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FINAL REPORT

PCR BASED MICROBIAL MONITOR FOR ANALYSIS OF RECYCLED WATER ABOARD THE ISSA: Issues and Prospects

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Summary

The monitoring of spacecraft life support systems for the presence of health threatening microorganisms is paramount for crew well being and successful completion of missions. Development of technology to monitor spacecraft recycled water based on detection and identification of the genetic material of contaminating microorganisms and viruses would be a substantial improvement over current NASA plans to monitor recycled water samples that call for the use of conventional microbiology techniques which are slow, insensitive, and labor intensive.

The union of the molecular biology techniques of DNA probe hybridization and polymerase chain reaction (PCR) offers a powerful method for the detection, identification, and quantification of microorganisms and viruses. This technology is theoretically capable of assaying samples in as little as two hours with specificity and sensitivity unmatched by any other method. A major advance in probe-hybridization/PCR has come about in a technology called TaqMan™, which was invented by Perkin Elmer. Instrumentation using TaqMan concepts is evolving towards devices that could meet NASA's needs of size, low power use, and simplicity of operation. The chemistry and molecular biology needed to utilize these probe-hybridization/PCR instruments must evolve in parallel with the hardware. The following issues of chemistry and biology must be addressed in developing a monitor:

- Early in the development of a PCR-based microbial monitor it will be necessary to decide how many and which organisms does the system need the capacity to detect. We propose a set of 17 different tests that would detect groups of bacteria and fungus, as well as specific eukaryotic parasites and viruses.
- In order to use the great sensitivity of PCR it will be necessary to concentrate water samples using filtration. If a lower limit of detection of 1 microorganism per 100 ml is required then the microbes in a 100 ml sample must be concentrated into a volume that can be added to a PCR assay.
- There are not likely to be contaminants in ISSA recycled water that would inhibit PCR resulting in false-negative results.

- The TaqMan PCR product detection system is the most promising method for developing a rapid, highly automated gene-based microbial monitoring system. The method is inherently quantitative. NASA and other government agencies have invested in other technologies that, although potentially could lead to revolutionary advances, are not likely to mature in the next 5 years into working systems.
- PCR-based methods cannot distinguish between DNA or RNA of a viable microorganism and that of a non-viable organism. This may or may not be an important issue with reclaimed water on the ISSA. The recycling system probably damages the capacity of the genetic material of any bacteria or viruses killed during processing to serve as a template in a PCR designed to amplify a large segment of DNA (>650 base pairs). If necessary vital dye staining could be used in addition to PCR, to enumerate the viable cells in a water sample.
- The quality control methods have been developed to insure that PCRs are working properly, and that reactions are not contaminated with PCR carryover products which could lead to the generation of false-positive results.
- The sequences of the small rRNA subunit gene for a large number of microorganisms are known, and they constitute the best database for rational development of the oligonucleotide reagents that give PCR its great specificity. From those gene sequences, sets of oligonucleotide primers for PCR and TaqMan detection that could be used in a NASA microbial monitor were constructed using computer based methods.

In addition to space utilization, a microbial monitor will have tremendous terrestrial applications. Analysis of patient samples for microbial pathogens, testing industrial effluent for biofouling bacteria, and detection of biological warfare agents on the battlefield are but a few of the diverse potential uses for this technology. Once fully developed, gene-based microbial monitors will become the fundamental tool in every lab that tests for microbial contaminants, and serve as a powerful weapon in mankind's war with the germ world.

Introduction

Safe water to drink and air to breathe are essential for human life. A critical aspect of air and water safety is the absence of pathogenic microorganisms; however the closed nature of spacecraft environments makes control of microbial contaminants all the more critical and difficult. That need is compounded by the attenuation of human immune system function due to long term exposure to microgravity.[1] To achieve control of microorganisms in spacecraft, NASA must develop environmental sensors capable of monitoring the microbial content of recycled air and water. Traditionally, analysis of environmental samples for microbial pathogens relied on culturing the organisms on suitable growth media or propagation of viruses in tissue culture cells. Such methods are costly, slow in that some species of bacteria may take as long as 2 weeks to culture, and in many cases ineffective. Perhaps 99% of all organisms in environmental samples may not be culturable.[2] Although the current plan for monitoring microbial contamination on ISSA will utilize culture methods, new technologies for microbial detection are under development that could let astronauts know in 2 hours instead of 1-14 days if there were dangerous pathogens in their air or water. The most promising of these technologies is based upon a technique called PCR, for polymerase chain reaction.

PCR is a powerful technique invented by Nobel laureate Kerry Mullis that allows enzymatic amplification of DNA segments *in vitro* through a succession of incubation steps at different temperatures.[3, 4, 5] Typically, the double-stranded DNA is heat-denatured, two oligonucleotide primers (the PCR primers) that are complementary to the 3' boundaries of the target DNA segment are annealed at low temperature, and then enzymatically extended by Taq DNA polymerase at an intermediate temperature. One set of these three steps is referred to as a cycle, and the instrument that repeatedly changes the temperature of a PCR sample is called a thermocycler. The PCR process is based on repetition of this cycle and amplify DNA segments, called amplicons, by 10^5 to 10^9 fold.

The technique is relatively new; however it is being used increasingly as a method of diagnosing and precisely identifying microbial contamination in environmental, clinical, and industrial samples. As with any new scientific technique, it is continually being refined and improved. This report is an evaluation of the state of PCR science as it applies to the needs of NASA to develop a microbiology monitor for use aboard spacecraft. We have evaluated the scientific literature; talked with scientists in academia, government, and industry; and using DNA informatics methods, designed a set of oligonucleotides that could be used to detect potential pathogens in recycled water.

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Section 1.
What Pathogenic Microorganisms Must a Spacecraft Microbial Water Quality Monitoring System Be Capable of Detecting?

NASA has spent ≈8 million dollars in the development and construction of a system to convert all of the waste water on the ISSA into potable water. In tests of NASA's water reclamation system at the Marshall Space Flight Center (MSFC) in Huntsville, Alabama, *Staphlococcus sp., and Pseudomonas picketti* were among the bacterial taxons identified from clean water ports.[1] Additionally, in a small scale PCR based analysis project DNAs *from Legionella sp., Salmonella sp.* and pathogenic *Escherichia coli* were amplified from clean water ports.[2] On the Russian space station Mir, cosmonauts had a high incidence of skin and gastro-intestinal infections. Clearly, current technology is incapable of completely controlling the occurrence of potential pathogens in space environments.

Currently, NASA plans to monitor ISSA air and potable water for microorganisms as described in the Table 1-1. Bacterial and fungal assays will be performed in flight by passing air or water through membrane filters and culturing filtered organisms on R2A and other media. Specific analysis for viruses and the listed air based organisms will be done on Earth.

Table 1-1. In flight microbiological limits for ISSA air and water.

Air Quality Requirements		Water Quality Requirements	
Total Bacteria	≤ 500 CFU/m³	Total Bacteria & Fungi	≤ 100 CFU/100 ml
Total Fungi	≤ 100 CFU/m³	Total Coliform Bacteria	0 CFU/100 ml
Branhamella catarrhalis	0 CFU/m³	Total Viruses	0 PFU/100 ml
Neisseria meningitidis	0 CFU/m³		
Salmonella spp.	0 CFU/m³		
Shigella spp.	0 CFU/m³		
Streptococcus pyogenes	0 CFU/m³		
Aspergillus fumigatus	0 CFU/m³		
Cryptococcus neoformans	0 CFU/m³		

Although the ISSA water quality requirements are tractable for culture based analysis, if it were technically feasible, spacecraft water should be tested for a more comprehensive list of potential pathogens. One of the important capabilities of PCR based methods for microbial analysis is the ability to identify defined targets. That specificity can theoretically be tailored to any taxonomic level, from species to kingdom. PCR conditions can be designed to specifically amplify almost any unique genetic element. Our consultants, Dr. James Barbaree and Dr. Joseph Gauthier, both experts in the area of water quality, constructed lists of potentially significant pathogens for which a comprehensive water quality monitor should test. Dr. Barbaree's list is comprehensive in its inclusion of all microorganisms that might be hazards in reclaimed water (Appendix B). Dr. Gauthier's list was much shorter and more directed towards the organisms likely to be encountered; however even it contained some organisms that probably would not be a risk in spacecraft water (Appendix A).

After evaluating the aforementioned two lists, several generations of tests on the ISSA water reclamation system at the MSFC, and consulting guidelines from the American Public Health Association[3], and U.S. Environmental Protection Agency[4], we compiled a consensus list of infectious agents and groups of agents that could be potential hazards in ISSA recycled water (Table 1-2). The most important microbial taxons are placed at the top of the list, i.e. all bacteria and fungi, *Legionella sp.*, enteric bacteria, and Gram positive bacteria. *Thiobacillus sp.* and *Pseudomonas sp.* (also an opportunistic pathogen) were included on the list because they are associated with fouling (biofilm production) in wastewater treatment processes, and thus indirectly could pose a health problem in spacecraft by damaging water processing systems.

Because long-term habitation of a microgravity environment results in diminution of immune system function, it is inevitable that most infections occurring in space will result from normal human flora being exchanged between crew members and from opportunistic environmental pathogens. It will be impossible to keep normal human

Table 1-2. Composite list of infectious agents that are potential hazards in ISSA recycled water for which a PCR based monitor should analyze.

Microorganism or Virus
1. Any Bacteria
2. Any Fungi
3. *Legionella sp.*
4. Enteric Bacteria
5. Gram Positive Bacteria
6. *Pseudomonas aeruginosa*
7. *Pseudomonas sp.*
8. *Mycoplasma sp.*
9. *Acinetobacter sp.*
10. *Listeria sp.*
11. *Thiobacillus sp.*
12. *Cryptosporidium*
13. *Candida albicans*
14. *Cryptococcus sp.*
15. Norwalk Virus
16. Hepatitis A Virus
17. Rotavirus

flora or ubiquitous microorganisms out of the ISSA. Infections, which may result in disease will probably come from contact with organisms not normally considered pathogens in a healthy adult population such as astronauts. There is a risk of making our list of probable pathogens too exclusive, accordingly we have included assays for microbes unlikely to cause problems such as *Mycoplasma* and *Acinetobacter.* Although spacecraft crews will undergo rigorous medical screening before launch to prevent potential carriers of microbial pathogens from infecting their colleagues in space, the same intense screening will not be applied to every technician who comes in contact with the spacecraft and its cargo as it is being prepared for launch. Many microorganisms and viruses can persist for long periods of time on surfaces, and an

infected launch site worker or insect vector could unwittingly contaminate a spacecraft days or weeks before launch with a pathogen such as a Gram positive bacillus, fungal spore, or enterovirus for which the astronauts are routinely screened.

In Section 7 of this report we present lists of PCR primers and probes designed to specifically detect a number of organisms not listed in Table 1-2. Because we envision PCR based microbial monitor technology will be used both in space and on Earth for water quality analysis, we included organisms in the primer design section which would need to be considered in terrestrial applications.

Although we list three viral pathogens, assays to detect viruses in ISSA water may be of little value for two reasons. First, because viruses are obligate parasites and can replicate only in host cells, no increase in viral titer can take place as a result of viral replication in the water. Any virions in the water system will have to have passed through the entire water purification process or have been deposited on the clean water side of the purification system. Viral titers should always be very low if not zero. Second, although PCR based methods can detect as little as a single nucleic acid template, sample concentration is necessary in order to effectively utilize PCR's great sensitivity[5] (see Section. 2). Currently available sample concentration techniques are based on filtration, and because viruses are so very small, current filtration methods are largely ineffective for collecting viruses. Environmental sampling methods have been reported that use filtration to concentrate viruses in sea water for detection by PCR;[6] however in the ultrapure low conductivity water generated by the ISSA water reclamation system, filter concentration of viruses would probably not be possible given the size and power consumption requirements of the ISSA. Nonetheless, one of the principle rationales for incorporating viral testing capability into any PCR based system would be for spin-off terrestrial uses where such a viral monitor could have numerous uses in both clinical and environmental settings.

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Section 2.
Current and Projected Methods for Pre-PCR Sample Concentration

Although PCR based methods are capable of detecting a single target organism or virion, it is essential that samples be concentrated in order to attain a high sensitivity per unit volume. NASA specifications call for detection of a single organism in 100 ml of water. However because PCR samples are typically 40 µl or less, without concentration the lower limit of detection is 25 PCR templates/ml because 1 template per 40 µl corresponds to 2500 templates per 100 ml sample. To attain a lower limit of detection of 1 microorganism in 100 ml it is necessary to concentrate any microorganisms in a 100 ml water samples so that they can all go into a 50 µl PCR reaction. This is a decrease in volume of at least 2500 fold.

Additionally, because we envision analyzing water for perhaps as many as 20 different microorganisms or groups of microorganisms it will be necessary to concentrate more than a single 100 ml sample of water if the 1 template per 100 ml lower limit of detection is to be achieved. For that sensitivity, each PCR sample will need a 100 ml water sample that had had any microorganisms present concentrated 2500 fold. The TaqMan™ technology for analysis of PCR products we propose NASA use (described in Section 4) can be configured to simultaneously test for 2 different templates in a single multiplex PCR reaction. Thus to assay for 20 different microbial taxons with the prescribed limit of detection, 10 multiplex PCRs would be needed and the potential PCR targets in 1 liter of water would need to concentrated into approximately 400 µl of water. Importantly, although 1 liter would be a large volume of water given the limitations of the ISSA, the sample concentration process need not consume more than 400 µl of that amount and event that water could be reclaimed after the PCR assays.

