA) plot for Enterotube II, cell morphology, Gram staining, and growth parameters for 13 isolates obtained from soils and waters from Oconaluftee in Great Smoky Mountains National Park.

Table 1. Growth parameters for a bacterial isolate obtained from the Oconaluftee River, Great Smoky Mountains National Park, North Carolina, including ranges that permitted growth on R2A adjusted to various levels of NaCl, pH, and incubated at varying temperatures as well as requirement for O_2.

Temperature				pH				NaCl				Oxygen	
4°C	25°C	37°C	45°C	3	5	7	9	0%	5%	10%	15%	20%	0%
0/3	3/3	0/3	0/3	3/3	3/3	3/3	3/3	3/3	0/3	0/3	0/3	3/3	3/3

Table 2. DNA sequence for the bacterial isolate obtained from the Oconaluftee River, Great Smoky Mountains National Park, North Carolina, using the Ribosomal Database Project (RDP II) and Basic Local Alignment Search Tool (BLAST) software programs including environments in which the most closely related organisms were found (percentages indicate the extent of match of the GSMNP isolate to each isolate or clone represented in the RDP II GenBank database).

	Sequence Matches	
	RDP II (% Similarity)	Environment Detected (geographic location)
1.	*Rhizobium sp.* (99.2)	*Robinia pseudoacacia* root nodules (Germany)
2.	*Rhizobium sp.* (99.2)	root mats of cucumber & tomato crops (?)
3.	*Rhizobium sp.* (99.2)	tomato leaf (Japan)
4.	uncultured bacterium (99.2)	flooded *Populus* microcosm (Germany)
5.	uncultured bacterium (99.2)	unspecified (reported in journal published in Korea)
	BLAST	
1.	*Rhizobium sp.* (98.7)	root mats of cucumber & tomato crops (?)
2.	*Rhizobium sp.* (98.7)	tomato leaf (Japan)
3.	uncultured bacterium (98.7)	flooded *Populus* microcosm (Germany)
4.	*Rhizobium sp.* (98.7)	*Robinia pseudoacacia* root nodules (Germany)
5.	uncultured bacterium clone (97.9)	aerosol from Class B biosolids applied to field (?, Researchers from CT)