Two basic strategies have been used for concentration of microorganisms: centrifugation and filtration. Centrifugation is unlikely to be suitable because of the large sample volumes that would need to be concentrated as well as the power and

space requirements for a centrifuge that could pellet bacteria and viruses from one liter of water. Accordingly, filtration is a much more tractable option for the necessary sample concentration. Bacteria and eukaryotic parasites such as *Cryptosporidium* are large enough to be concentrated using filtration methods; however viruses are too small to be efficiently filtered using standard technologies and as a result are usually concentrated by centrifugation or vortex flow filtration.[1]

Using filtration, single cells of microorganisms in 100 ml water samples can be detected by PCR.[2,3] Samples were concentrated onto filters and the DNA of the microorganisms was released by freeze-thaw cycling prior to PCR. PCR can be performed without removing the filters. The choice of filtration media is critical. PCR amplification is unaffected by polyvinylidine fluoride filters and polytetrafluoroethylene filters, marketed by Millipore as Durapore® and Fluoropore® filters respectively. Cellulose acetate and nitrocellulose filters inhibit PCR amplification, presumably because DNA binds to the filter matrix.[2,3]

Filtration of water aboard the ISSA

Development of a system of filtration for use on the ISSA may prove to be problematic. Any filtration system must have a number of characteristics consistent with the NASA prescribed characteristics for a microbial monitor as well as for incorporation into a PCR based system:

- The filtration process must integrate with the ISSA water system and the PCR processor.
- The system must use minimal amounts of power, space, and water.
- If possible the filtration system should be fully automated, so that zero or minimal ISSA crew effort is expended to make it function.
- The filtration system will need to be kept sterile so that no microbial contamination from outside the water system becomes a source of false positive PCR results.

One liter water samples will need to be taken at some defined interval, perhaps daily, from the clean water side of the ISSA water system. Where should the water collection site be? Environmental detection of *Legionella* is usually done at all of the end use ports because those bacteria may exclusively colonize one site such as a shower head. On the ISSA it may be possible to collect water from every port; however that would require active crew involvement in the microbial monitoring process. Even if all the water was collected from a single port, probably the drinking water port, development of an instrument that would collect, filter, and recycle one liter of water on a daily basis and then transfer the filter to the PCR processor would be an elaborate and expensive project. Alternatives that require crew involvement could probably be developed using modifications of existing technology. For instance, an astronaut could collect the liter of water into a manifold that holds 10 filters. Thus 100 ml could be forced by compressed air or vacuumed through the filters (the ISSA does have a vacuum source for use in the hygene system), and then the water could be returned to the stainless steel bellows tanks or used directly. The filtration would constitute an additional purification step. Once the filtration was complete, an astronaut would then aseptically transfer the filters to the PCR sample tubes. Aseptic transfer so that no microbes form outside the water system contaminate the PCR samples could be difficult to accomplish; however methods and an appropriate apparatus should be possible to devise.

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Section 3.
Does the MSFC Water Reclamation System Introduce Chemicals into Water That Would Inhibit a PCR-Based Microbial Monitor?

Can PCR be done on water reclaimed by the system designed for use aboard the ISSA? Yes, in analyses performed on samples from the Stage 7 and Stage 8 test of the water reclamation system at MSFC, PCR was shown to be an effective and sensitive tool to monitor microbial contaminants.[1] Are there chemicals in the reclaimed water that inhibit PCR assays? That question must have the qualified answer of probably not, but we do not know for sure. The principle reason there are not likely to be any inhibitors of PCR is that as a result of the high efficiency of the water reclamation system, the recovered water is extremely clean. Chemical analysis of the MSFC reclaimed water for a great number of elements showed only iodine, which is used as a biocide, is present in greater than mg/L amounts (Table 3-1). We cannot be sure because although PCR analysis of the Stage 7 and 8 samples was successful, the scientist who performed those tests, Dr. Asim Bej of the University of Alabama at Birmingham, stated no effort was made to determine if there were PCR inhibitors in the water that would make the tests less sensitive.[2]

There are a number of chemicals that have been reported to inhibit the PCR enzymes; however none of the chemicals identified in the MSFC recycled water are present at concentrations known to inhibit PCR. There are no reports in the literature documenting the effect of iodine on PCR. The aforementioned work by Asim Bej on the Stage 7 and 8 samples of MSFC recycled water suggests iodine is inconsequential.[1] Another potential contaminant whose effect on PCR has not been reported is silver. The Russian space program employs silver as a biocide in its water reclamation system.[3] Solubilized metals can affect PCR. High levels of iron have been reported to inhibit Taq DNA polymerase, the PCR enzyme; however no other metals have been reported to affect PCR.[4]

Development of a PCR based microbial monitor for the ISSA should have as one its initial steps experiments to determine if the MSFC recycled water contains inhibitors of

PCR. Additionally, the affects on PCR of iodine concentrations greater than the 2.3 mg/L average value found in the MSFC recycled water, and silver in the concentration range found in Mir recycled water should be tested.

Table 3-1. Chemicals identified in the MSFC recycled water.[1]

Parameter	Units	Detected Average
(Z)-9-octadecan-1-ol	µg/L	6.6
1-methyl-2-piperdinone	µg/L	14
1-methyl-2-pyrrolidinone	µg/L	226
2-ethyl-12-hexanol	µg/L	8.9
toluene	µg/L	3
acetic acid	mg/L	0.21
ß-hydroxy butyric acid	mg/L	0.32
ethanol	mg/L	0.54
formaldehyde	mg/L	0.1
glycolic acid	mg/L	0.2
oxalic acid	mg/L	0.9
propionic acid	mg/L	0.32
aluminum	mg/L	0.6
barium	mg/L	0.01
calcium	mg/L	0.06
chloride	mg/L	0.08
fluoride	mg/L	0.06
iron	mg/L	0.01
manganese	mg/L	0.008
nickel	mg/L	0.03
nitrate	mg/L	0.16
phosphate	mg/L	0.47
potassium	mg/L	0.21
sodium	mg/L	0.63
sulfate	mg/L	0.22
residual iodine	mg/L	2.3
iodide	mg/L	0.64
conductivity	µohm/cm	5.6
pH	pH units	7 (4.4-8.5)
total organic carbon	mg/L	0.59

Analyzed for, but not detected		
Cadmium	Lead	Magnesium
Copper	Selenium	Silver
Molybdenum	Arsenic	Chromium
Zinc		

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Section 4.
Current and Projected Methods for Quantitative Analysis of Post-PCR Products

Gene-based microbial analysis: PCR

Since PCR's invention in 1985 as a method for the prenatal diagnosis of sickle cell anemia,[1] PCR has rapidly become the basic tool in all types of genetic diagnosis. For detection of low levels of microbial contamination in almost any kind of sample, PCR based methods are unsurpassed in speed, specificity, and sensitivity. PCR is based on the concept that repetition of a DNA extension reaction bounded by two synthetic oligonucleotide primers would generate a large quantity of any specified DNA sequence. Culture based microbial analysis relies on the reproduction of individual organisms until sufficient progeny exist to constitute a colony that can be easily detected, and identified based on a phenotype. Similarly, PCR based microbial monitoring replicates a specific segment of a target microbe's genome to a concentration sufficient for detection and characterization. As the number of colonies on a bacterial assay plate is a quantitative function of the number of that bacteria in a sample, so can the number of copies of a PCR amplified DNA sequence be a function of number of those sequences in the sample prior to PCR. It is important to note that because the efficiency of amplification varies among different templates and primer sets, so quantitative PCR assays must be evaluated independently.

In most current PCR applications, to analyze post-PCR products for amplified DNA sequences, called amplicons, there are two basic methods. Most simply, the PCR products are size fractionated by gel electrophoresis, stained with a fluorescent dye, and any amplicons present are visualized by exposing the gel to UV light. An alternative and vastly more sensitive method, often referred to as Southern blotting and hybridization, fixes any amplicons present to a substrate, usually after gel fractionation. The double stranded DNA amplicons are then denatured and the substrate, usually a nylon membrane, is incubated with a fluorescently or radioactively labeled oligonucleotide probe. The probe specifically hybridizes to a complementary sequence of any amplicons present and the amplicons are visualized by detecting the

bound probe using either radioactivity or fluorescence detection methods. Thus probe-hybridization/PCR offers increased sensitivity and specificity over direct analysis of PCR products; however the time (hours to days) and technical requirements of both methods of post-PCR product analysis make them unsuitable for NASA's needs.

Although these gel electrophoresis based methods for post-PCR analysis are in wide use in research and diagnostic labs, the techniques are too slow, and labor intensive for both NASA's needs, and to fulfill the promise of PCR as a rapid, highly automated diagnostic tool. For gene-based diagnostic technology to work as an effective microbial monitor the analysis of post-PCR products will have to advance beyond gel separation based methods. Otherwise alternative technologies such as described below, that do not rely on PCR, will need to be developed.

Alternative Gene-based diagnostic methods

DNA probe-hybridization techniques are under development that should lack some of the problems of speed and labor intensiveness characteristic of standard probe-hybridization/PCR. NASA has funded two of these efforts via Small Business Innovation Research contracts. Both methods rely on hybridization of fluorescently tagged oligonucleotide probes to bacterial ribosomal RNA (rRNA) molecules. BioTechnical Resources L.P.'s direct hybridization method can detect 10^4 bacteria in about 8 hours.[2] Although the method is simple and low-tech, its sensitivity is unsuitable for NASA's stated needs. Many probe-hybridization/PCR based methods can detect a single organism.[3] Genometrix Inc. is developing silicon microchips on which arrays of different oligonucleotides probes for rRNA sequences are bound at specific addresses. The rRNAs of any bacteria in a sample would specifically hybridize to their complementary probe on the microchip. Next, in a second hybridization step, labeled oligonucleotide probes would anneal to the bacterial rRNAs already bound to the microchip. A charged-coupled device (CCD) detector would then determine which locations on the chip had the tagged oligonucleotide attached. Genometrix predicts they will be able to detect 1000 rRNA molecules. No amplification is necessary because each bacterium contains 100-1000 ribosomes.[4] *Although this revolutionary*

direct hybridization technology is theoretically fast and sensitive enough to meet NASA's specifications for bacteria (although not viruses), it is unproven technology that may be many years from implementation. When this technology matures, it will have several major advantages over PCR based methods. Because it does not require amplification of a nucleic acid template, the risk of false positive results due to contamination is greatly reduced. Although this hybridization to a silicon chip technology would have limited sensitivity for viruses because each virion would have only one hybridization target, the method could be used in concert with PCR to allow sensitive detection of viruses.

Analysis of Post-PCR Products: Electrochemiluminescence

A system for analysis of PCR products has been reported that does not employ the standard methods of gel separation of products, or binding to the PCR products to filters followed by hybridization with radiolabeled or fluorescent probes. The method is based on the incorporation of a biotinylated oligonucleotide as a primer, with the inclusion of a labelled oligonucleotide. Oligonucleotides are labeled with an N-hydroxy succinimide ester of tris-bipyridine ruthenium (II) dihexafluorophosphate (Origen-label) by modifying the 3' and 5' ends of the oligonucleotide probes. The assay makes use of the inherent thermal stability and absence of polymerase activity on such probes to allow the PCR and probe hybridization to be completed automatically on the thermocycler. The assay is concluded by the addition of PCR samples to streptavidin beads on an electrochemiluminescence analyzer for binding and analysis.

Although electrochemiluminescence is an improvement in post-PCR analytic methods, in its current form the method is still cumbersome in that it requires addition of reagents after the PCR and the PCR products must be transferred from the cycler to a different instrument for analysis. This method, like a similar approach developed by Roche Molecular Systems for cystic fibrosis testing called "reverse dot,"[6] although amenable to quantitative analysis of PCR products, is insufficiently automated to afford the low technician effort NASA will need for monitor microorganisms in space vehicles. A

different system for combining PCR and post-PCR product analysis that we believe has the potential to meet NASA's needs for a microbial monitor is described below.

TaqMan™ PCR

This is a new method that combines probe-hybridization and PCR while eliminating the time consuming steps of electrophoresis and/or blotting of the post-PCR products. TaqMan employs a probe technology that utilizes the 5'-3' endonuclease activity of *Taq* DNA polymerase,[7] to allow direct detection of PCR amplicons by the release of a fluorescent reporter during the PCR (Figure 4.1).[10] The trademark TaqMan name is a

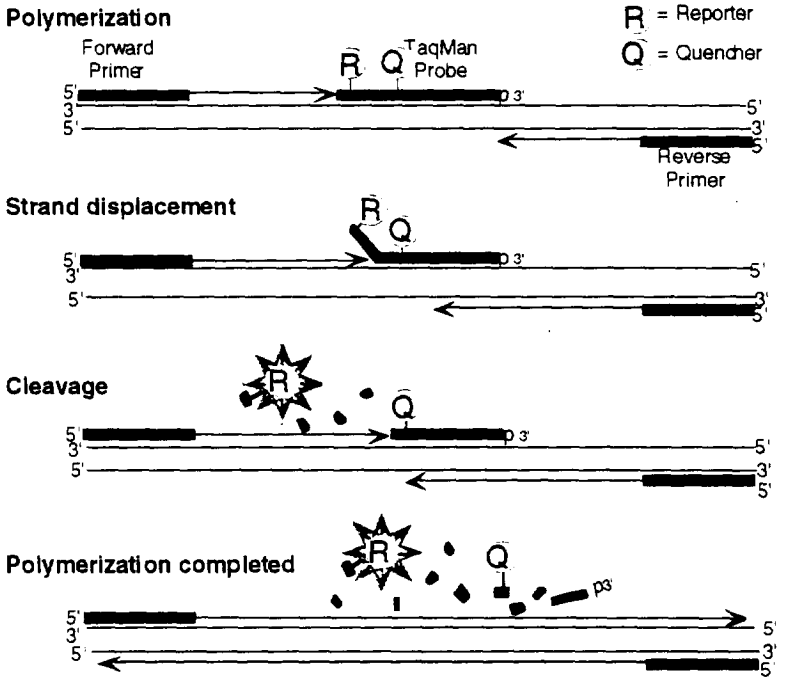

Figure 4-1. *Taq* DNA polymerase activity in TaqMan PCR. In a single cycle of PCR, the initial steps are template denaturation and annealing of that denatured DNA template with the forward and reverse primers, as well as the tagged TaqMan probe(both steps not depicted). After which, the enzyme's polymerization dependent 5'-3' endonuclease activity frees the reporter dye from the neighbor effects of the quencher dye, so it can produce a signal that is proportional to the PCR amplification. Cleavage of the TaqMan probe does not affect forward primer extension. (Modified from TaqMan™ Reagent Kit Protocol, Perkin Elmer/Applied Biosystems).[9]

oligonucleotide with a 5' reporter dye, an internal quencher dye, and a 3' blocking phosphate. The reporter dye, for which there are three different fluorescein options, is covalently bonded to the oligonucleotide's 5' end. A rhodamine quencher dye is similarly linked four to thirteen nucleotides 3' to the fluorescein reporter. To prevent the TaqMan probe from extending during PCR, there is a 3' phosphate instead of a 3' hydroxyl group. So long as the reporter and quencher are held in close proximity by the oligonucleotide, its fluorescence is quenched, principally by Förster-type energy transfer.[10] During PCR, if the TaqMan probe's target is present, the probe anneals between the two PCR primer sites. As *Taq* DNA polymerase extends from the PCR primer annealed to the same DNA strand as the probe, its 5'-3' endonuclease activity sequentially digests the probe's nucleotides. *Taq* DNA polymerase does not digest free probe (Figure 4.2). In every cycle, as the probe is displaced from the template, the PCR primer extends without interfering with the exponential accumulation of amplicon. Thus the reporter dye is liberated from the quencher and can now fluoresce when excited. Fluorescence increases in direct proportion to amplification of the PCR target. As with all probe-hybridization/PCR, the TaqMan's specificity is a result of the

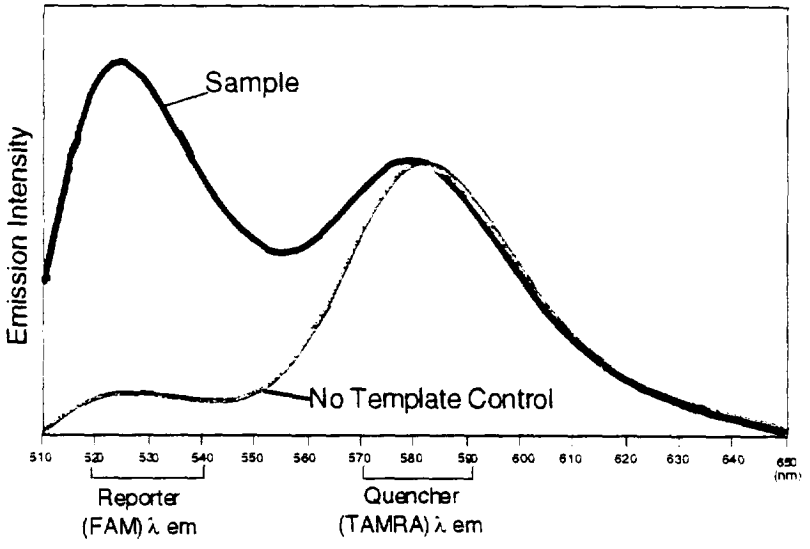

Figure 4-2. Two TaqMan emission scans post PCR, Sample and No Template. The reporter dye is 6-CA fluorescein (FAM) and the quencher dye is 6-Carboxytetrame rhodamine (TAMRA). (Adapted from the TaqMan™ Reagent Kit Protocol, Perkin Elmer/Applied Biosystems).[9]

requirement for primer and probe complementarity to the target DNA before any amplification and probe cleavage take place. Unlike other probe-hybridization/PCR methods, TaqMan PCR has no laborious post-PCR product analysis steps. *The entire reaction takes place in a single tube, and everything happens at once.* The samples and reagents are mixed, sealed in a reaction tubes, and then placed in a thermal cycler for amplification. To enhance specificity and minimize the risk of carry-over contamination the method employs the hot start method and UNG/dUTP.[11] In the system's present version at the conclusion of the PCR, aliquots of the amplified samples are transferred to microtiter plates for analysis in a luminescence spectrometer. Detection of all 96 wells takes only 7 minutes. The assay's results are expressed as the comparison of the increase in reporter dye fluorescence with that of a no template control. The ratio of reporter fluorescence to quencher fluorescence in the sample and no template control, ΔRQ, is proportional to the number of DNA templates in a sample.[12]

TaqMan is a great leap in PCR technology. It has to major improvements over gel-based post-PCR analytic methods, and both of these advances are essential to meeting NASA's needs for a microbial monitor for the ISSA.

- Samples are analyzed directly and in just a few seconds, as opposed to being transferred to a gel and electrophoretically analyzed.
- TaqMan is an inherently quantitative technique. Within a range of template concentrations, the TaqMan signal will be proportional to the amount of template present. Thus the number microorganisms in a sample can be quantitated.

In its present format, the TaqMan system requires that samples be manually transferred from a thermal cycler, where the PCR amplification is performed, to a fluorescent plate reader for analysis of the reactions. The next generation of TaqMan instrumentation, which Perkin Elmer/Applied Biosystems will begin field testing in the next year, can analyze samples directly in the PCR tube, thus eliminating the need for sample transfer. Additionally, because the next generation machine can analyze

samples in the reaction tubes, the progress of the PCRs can be monitored after each thermal cycle. This will improve the quantitative effectiveness of the instrument, because when a PCR template is present at high concentration during later cycles of a PCR, as reagents are consumed in the reaction, the efficiency of the PCR declines. Monitoring of the amplicon accumulation after each cycle permits template quantitation during the linear phase of the PCR.

The current TaqMan system being marketed by Perkin Elmer/Applied Biosystems consists of a thermal cycler, a fluorescent plate reader, and a dedicated computer. The next generation TaqMan instrument is even larger, and has significant power requirements. Because of the space and power limitations on ISSA the monitor must be small and energy efficient. Efforts at creating smaller instruments for gene-based diagnostics using microfabricated devices are ongoing in a number of laboratories.[4]

Microfabricated DNA Analysis System

A prototype miniaturized PCR thermal cycler was developed by researchers at Lawrence Livermore National Laboratory (LLNL) in conjunction with Roche Molecular Systems and Perkin Elmer/Applied Biosystems.[13, 14, 15(Appendix C)] Fabricated on a 3 inch by 5 inch Plexiglas platform, the unit consists of up to three PCR reaction chambers, a thermocouple converter chip reaction controller, and 4 nine-volt batteries to run the heaters and the control electronics. The reaction micro-chambers, made from an anisotropic etched silicon cavity with one or two medium low stress silicon nitride membrane windows, are typically 5 to 10 mm^2, 0.5 mm deep, and contain embedded polysilicon resistive heaters. The windows are designed for use in detection of PCR products. This device has been used to detect cystic fibrosis causing mutations on human DNA in a multiplex reaction simultaneously amplifying segments from eight different targets on the human genome. M. Allen Northrup, principal investigator of the LLNL group, envisions this technology evolving into a hand held PCR system that can take a sample, perform the PCR thermal cycling, and then analyze the sample by monitoring micro-electrochemiluminescence through the silicon nitride membrane windows in the reaction micro-chambers. His group has built a real-time fluorescence

monitoring system that uses laser excitation and CCD camera surveillance of the PCR progress. In collaboration with Dr. Rosemary Smith, of the University of California at Davis, the LLNL researchers are exploring the use of electrochemiluminescence with ruthenium labeled oligonucleotide probes[5] as a method to assay PCR amplification in the reaction tube. Ultimately, instruments consisting of large arrays of as many as 1000 individually controlled reaction chambers could be built. Northrup's January 1995 report to the Advanced Research Projects Agency (ARPA) is included with this report as Appendix C.

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Section 5.
Possible Methods of Avoiding False-Positive Results Due to the Detection of Dead Organisms or Free DNA.

Unlike culture based microbial diagnostic assays, which function by detecting an increase in the number of whole organisms or virions, PCR can amplify intact DNA from a living bacterium or infectious virion as effectively as from a dead microbe or even from solubilized DNA. Sixteen weeks after being killed by boiling, *E. coli* can be detected by PCR as effectively as before inactivation.[1] This limitation of gene based monitoring might be addressed in several different ways that could meet NASA's needs for monitoring water quality on spacecraft.

- Determine if PCR targets from nonviable microorganisms elute from the ISSA water reclamation system.
- Determine if microbial monitoring could be based on the observation of population growth changes in the ISSA water collection tanks.
- The PCR target could be short lived molecules of messenger RNA (mRNA) instead of DNA.
- Evaluate the use of vital dye staining, which would determine how many bacteria, fungi, or protozoans are respiring in a sample, in concert with PCR based assays.

Do nonviable organisms elute from the ISSA water reclamation system?

Although we know PCR is blind with respect to whether organisms are alive or dead, we do not know if or how long the DNA from organisms inactivated by the MSFC water reclamation apparatus can still be amplified by PCR. That water reclamation system's penultimate step in generating potable water is a catalytic oxidation system. Designed to completely oxidize any organic molecules that have made it past the upstream components of the water reclamation system (mixed bed resins provide growth media for many bacterial species), *the catalytic oxidation system should completely mineralize soluble nucleic acids.*[2] Nonetheless, previous PCR analyses of MSFC reclaimed water detected more species of bacteria than were found using culture based methods.[3] That suggests the PCR assays detected a great many nonviable cells

or DNA released from lysed cells; however, that result could be due to the greater sensitivity of PCR based assays relative to culture and the fact that many microorganisms when handled roughly are viable but not culturable (notably *Legionella sp.*[4]).

A recent test of the capacity of the MSFC water reclamation system to eliminate infectious viruses may have laid the groundwork to address the issue of nonviable microbes passing through the system as intact PCR targets. In January 1995, MSFC Chief Microbiologist, Ms. Monsi Roman, and Dr. Christon Hurst of the U. S. Environmental Protection Agency conducted a test in which they added a mixture of $\approx 10^8$ plaque forming units of four different bacteriophages into the water reclamation system intake. During 5 days of system operation, no infectious bacteriophage eluted from the system's clean water ports.[5] To date those samples have only been tested in infectivity assays. Ideally, PCR should be used to analyze those samples for bacteriophage DNA/RNA. Because the nucleotide sequences of all of the bacteriophage used have been published, it should be possible to develop effective PCRs to answer this question. If phage genomes are detected in the clean water in the absence of infectious particles then there is proof that nonviable organisms/viruses passing through the system can generate a false positive result for contamination. Thus any gene based assay system will be to some extent blind as to whether any virus detected is viable or nonviable. If no detectable bacteriophage is found in the clean water by PCR, one can still not rule out the possibility that the mixed bed resins in the system so retarded the virus that in the short test of 5 days, no bacteriophage had time to complete passage through the system. The experiment outlined below addresses that possibility.

Are intact target nucleic acid sequences are available for PCR amplification from or in nonviable cells and virions after passage through the MSFC water reclamation system's catalytic oxidation stage? Different bacterial, viral, protozoan, and fungal samples could be exposed to the system's multiple disinfection procedures, i.e. heat, 250°F for 20 minutes, and/or the 2 ppm iodine imparted to the water by the system's

microbial check valves.[3] One would need to investigate a variety of microbes because different species may respond differently to the inactivation treatments. This could be the result of differences in cell wall or capsid structure or it could be a function of the size of the PCR amplicon.[6] The genomic templates for large amplicons may be more susceptible to damage as a result of germicidal treatment than small templates due to the random nature of the effects of germicidal treatment. After either or both of those treatments the samples would be passed through the catalytic oxidation stage of the water reclamation system and the resulting water would be analyzed by both PCR and culture. Aliquots of the microbial samples should be analyzed by PCR before the heat and/or iodine treatments and between heating/iodination and catalytic oxidation. The PCR data from the three different stages of the water decontamination process would show the extent to which nonviable microbes can be detected by PCR after passage through the MSFC water reclamation system.

Can microbial growth be monitored as a way of bypassing false positive PCR results?

If nonviable microbes contribute significantly to the amount of DNA amplified by PCR of water samples from the MSFC water reclamation system, we would suggest attempting to use a PCR based microbial monitoring system for analysis of recycled water for pathogens to focus on changes in microbial concentration with time that are indicative of increasing populations in the processed water collection tanks. This analysis of microbial population growth approach should work despite the indefinite lifetime of nucleic acid sequences in nonviable cells as demonstrated by Josephson, *et al.*[1] Although there would be a continual influx of low levels of nonviable cells from the water reclamation system, use of the recycled water should result in a continual outflow of the nonviable cells. Thus the contribution of the dead organisms to the estimated microbial load of the collection tank should reach a steady state. Only the presence of microbial growth in the tank should disturb that equilibrium. Obviously, this approach would not work for analysis of viruses because they are obligate parasites and cannot replicate outside of their hosts.

mRNA instead of DNA as a PCR target

One of the main reasons PCR cannot distinguish between viable and non-viable organisms is the great stability of DNA. There is another potential gene target molecule that is much more fragile and short-lived called mRNA. The half life of *E. coli* mRNA is only 30 minutes in a living organism, and presumably much shorter in a dead organism. Similarly, soluble RNA is rapidly degraded in environmental waters and thus is In a pre-PCR step, mRNA can be enzymatically copied using reverse transcriptase.[7] The combination of reverse transcription and PCR, called RT-PCR, has been successfully used to detect mRNA in both eukaryotes and bacteria, and in fact is the only way to detect viruses with RNA genomes such as polio, rotaviruses, and Norwalk viruses. Detection of a short-lived molecular species that can only be made by viable microorganisms would theoretically be the same as detecting only viable organisms..

Unfortunately, at least for bacteria, this would be much more difficult than standard PCR. Although RT-PCR would be required for the detection of RNA viruses (influenza and Norwalk for example), the additional effort might not be feasible or practical bacteria. The half life of mRNA would need to be determined for each species analyzed, along with the average concentration of target mRNA in each cell. Additionally, it would be necessary to eliminate any potential DNA templates in a sample using DNA specific nucleases, and that step could prove to be very difficult.

Several research groups have investigated the possibility of using an RT-PCR approach to discriminate between viable and non-viable microorganisms, however no one has developed an assay that works yet. Dr. Ian Pepper, at the University of Arizona, and Dr. Asim Bej, at the University of Alabama at Birmingham, have both been able to detect bacterial mRNA; however neither see the technology as a method of detecting only viable organisms[1,8,9,10] Scientists at Perkin-Elmer's Applied Biosystems Division said they had experimented with RT-PCR as a tool to screen for

viable bacteria and had abandoned the effort because they felt it could never be made to work.

Vital dye staining as a complement to PCR for discrimination of viable organisms.
It may be possible to use vital dyes to detect the presence of live bacteria and protozoa. Vital dye staining is an established technology that could be coupled with the TaqMan PCR (section 4). Thus one could estimate the total number of respiring microorganisms in a sample with the vital dyes, as well as speciate and enumerate the viable and the nonviable microorganisms present using TaqMan PCR. The TaqMan PCR detection system's LS-50B fluorescent plate reader would analyze both the vital dye samples and the TaqMan PCRs.

Viability staining could be achieved through the use of several vital dyes to determine which is most suited to these investigations. Potential dyes include the redox dye 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), and acridine orange to directly observe respiring microorganisms. In the case of INT the reducing power of the electron transport system converts INT into insoluble INT-formazan crystals that accumulate in metabolically active bacteria.[11] Microscopically the INT-formazan deposits are observed as red deposits under bright field microscopy. The INT method has been successfully combined with the acridine orange direct count method to simultaneously enumerate total and viable bacterial concentrations.[12] A method developed by Kogure, *et al.*,[13] also allows for the simultaneous enumeration of both total and viable cells. This method utilized nalidixic acid, a gyrase inhibitor, and yeast extract as a nutrient source. The nalidixic acid prevents cells from dividing while they continue to metabolize the yeast extract and enlarge, dead cells will be unable to utilize nutrients and remain "normal-size". However, there are a number of problems with this method. For example, not all cells are sensitive to the effects of nalidixic acid and not all cells are capable of utilizing yeast extract as a food source. In addition the metabolic rate of microbial pathogens varies which may cause some cells to swell to various sizes

making enumeration difficult. Finally, some bacteria such as *Legionella* are resistant to the effects of nalidixic acid, therefore the Kogure method is not a viable option.[13]

Recently a fluorescent redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), has successfully been used to directly visualize actively respiring bacteria. The oxidized CTC dye is almost colorless and nonfluorescent, however once the dye is reduced via the electron transport system, it becomes fluorescent, insoluble CTC-formazan compound that accumulates intracellularly.[14] Based on published studies with other microorganisms, the dye should provide valuable viability information that would complement the PCR data.

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Section 6.
Current and Projected PCR Quality Control Techniques.

A critical aspect of a PCR based microbial monitor will be a set of quality control measures. Methods must be in place that will insure the following:

- That the assay is functioning according to specifications.
- That reagents are prepared, aliquoted, and stored so that the microbial monitor can function effectively throughout long space missions.
- That samples are not contaminated with microbes from outside of the water reclamation system or with PCR amplicons from earlier reactions resulting in false positive results.

The first two items on this list should be easily attainable. Development of effective internal control reactions has been done for other microbial detection assays; adaptation of that technology to NASA needs should be straightforward. Methods have been reported that would permit long term storage of reagents that have been assayed and aliquoted so that only the sample and water would need to be added prior to assay. Unfortunately, the problem of false positive results due to contamination may prove to be one of the most difficult aspects of developing a PCR based microbial monitor. Diagnostic PCR labs strive to avoid contamination problems through devotion to fastidious technique and laboratory practice as well as through a number of structural and procedural safeguards (Table). Any PCR based instrument used to monitor microorganisms aboard the ISSA will need to incorporate these procedures into the systems design.

Table. Guidelines for the operation of a PCR laboratory. adapted from 1

- Establish separate pre- and post-PCR work areas with dedicated supplies and reagents.
- Carefully plan experiments: do not enter the pre-PCR area after handling amplicons or target DNA.
- Use plugged pipet tips or positive -displacement pipettes.
- Use aliquots of all reagents to limit handling.
- Incorporate enzymatic or chemical methods to control amplicon carryover.
- Always use a low-copy number (10-50 templates per PCR) of positive controls, a large number of negative controls, and reagent controls with every amplification.

Control reactions to confirm PCR effectiveness.

A positive control will be incorporated into every sample to insure the PCR worked properly. Reactions could fail because of contamination of the sample with inhibitors, degradation of one of the enzymes or other reagents, or problems with the instrument. An effective internal positive control that is designed to generate a fixed amount of PCR amplicon can provide a quantitative assurance that the PCR system and individual reaction are performing to design specifications.

Included in the reagents used for each PCR will be 10-50 copies of part of the human ß-actin gene, as well as primers and a TaqMan probe that will generate and allow monitoring of the synthesis of an amplicon from the human ß-actin gene.[2,3] Although several different genes are commonly used as an internal positive control molecules, Perkin Elmer Corporation developed the TaqMan system with the intent of using the ß-actin gene for that purpose. As mentioned previously, the TaqMan system can do multiplex PCR because there are three different reporter dyes for labeling probes. Thus every PCR tube will contain two TaqMan probes specific for different microorganisms or groups of microorganisms, plus a probe specific for the ß-actin amplicon. Each of the three probes will be labeled with a different reporter dye.[4]

In addition to the positive controls, every set of PCRs would also include a number of negative controls. Negative control reactions are necessary for confirmation that PCR amplicon carry over is not generating false positive results and to serve as a baseline value for the TaqMan system. With TaqMan each sample being assayed has two tubes containing only the reagents and no sample. Thus, if the ISSA microbial monitor assays for 20 different microorganisms or groups of microorganisms in 10 multiplex PCRs, then an additional 20 negative control PCRs will be required also.

Reagent storage

To simplify the microbial monitor, it will be critical that most reagents be prepared and aliquoted on earth and then stored, potentially for months or years, until needed. In its current configuration, the TaqMan system is designed to assay samples in a 96-well

tray format. Although a full 96-well tray would not be needed to analyze water samples for 20 different kind of PCR targets, NASA should design its PCR based microbial monitor to use a multi-well tray. Reagents could be pre-loaded into multi-well trays on earth so that enzymes, primers and dNTPs are segregated until the reaction is heated, thus preventing reagent degradation due to PCR reactant assembly and storage prior to thermal cycling. One method for accomplishing this is encapsulation of subsets of the PCR reagents in special agarose beads so that they can be stored for long periods of time.[5] G. K Smith, of the University of Houston, believed his microencapsulation methods could be refined to meet the PCR reagent storage needs of an ISSA microbial monitor.[6]

By pre-encapsulating aliquoted amounts of all the components of the PCR except the sample to be assayed , the quality control criteria for diagnostic PCR can largely be addressed. A method and instrumentation will need to be developed to transfer the samples to be analyzed from the filtration system (see Section 2) to a multi-well tray. Perhaps a 10-filter manifold (one filter for each multiplex PCR) could be used to insert filters directly into the multi-well tray containing the reagents prior to thermal cycling. For this to work, procedures would need to be included to release of the DNA or RNA form any microorganisms on the filters without damaging the PCR reagents. Two of the most simple methods for liberation of the nucleic acids from bacteria and viruses prior to PCR are boiling and repeated cycles of freezing and thawing.

Control of carry over contamination that could yield false positives.

The sensitivity advantage that PCR contributes to the detection of microorganisms can also potentially be a major disadvantage. Previously amplified DNA that is replication competent can be carried over and can serve as a template in later amplifications, resulting in false positives. The capacity of single molecule amplification requires special methods be used to insure accurate results. Several approaches utilizing either chemical or enzymatic methods to minimize PCR product carryover have been described.[7] Analysis and comparison of these methods indicates the most effective

method for spacecraft use uses uracil N-glycosylase (UNG) to degrade any contaminating PCR amplicons present in a reaction before the onset of PCR.

UNG is an *E. coli* enzyme that modifies DNA containing uracil so that it can later be degraded by heating. By substituting dUTP for dTTP in the PCR, the resulting amplicons are susceptible to UNG degradation.[8] A 2 minute incubation at 50° is sufficient to modify any contaminating amplicons as well as any mis-primed or non specific products produced prior to specific amplifications, but not degrade native nucleic acid templates. At the end of the 2 minute treatment a 10 minute incubation at 95° completes the degradation of uracil containing DNA, inactivates the UNG, and denatures the template DNA prior to thermal cycling. The procedure actually enhances the quality of the PCR by eliminating any misprimed reaction products that result from the primers annealing incorrectly to templates at low temperature during the mixing of reagents prior to thermal cycling.[9]

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Section 7.
Prediction and Analysis of PCR Primers and TaqMan Probes for the Detection of Microorganism Contaminants in Environmental Samples

Detection of microbiological organisms contaminating environmental samples using TaqMan PCR technology will require primer and probe oligonucleotides to be defined for each organism or group of organisms to be detected. The basis of primer and probe definition is through analysis of available genomic sequence data for the organisms in question. Following the initial step of constructing a list of organisms to be detected, genomic sequences for these organisms are obtained from sequence databases, and then analyzed using parameters appropriate for designing functional primers and probes. All of these steps are computer-based, and result in a library of primer and probe oligonucleotide sequences that have the potential of providing relatively specific and sensitive detection of the desired microorganisms. While use of computers for oligonucleotide design can greatly facilitate construction of an oligo library, these primers and probes will need to be tested empirically in the laboratory to ensure that they work "as advertised". If not, additional oligo sequences will need to be defined. A reiterative process of computer prediction and laboratory testing is the most efficient means available for deriving the basic library of oligonucleotides necessary for environmental monitoring.

Below we discuss some of the considerations that are involved in the process of primer and probe prediction. These include determination of sequences to be detected; computer analysis of these sequences prior to oligo prediction; and analysis of the resulting oligonucleotide library. These methods were then used to predict primer and probe combinations for both a prokaryotic and eukaryotic data set of potential microorganism contaminants.

Genomic Sequences to be Detected

Choice of the particular genomic sequence to be detected is the first critical step in the process of primer and probe design. A wrong choice can lead to high background levels–low specificity (e.g., detection of normal microbiological flora) and low

sensitivity (failure to detect the desired organism). It has been estimated that the determination of the total diversity of microorganisms in environmental samples using culturable plate counts greatly underestimates the true level of diversity by over 90% (Amann, et al., 1995). These authors propose that using methods based upon detecting the presence of ribosomal RNA genes, a much more accurate analysis of the true levels of microorganism diversity can be obtained. The same reasons that make ribosomal RNAs useful in a study of microbiological diversity make them a good candidate for detection in a PCR-based environmental monitor.

Ribosomal RNA Genes

Ribosomal genes are universally present in the cells of all living organisms since they are critical to the process of protein synthesis. Ribosomes consist of two subunits that contain a combination of protein and structural RNAs. The sequences of the large subunit ribosomal RNA and in particular the small subunit ribosomal RNA (ssu rRNA) have been determined for a large number of different prokaryotic and eukaryotic organisms. The availability of these sequences has allowed a significant amount of work to be done in analyzing the biological features and evolution of these sequences between different species (Hillis, et al., 1991; Neefs, et al., 1991). The properties listed below contribute to the usefulness of these genes for detection of environmental contaminants:

- Sequences are present in all living organisms
- Genes contain multiple genomic copies undergoing concerted evolution
- Sequences have undergone variable rates of evolutionary change
- Primers and probes can be defined for hierarchical detection of microorganisms
- Sequences and alignments for most organisms are currently available through the Ribosomal Database Project (RDP) (Maidak, et al., 1994) and Genbank (National Center for Biotechnology Information–National Institutes of Health)

Having available such a large database of genetic sequence information for such a broad range of organisms allows a thorough analysis of the potential specificity of any potential primer and probe combination. Oligonucleotides can be designed with low specificity, but high sensitivity allowing detection of a broad range of organisms using

a single "universal probe". Alternatively, primer and probe combinations can be designed that are very specific, detecting the presence of only one particular pathogen. This provides the capability to design hierarchical probes that initially screen for gross contamination by microorganisms using universal probes, and then, if such contamination is present, the sample can be screened for the presence of particular pathogens using very specific primer and probe combinations. This technique has already been demonstrated using probes derived from small ribosomal RNAs that are designed to detect pathogenic bacteria in cerebrospinal fluid (Greisen, et al., 1994).

The property of these RNAs that provides this capability to detect either broad groups or specific organisms is the variable rates of evolution that these sequences have undergone over time. Certain regions of the ribosomal RNA genes have remained relatively conserved among species (probably due to functional constraints), while other regions show high variability when sequences from different species are compared (Hillis, et al., 1991). These regions have been mapped and correspond to specific regions of the predicted secondary structures of these molecules (Neefs, et al., 1991) (See Figures 7.1-7.4 below). This variable rate of evolutionary change can be exploited for primer and probe design purposes. The highly conserved regions are used to construct universal, or genus-specific probes, while the variable regions provide the necessary specificity to construct species-specific probes (Greisen, et al., 1994; van Kuppeveld, et al., 1992).

Other genes for PCR-based detection

While small ribosomal RNA genes can be used to detect a broad range of organisms, it may be useful to design probes based upon other genomic sequences. Detection of particularly pathogenic organisms may be best accomplished by designing probes to detect the genes specifically involved in the pathogenic mechanisms of these organisms. Examples are the toxin genes in strains of *Shigella* and *E. coli* (Stacy-Phipps, et al., 1995; Read, et al., 1992; Yavzori, et al., 1994; Sethabutr, et al., 1993). These authors have used PCR primers and oligonucleotide probes to detect the

presence of a number of the different toxin genes that have been identified in various strains of these species.

Another reason for utilizing non-rRNA sequences for PCR-based detection schemes, is that the ribosomal RNAs of several species have either not been sequenced, or sequenced to a limited extent. Currently, rRNA sequences for several *Klebsiella*, *Shigella*, and *Salmonella* species among others are absent or incomplete. Inclusion of primers and probes for these species using the ssu rRNA scheme will be dependent on new sequence information as it becomes available. Detection of these organisms will generally need to be based upon other species-specific gene sequences that are in the database; though the evolutionary history of these organisms does predict that they should be detectable by at the very least, the universal primer and probe sets, and possibly by some of the more specific primer and probe combinations (e.g. *Shigella, Salmonella,* and possibly *Kelbsiella species* should be detectable by the Enteric probe described below due to the close relatedness of these organisms to *E. coli*).

Currently several organisms are detected in PCR-based assays using probes not based upon ribosomal RNAs. Two examples are detection of *Legionella pneumophila* (Paszko-Kolva, et al., 1995) and enterotoxigenic E. coli. (Stacy-Phipps, et al., 1995). When appropriate, comparisons will need to made empirically to test the specificity and sensitivity of detection using these currently defined primers to newly defined ssu rRNA-based primers and probes.

Finally, viruses, which have no ribosomal RNA genes since they utilize the host cell's protein synthesis machinery, need to have a separate library of primers and probes designed for their detection. Primers and probes have already been defined and tested for most of the viruses that would need to be in an environmental monitor. These include the enteroviruses (Straub, et al., 1994), adenoviruses (Rousell, et al., 1993), rotaviruses (Sethabutr, et al., 1992) and Norwalk virus (DeLeon, et al., 1992; Jiang, X. et al., 1992).

Primer and Probe Prediction

Using the list of organisms discussed in Section 1, the process of designing primers and probes proceeded as follows:

- Sequences were obtained from both the RDP and Genbank Databases
- Sequence alignments from the RDP were refined, and new sequences were added to the alignments
- Evolutionary relationships between the organisms were inferred based upon the aligned ssu rRNA sequences, and a rough evolutionary tree was constructed
- The organisms were grouped into a detection hierarchy
- Conserved and variable regions within the aligned genes were mapped
- Primer and probe sequences were determined based upon the sequence conservation necessary to detect the desired group of organisms
- These primer and probe combinations were analyzed by computer programs for the desired primer and probe characteristics consistent with optimum TaqMan-PCR detection

As a final critical step, these primers and probes must be tested in the laboratory to ensure that the computer-predicted characteristics actually result in a reliable detection system. This process is designed to provide the most efficient means of combining computer analysis and laboratory testing to establish a library of primers and probes. Each of these steps is described in more detail below, along with the results.

Desired Primer and Probe Characteristics

To design primers and probes that will be optimized for TaqMan-based PCR detection, it is necessary to follow a number of guidelines for probe design. These guidelines attempt to ensure that the desired sensitivity, specificity, primability, and overall usefulness of the oligonucleotides are optimized for the established reaction conditions. Some of the parameters that are known to be important in PCR primer design are as follows (McPherson, et al., 1992):

- Specificity for the desired target
- Appropriate melting temperature (formation of stable duplexes)

- Lack of internal secondary structure (dimers and hairpin loops)
- Lack of secondary structure formation with other primers and probes
- GC content between 40 and 60%
- Avoidance of long runs of a single base

and these additional parameters for TaqMan probe design (Livak, et al., 1995):

- No G at the 5' end
- Add a T at the 3' end if not normally present for attachment of the TAMRA quencher
- Located from 1 to 100 bases to the 3' end of the PCR primer
- Melting Temperature at least 5° C higher than the PCR primers

Computer analysis was used to screen potential PCR primer pairs and TaqMan probes to ensure compliance with the above criteria.

Data Analysis

Data collection

As indicated above, the basic genomic sequence information necessary for this project is available through databases that provide public Internet access to the desired sequence data. The Genbank database is the main US repository for sequence data. It is maintained by the National Center for Biotechnology Information (NCBI) under the auspices of the National Library of Medicine, a part of the National Institutes for Health. We maintain tools for searching and retrieval of sequences from this database, as well as maintaining a local copy of the complete database for internal use. In addition, the Ribosomal Database Project (RDP) at the University of Illinois (Maidak et al., 1994) maintains a subset of this database pertaining to ribosomal RNA sequences. This database includes pre-aligned sequences and predictions of evolutionary relationships that greatly facilitate using this information for primer and probe prediction. Genbank and RDP data were obtained through anonymous FTP.

Sequence Analysis

General sequence analysis tools are provided by a comprehensive package of sequence analysis programs published by the Genetics Computer Group (GCG) of Madison, Wisconsin (Devereux, 1994). This package has tools that allow simple pattern recognition, multiple sequence alignment, evolutionary analysis, and most other programs necessary for sequence analysis. This package provided the basic core of analysis tools used in this project.

Evolutionary analysis

In addition to the GCG programs, several other programs were used for evolutionary analysis of aligned sequences. These include Clustal (Higgens, 1991); Phylip (Felsenstein, 1994); and Phylogenetic Analysis Using Parsimony (PAUP) (Swofford, 1993). Evolutionary analysis of the sequence information was an important step in determining which groupings of microorganisms can be effectively detected with a single primer and probe combination.

Primer/Probe analysis

Prediction and analysis of PCR primers and TaqMan probes was accomplished using the OLIGO program from National Biosciences, Inc. (Wojciech, 1994). This program predicts and analyzes oligonucleotides that satisfy the criteria outlined above for optimal PCR and probe characteristics.

Primer and Probe Prediction

Listing of organisms to be detected

The microorganisms listed in table 7.1 formed the basic data set from which a series of PCR primers and TaqMan probes were derived for environmental monitoring. This list of organisms does not include all of the organisms indicated in section 1 as being desirable for detection. This is due to the lack of ssu rRNA sequence information for some microorganisms. As additional sequence information becomes available, additional organisms can be analyzed using the procedures followed below. Never-the-less, contamination by many of the organisms not listed (such as *Klebsiella pneumoniae*, and *Shigella* species) should be detectable by the universal PCR

primers and TaqMan probes listed below. In addition, references were provided above for the detection of additional organisms, including viral contaminants, using PCR and probe-based methods not dependent on rRNAs.

Table 7.1. Microorganisms Analyzed

Prokaryotic Organism	Abbreviation
Acinetobacter	*
Alteromonas	*
Bacillus coagulans	B-coagu
Burkholderia cepacia	Bur-cep
Burkholderia pickettii	Bur-pick
Corynebacterium	
Enterococcus avium	Eco-avi
Entercoccus faecium	Eco-fcm
Enterococcus faecalis	Eco-fae
Escherichia coli	E-coli
Legionella pneumophila	Leg-pne
Listeria	*
Micrococcus luteus	Mic-Luteus
Mycoplasma fermentans	M-ferme
Mycoplasma hominis	M-homin
Mycoplasma pneumonia	M-Pneum
Pseudomonas aeruginosa	Ps-aeru
Salmonella cholera	S-chole
Salmonella dublin	S-dubli
Salmonella enteritidis	S-enter
Salmonella paratyphi	S-parat
Salmonella typhi	S-typhi
Staphylococcus aureus	Stp-aureus
Staphylococcus epidermidis	Stp-epider
Staphylococcus haemolyticus	Stp-haemo
Staphylococcus hominis	Stp-homin
Staphylococcus saprophyticus	Stp-saprop
Staphylococcus warneri	Stp-war
Streptococcus bovis	Stc-bovis
Streptococcus equinis	Stc-equins
Thiobacillus ferrooxidans	Thb-fer
Ureaplasma urealyticum	Upl-ure
Vibrio cholerae	V-chole
Vibrio parahaemolyticus	V-parah
Vibrio vulnificus	V-vulni

Eukaryotic Organism	Abbreviation
Aspergillus fumigatus	Asp-fuki
Candida albicans	Cnd-albc
Cryptosporidium parvum	Crp-parv
Cryptococcus neoformans	*
Entamoeba histolytica	Ent-hist
Girardia lamblia	Gir-lamb

* These organisms are not displayed in the sequence alignment or analyzed for Figure 7.4 (see below), but were analyzed for detectability using the primer and probes oligonucleotides indicated in Table 7.2.

Alignment

The sequences of the ssu rRNAs for these sequences were obtained from the RDP and Genbank databases. These sequences were reformatted as necessary for use in subsequent analyses. The RDP also provided sequence alignments and evolutionary trees for these RNAs. Where necessary, these alignments were refined, and additional sequences added that were not present in the RDP database. Programs in the GCG package were used for these purposes.

The alignment of the ssu rRNA sequences is shown in Figures 7.1 (prokaryotic) and 7.2 (eukaryotic). Gaps have been introduced into the sequences to account for evolutionary changes due to insertions and deletions into sequence lineages. Gaps are represented by dashes. Also shown are the positions of the variable regions that are interspersed with more conserved sequences as these RNAs evolved (see below). The positions of the predicted set of PCR primers and TaqMan probes is also shown (see below). The eukaryotic alignment includes Human and *E. coli* ssu rRNA sequences for reference purposes.

Ribosomal RNA Secondary Structure and Sequence Conservation

As discussed above, one of the features of ssu rRNAs that make them particularly suitable for environmental monitoring are the conserved and variable sequence features that are interspersed throughout these genes (Hillis, et al., 1991; Neefs, et al.,

1991). These RNAs must form secondary and tertiary structures to function as components of the protein-synthesizing ribosomes. Certain features of these RNAs must be maintained for functional purposes, while other features need not be strictly conserved, and can vary. This results in alternating patterns of conserved and variable domains seen when comparing ssu rRNA sequences from different species. Figure 7.3 shows the predicted secondary structure for the *E. coli* ssu rRNA, and the conserved and variable region domains. Conserved features can be utilized to derive universal PCR primers and TaqMan probes that will bind to, amplify, and detect ssu rRNAs from a wide variety of organisms, while additional TaqMan probes can be designed from the more variable regions that would be very specific and detect only one particular species.

Figure 7.4 is a graph showing the extent of evolutionary change for three separate groups of sequences. The top, blue shaded graph is for the alignment of all of the prokaryotic organisms indicated in table 7.1. The middle, pink shaded graph analyzes the gram-negative organisms from the above list, and finally, the bottom, yellow shaded graph shows the similarity among the *Mycoplasma* species. To generate this data, the aligned set of sequences were grouped according to their evolutionary relationships (see below), and then the program MacClade (Maddison and Maddison, 1992) was used to calculate the extent of evolutionary change at each position in the sequence alignment. The Y-axis is proportional to the number of sequence changes that have occurred at each alignment position as these sequences (organisms) have diverged over the course of evolutionary history. The greater the divergence, the greater the number of evolutionary changes, and the higher the value seen on the Y-axis. As can be seen, as the set of organisms analyzed is reduced to those that are more closely related, the extent of sequence identity and evolutionary conservation increases. Never-the-less, the variable rates of evolution can be clearly seen even among just the *mycoplasma* group by noting the interruption of highly identical (conserved) regions with extremely variable regions. This information provided the basis by which the location of potential PCR primers and TaqMan probes were determined that could be used to detect specific groupings of organisms.

Sequence Evolution

The sequence alignments in Figures 7.1 and 7.2 were used to construct the evolutionary trees in Figures 7.5 (prokaryotic) and 7.6 (eukaryotic). These trees show the evolutionary relationship between these organisms as calculated by Maximum Likelihood methods using Phylip (Felsenstein, 1994) and fastDNAml (Olsen, 1994). The trees displayed are based upon data obtained from the RDP (Maidak, et al., 1994). These relationships were confirmed using additional analysis methods based upon maximum parsimony using PAUP (Swofford, 1993), and neighbor-joining using Clustal (Higgens, 1991) and GCG (Devereux, 1994), These trees are shown only to indicate approximate evolutionary relationships between these organisms. No attempt was made to clearly define the branching order between closely related sequences (and thus define the common ancestry and evolutionary lineages of these organisms).

The length of the horizontal branches are proportional to the extent of sequence divergence among these sequences. Therefore, these figures show both the inferred evolutionary relationships and the extent of evolutionary change. For the purposes of environmental monitoring by PCR, we are only concerned with the sequence relationships and how these organisms can be grouped together. The prokaryotic evolutionary tree clearly shows the division between gram-negative and gram-positive organisms. Other relationships are as expected, and these groupings formed the basis of determining primer/probe combinations that could be used in a hierarchical detection scheme.

Primer and Probe Prediction

Using the data from the above analyses, a set of PCR primers and TaqMan probes were predicted that could be used in a PCR-based environmental monitor. These primers and probes were predicted with the aid of the OLIGO program (Rychlik, 1994) along with direct visualization of the alignment—looking for regions showing the appropriate conservation and/or divergence necessary for the indicated specificity. OLIGO was initially used to derive a set of compatible PCR primer pairs that meet all of the criteria indicated above. Each of these primer pairs were than located on the

sequence alignment and visually analyzed to determine primer pairs that would best satisfy the criteria of providing a set of universal primers for amplifying prokaryotic sequences, and another set for eukaryotic sequences. After these sets of universal PCR primer pairs were established, a combination of OLIGO and direct visualization was again used within the confines of the PCR-amplified product, to predict sets of TaqMan probes that again satisfy the criteria outlined above for optimal probe design. The primers and probes that resulted from this analysis meet the above criteria to the extent possible for optimal activity. Empirical testing will of course need to be performed to ensure the adequacy of these oligos for their intended purpose. This includes assaying for the desired sensitivity to amplify and detect the indicated organisms, and the desired specificity in only detecting the intended group of organisms.

The location of the PCR primers and the TaqMan probes are indicated on the sequence alignments in figures 7.1 and 7.2. The sequences of these primers and probes, their locations, and their predicted melting temperatures (T_m) are listed in table 7.2.

Table 7.2. PCR Primers and TaqMan Probes

Prokaryotic

Name	Sequence	Location	Description	T_m °C
PCR Primers				
U	GGGGAGCAAACAGGATTAGA	E-coli:773U20	Universal Upper	64.0
L	AAGGGCCATGATGACTTGAC	E-coli:1193L20	Universal Lower	64.1
Probes				
Uni	CCTGGTAGTCCACGCCGTAAACGAT	E-coli:796U25	Universal	76.8
GmP	TGAGTGCTAAGTGTTAGGGGGTTTCCt	Stp-aur:U828	Gram Positive	73.7
Enteric	TCGACTTGGAGGTTGTGCCCTTGAGt	E-coli:822U25	*E. coli, Vibrio, Salmonella sp.*	77.8
Legion	TGAAAATAATTAGTGGCGCAGCAAAt	Leg-Pne:842U25	*Legionella sp.*	72.9
Burk	TTGTTGGGGATTCATTTCCTTAGTAACt	Bur-Cep:824U27	*Burkholderia*	71.2
Ps	TCCTTGAGATCTTAGTGGCGCAGCT	Ps-Aeru:833U25	*Pseudomonas sp.*	75.2
Thb	TGGGTACTAGACGTTGGGAGGTTTAt	Thb-Fer:661U25	*Thiobacillus*	70.9
Myco	TAACTAACGAAAGGGGTTGCGCTCGt	Upl-Ure:1094L25	*Mycoplasma sp.*	77.2

Eukaryotic

Name	Sequence	Location	Description	T_m °C
PCR Primers				
U	ACATCTAAGGAAGGCAGCAG	Crp:371U20	Universal Upper	61.8
L	CGATCCCCTAACTTTCGTTC	Ent:952L20	Universal Lower	63.8
G-U	ACATCCAAGGACGGCAGCAG	Gir:322U20	*Girardia* Upper	70.3
G-L	GCCTTCGCCCTTGATTGACA	Gir:713L20	*Giardia* Lower	70.4
Probes				
Fungi	CTTTTGGGTCTCGTAATTGGAATGAt	Asp:489U25	*Aspergillus, Candida, Cryptococcus*	71.2
Crp	CAATACAGGGCCTAACGGTCTTGTAt	Crp:440U25	*Cryptosporidium*	71.4
Ent	TGTTCCTTTTAATCCTTCTCTCGAAt	Ent:827L25	*Entamoeba*	68.6
Gir	CGGTCTCGGCGGGATCATCCTGTTT	Gir:656L25	*Giardia*	82.1

Table 7.2. PCR Primers and TaqMan Probes. The composition of the predicted optimal PCR primers and TaqMan probes are listed for prokaryotic and eukaryotic monitoring. The oligo sequences are written 5' to 3' in the orientation necessary for synthesis. Therefore for upper strand oligos, the indicated sequence is the same as what would be seen in the sequence alignments (Figures 7.1 and 7.2), while for lower strand oligos, the sequence shown represents the reverse-complement of the sequence in the sequence alignments.

A lower case t at the 3' end of a probe sequence indicates the necessary addition of a non-templated T to the end of the probe to which the TAMRA quencher will be added. The fluorescent reporter dye should be added to the base at the 5' end of the probe sequences.

The oligo location indicates the organism from which the sequence information was derived, the number of the sequence base (this number excludes gaps introduced for alignment purposes) at the left-most position of the oligonucleotide as the sequence is viewed in the 5' to 3' direction of the rRNA. Therefore for oligos derived from the upper DNA strand (U in the location designation), this number represents the base at the 5' end of the oligonucleotide. For oligos derived from the lower DNA strand (L in the location designation), this number represents the base at the 3' end of the oligonucleotide. The L or U designation in the location is followed by a number indicating the length of the oligonucleotide.

The melting temperature—T_m of each oligo is predicted using the nearest-neighbor method as implemented by the program OLIGO. These are indicated for reference purposes and are useful in comparing the melting temperature properties of one oligo to another, but the actual melting temperatures will vary with reaction conditions, and will have to be determined empirically.

The PCR primers consist of a set of universal forward and reverse oligos that should be able to amplify DNA from any of the prokaryotic organisms, and another set for the

eukaryotic microorganisms with the exception of *Giardia*. An alternate set of PCR primers was necessary for *Giardia* due to the extensive divergence of it's ssu rRNA sequence from the other eukaryotic organisms.

The TaqMan probes consist of a number of different probes designed to detect particular groupings of organisms based upon similarities in specific regions of their ssu rRNA sequences. These groupings are shown in figures 7.5 and 7.6. along with the intended targets for each of the TaqMan probes. The prokaryotic probes are designed to detect either all of the organisms using a universal probe; a probe for gram-positive organisms; a probe for *mycoplasma* species; a probe to detect gram-negative enterics including *E. coli*, *Vibrio*, and possibly *Salmonella* species; a *Legionella*-specific probe; two probes specific for different species of *Burkholderia* and *Pseudomonas*; and a *Thiobacillus*-specific probe.

In addition to the organisms specifically analyzed in Figures 7.1 and 7.2, the universal probe should also detect most other organisms that might be of concern as environmental contaminants. The universal prokaryotic probe falls within an extremely conserved domain of the prokaryotic ssu rRNAs. All prokaryotic organisms examined, including many organisms not specifically mentioned here, should be detected by this probe. We specifically looked at the ability of the universal probe to detect several organisms that may prove to be rare environmental contaminants, but would be important, never-the-less, to be detected by an environmental monitor. These include *Listeria*, *Corynebacterium*, *Acinetobacter*, and *Alteromonas* species. All of these organisms should be detected by the universal probe. If deemed necessary, probes specific for the detection of these, and other possible environmental contaminants can be designed and tested, using the same procedures outlined in this report.

The *Legionella* probe should efficiently detect all types of *Legionella pneumophila*. Sequence analysis also indicates that it may also function as a universal *Legionella* probe detecting other Legionella species as well. Only empirical testing will ensure the applicability of this probe as a universal *Legionella* probe. Alternatively, universal

primer/probe combinations already described in the literature may be used as desired (Paszko-Kolva, et al., 1995).

The *Pseudomonas* probe should efficiently detect *Pseudomonas aeruginosa.* Sequence analysis also indicates that it might function as a universal *Pseudomonas* probe detecting other *Pseudomonas* species as well. The diversity of ssu rRNA sequences between different *Pseudomonas species* makes prediction of a universal *Pseudomonas* probe difficult. Only empirical testing will ensure the applicability of this probe as a universal *Pseudomonas* probe.

For the eukaryotic microorganisms, a universal fungi probe was designed to detect the presence of various Fungi including *Aspergillus, Candida,* and Cryptococcus species. Cryptococcus is not specifically listed in Table 7.3 or Figure 7.2, but analysis of Cryptococcus ssu rRNA sequences indicates that it should be detected using this probe. Specific probes were also designed to detect *Cryptosporidium, Entamoeba,* or *Giardia species* . It was not possible to design a universal eukaryotic probe due to the more extensive divergence of these ssu rRNA sequences in comparison to the prokaryotic sequences.

Primer and Probe Analysis

To ensure to the extent possible that the set of primers and probes predicted above satisfy the criteria for sensitivity and specificity of detection, a feature of the OLIGO program was used to quantify the ability of each of the oligos to hybridize to the different ssu rRNAs. OLIGO includes a priming efficiency (PE) statistic that attempts to infer the binding probability of a specific oligo to a specific sequence. The PE statistic includes analysis of base content, sequence mismatches, duplex stability, and terminal stability of the oligo. Table 7.3 lists the PE for all prokaryotic and eukaryotic primers and probes, along with the intended PCR product size and location for each of the ssu rRNA sequences.

Table 7.3: PCR Product; PCR Primer; and TaqMan Probe Statistics

Prokaryotic	*PCR Product*			*PCR Primers*		*TaqMan Probes*							
Organism	*Size*	*Start*	*End*	*U*	*L*	*Uni*	*GmP*	*Enteric*	*Legion*	*Burk*	*Ps*	*Thb*	*My*
			Max. P.E.:	*440*	*437*	*562*	*552*	*540*	*538*	*533*	*542*	*507*	*566*
B-coagu	439	774	1193	*311*	*290*	*562*	*448*	109	87	65	95	97	*389*
Bur-cep	435	767	1182	*440*	*368*	*465*	138	66	165	*533*	128	91	*403*
Bur-pic	435	725	1140	*440*	*368*	*465*	152	80	165	*533*	128	91	*384*
Eco-avi*													
Eco-fcm	440	792	1212	*440*	*290*	*562*	*420*	197	*212*	120	0	64	*413*
Eco-fae*													
E-coli	440	773	1193	*440*	*437*	*562*	123	*540*	170	81	130	13	*379*
Leg-pne	442	773	1195	*440*	*437*	*456*	140	85	*538*	71	105	86	*404*
Mic-Luteus	443	752	1175	*381*	*290*	*291*	174	97	114	67	158	115	*340*
M-ferme	426	768	1174	*440*	*245*	*249*	108	14	190	147	*282*	110	*414*
M-homin	377	766	1123	*440*	*245*	*562*	101	38	178	84	207	32	*442*
M-Pneum	418	771	1169	*338*	*290*	*410*	91	31	138	65	108	77	*466*
Ps-aeru	440	767	1187	*440*	*437*	*562*	144	205	*255*	96	*542*	63	*412*
Salmonella*													
Stp-aureus	442	781	1203	*374*	*290*	*562*	*542*	24	149	163	203	31	*371*
Stp-epider	440	782	1202	*374*	*290*	*562*	*542*	24	155	65	203	31	*371*
Stp-haemo	440	773	1193	*374*	*290*	*562*	*542*	142	149	65	203	31	*371*
Stp-homin	440	773	1193	*374*	*290*	*562*	*542*	24	149	65	203	31	*371*
Stp-saprop	442	754	1176	*374*	*290*	*562*	*542*	131	149	65	203	88	*371*
Stp-war*													
Stc-bovis	440	781	1201	*440*	*290*	*562*	*313*	192	167	137	108	184	*459*
Stc-equins	440	675	1095	*440*	*290*	*512*	*303*	93	184	139	*268*	190	*460*
Thb-fer**	308	614	902	*440*	*298*	*562*	150	98	176	44	0	*507*	*372*
Upl-ure	415	772	1167	*383*	*290*	*410*	0	11	126	38	143	158	*566*
V-chole	441	771	1192	*440*	*437*	*562*	122	*375*	181	17	21	13	*366*
V-parah	441	771	1192	*440*	*437*	*479*	122	*378*	181	33	103	58	*366*
V-vulni	441	771	1192	*440*	*437*	*456*	122	*393*	181	65	103	64	*366*

Eukaryotic	*PCR Product*			*PCR Primers*				*TaqMan Probes*			
Organism	*Size*	*Start*	*End*	*U*	*L*	*G-U*	*G-L*	*Fungi*	*Crp*	*Ent*	*Gir*
			Max. P.E.:	*420*	*443*	*465*	*476*	*508*	*523*	*490*	*594*
Asp-fumi	583	408	971	*383*	*443*	*388*	108	*508*	24	121	204
Cnd-albc	570	408	958	*387*	*443*	*388*	108	*439*	*295*	204	79
Crp-parv	557	371	908	*420*	*443*	*338*	108	*323*	*523*	153	92
Ent-hist	567	405	952	*383*	*443*	*304*	175	111	92	*490*	93
Gir-lamb	411	322	713	*339*	11	*465*	*476*	51	98	0	*595*
Human	591	458	1029	*387*	*278*	*388*	116	*320*	*260*	95	78
E-coli	443	338	761	*279*	100	*234*	112	82	7	42	103

* No sequence information available within the PCR primer region

** Limited sequence information within the PCR primer region

Table 7.3: PCR Product; PCR Primer; and TaqMan Probe Statistics. For each prokaryotic and eukaryotic organism listed in table 7.1, the size and the start and stop positions (numbered excluding alignment gaps) of the expected PCR product using either the universal prokaryotic or eukaryotic primers is shown. For *Girardia lamblia*, the Giardia-specific PCR primers are used.

For each of the different PCR primers and TaqMan probes, the priming efficiency (PE) value as calculated by the program OLIGO is shown for each organism. The higher the PE value, the greater the chance that the indicated oligo will hybridize to the indicated sequence. Values above 250 are highlighted in bold type.

These PE statistics provide a rough guide as to the potential sensitivity and specificity of each of the primers and probes. As indicated previously, all of these combinations will need to be tested empirically because the PE values may not necessarily represent the true ability of some of the probes to function as intended. For example, the *mycoplasma*-specific probe shows high PE values for all of the prokaryotic ssu rRNA sequences. Even though the *mycoplasma* sequences show the highest values, it might be assumed that this probe would act more as a universal probe rather than a *mycoplasma*-specific probe. In this instance the PE values may be misleading. For a TaqMan probe to function, it is important that the 5' end of the probe be efficiently base-paired to the sequence template to allow for the nuclease activity of the Taq polymerase to cleave the 5'-fluorescently-labled base of the probe away from the rest of the probe oligo and the TAMRA quencher on the 3' end. The *mycoplasma*-specific probe shows a fair degree of homology to non-*mycoplasma* sequences at the 3' end of the probe. Much less homology exists at the 5' end of this probe to non-*mycoplasma* sequences. Therefore, we would predict that in spite of the high PE values for non-*mycoplasma* sequences, this probe may still function specifically to detect only ssu rRNAs from *mycoplasma* species.

Limitations of Computer Prediction

All of the analyses performed for section 7 rely on translating molecular biological knowledge into computer programs that try to make biological predictions based upon our current understanding of biological processes. While these programs provide a useful basis to make the sorts of predictions seen above, the limitations of these predictions must always be considered. The process of primer and probe prediction is necessarily a reiterative one in which the initial computer-predicted oligos are tested in the laboratory by using them to detect samples of actual microorganisms under conditions that come as close as possible to those utilized by an environmental monitor. Following the initial round of laboratory testing, primer and probe sequences will need to be refined as necessary, and the testing repeated until the desired characteristics are obtained. This process should eventually lead to a functional and efficient monitor for the detection of microorganisms in environmental samples.

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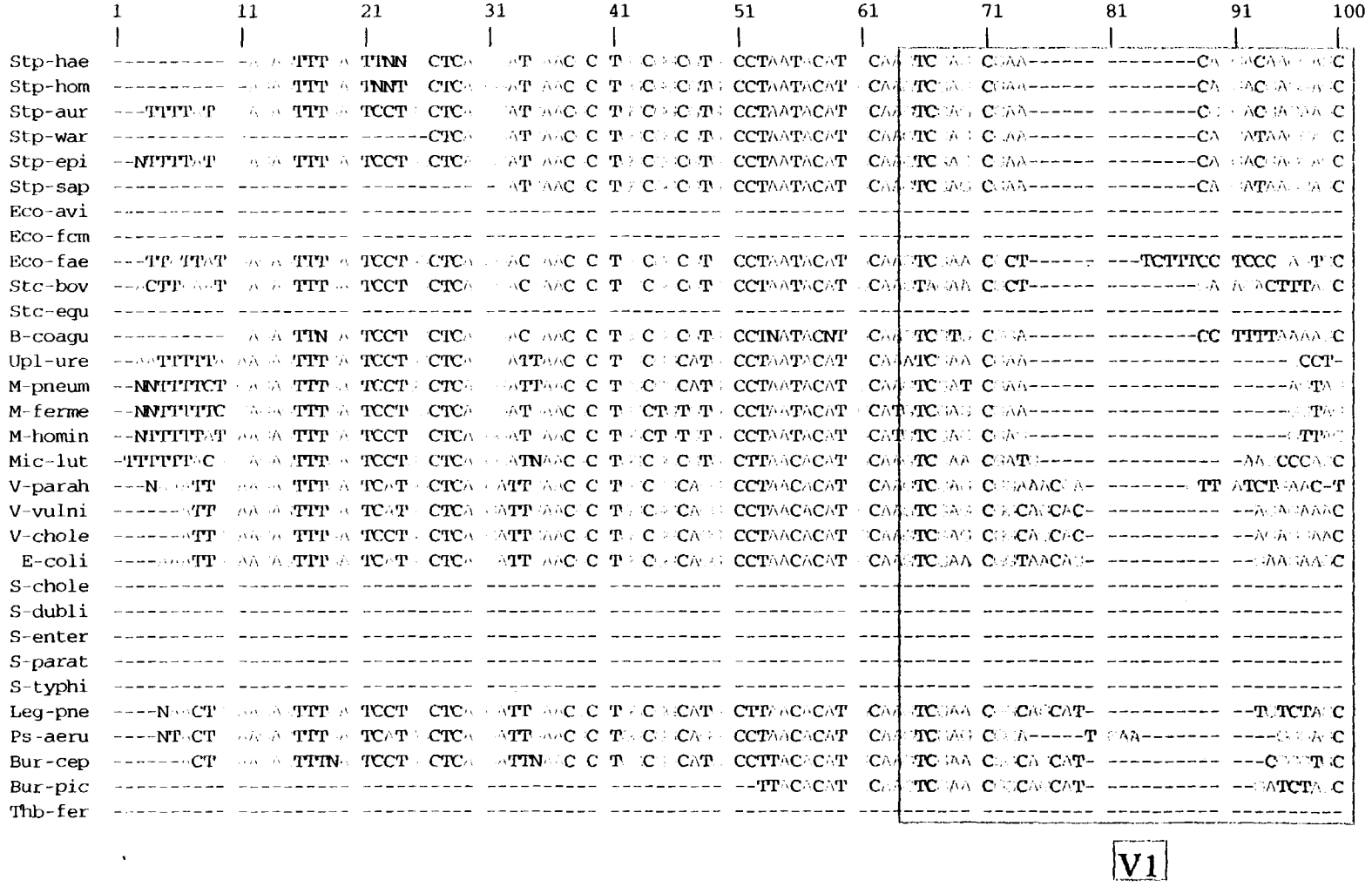
Figure 7.1. Alignment of prokaryotic ssu rRNA sequences.

Alignments of the ssu rRNA sequences of the prokaryotic microorganisms listed in table 7.1 are shown. Gaps in an alignment position are indicated by dashes. Numbering across the top of the alignment include gapped positions.

The variable regions of the ssu rRNAs are shaded in gray, and labeled in red. These correspond to the variable regions discussed in Neefs, et al., 1991.

The position of the upper and lower PCR primers are shaded in light blue. The position of the TaqMan probes listed in table 7.2 are also indicated. The numbering has been altered due to the inclusion of gaps in the numbering of the alignments.

Data used in preparing this figure were derived from the Ribosomal Database Project (RDP) accessed at the University of Illinois in Urbana, Illinois via FTP (Maidak, et al., 1994). Some of these sequences were taken from release 4.1 of the RDP, October, 1994.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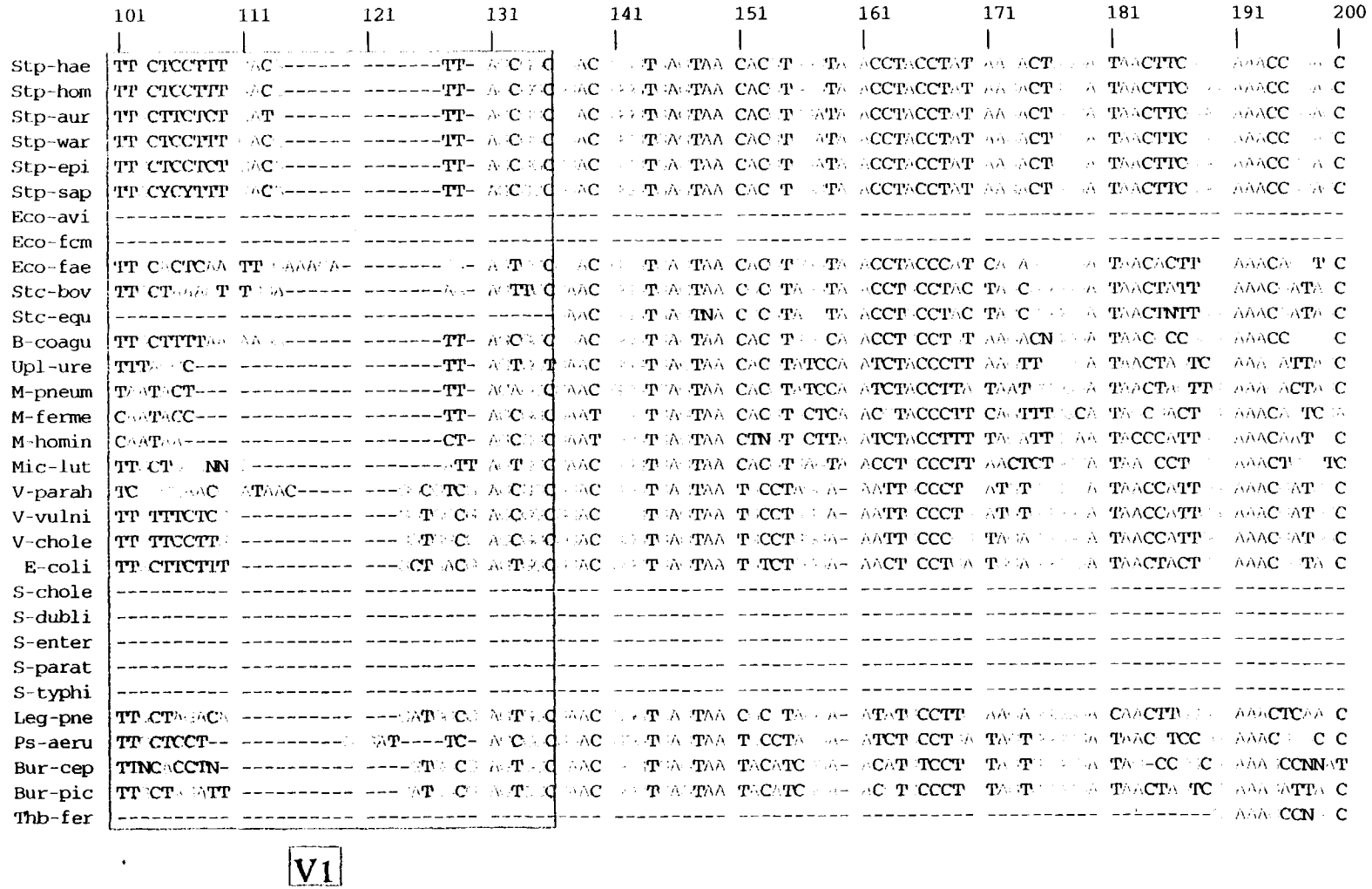
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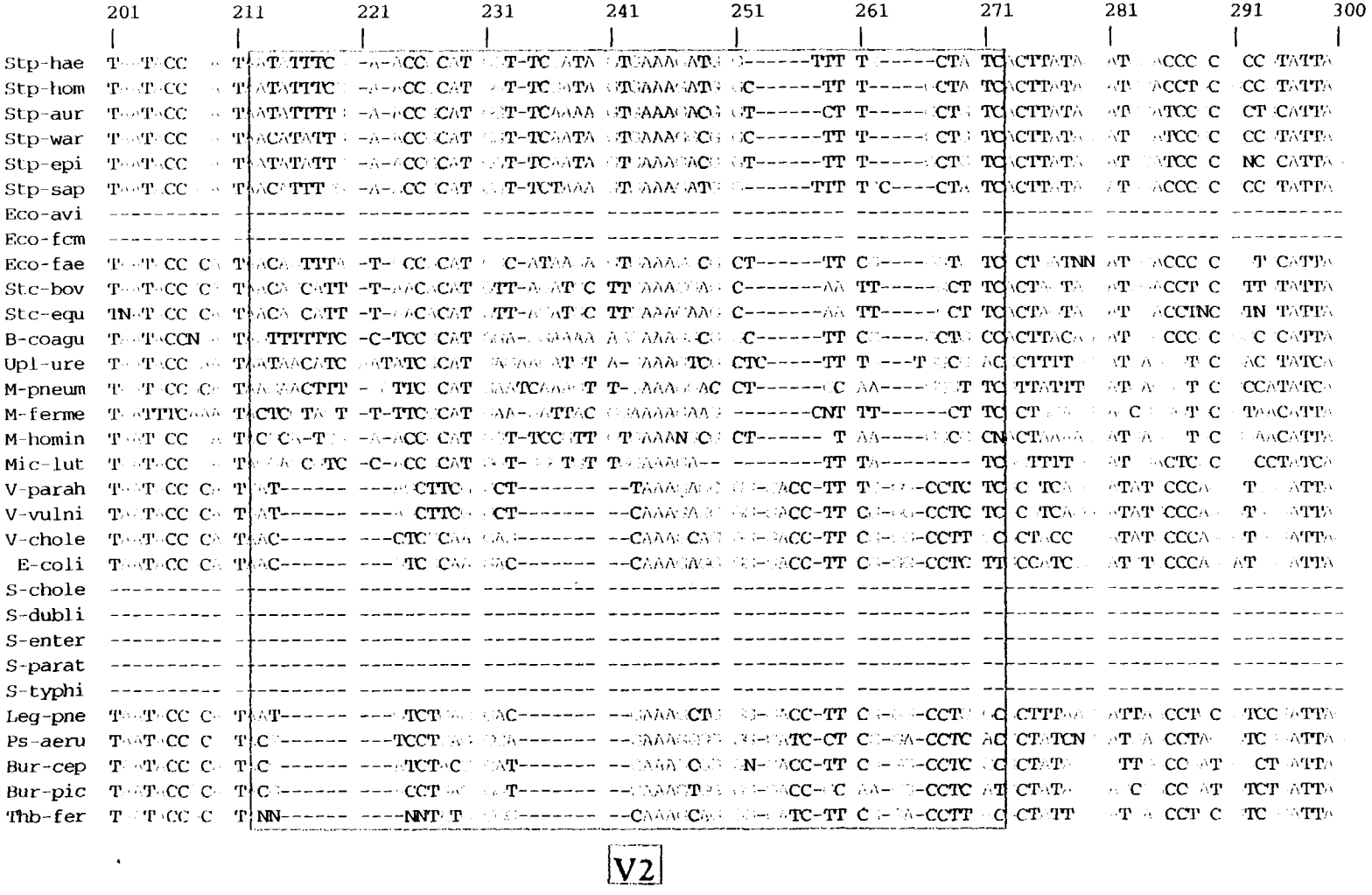
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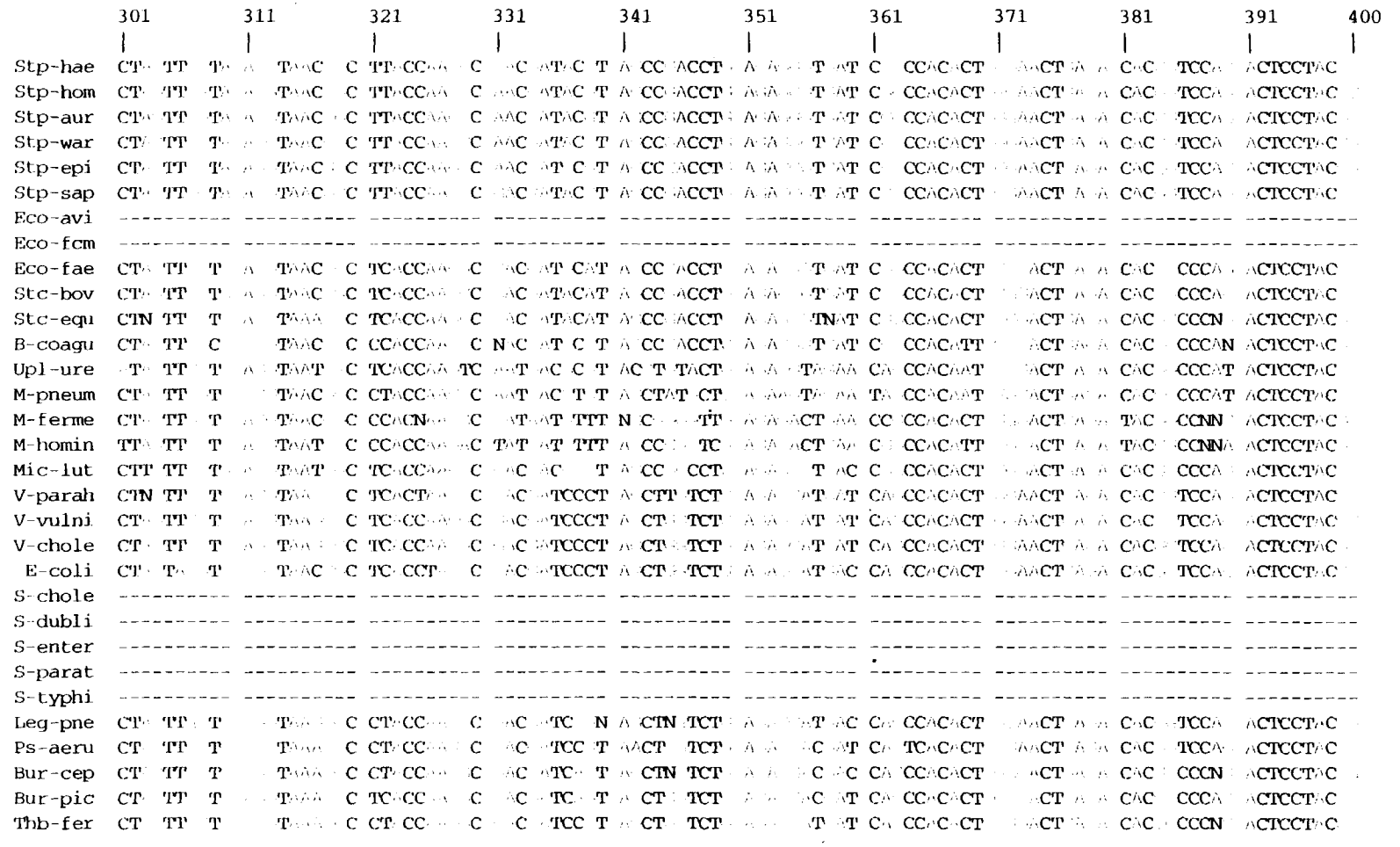
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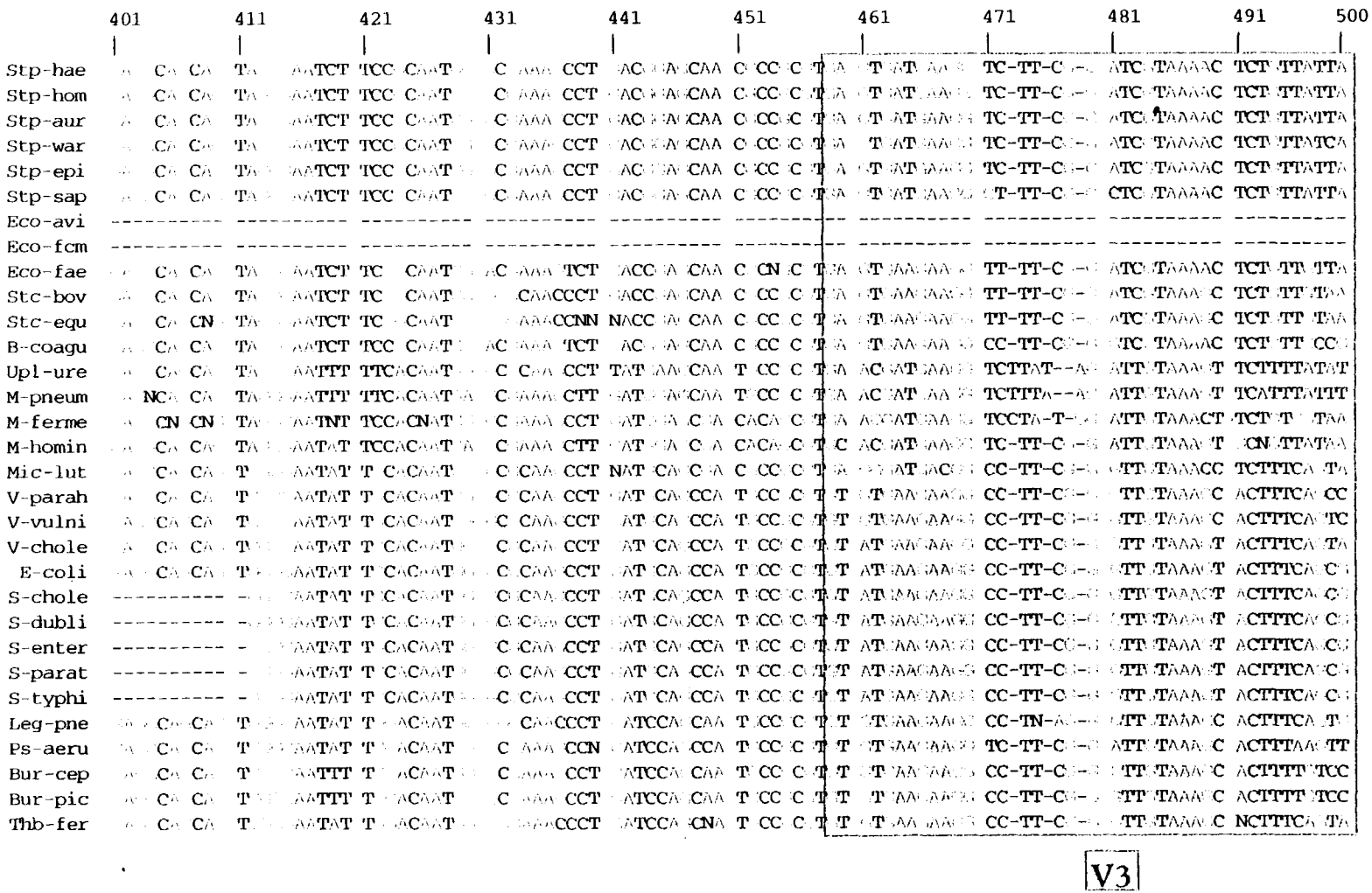
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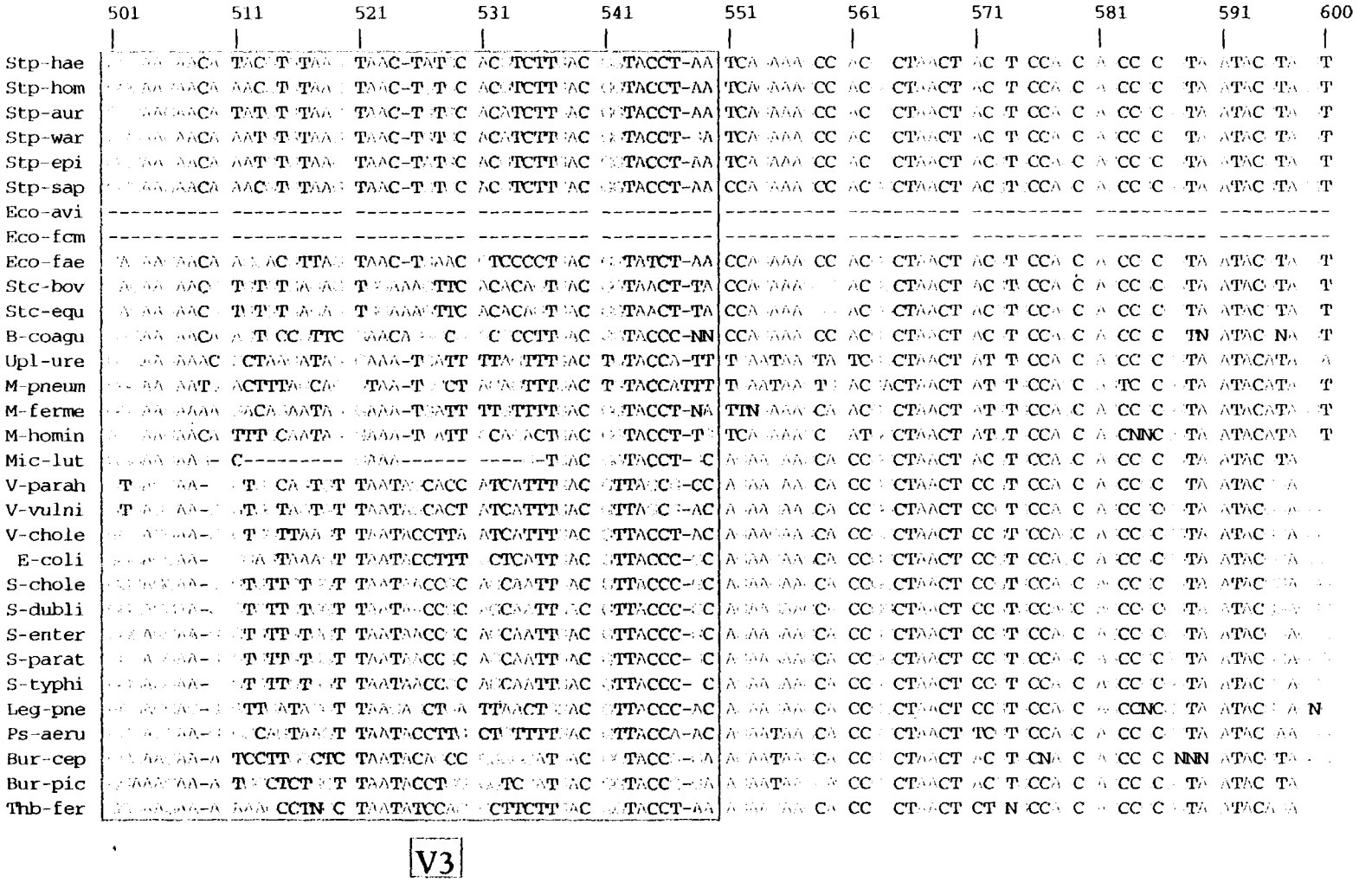
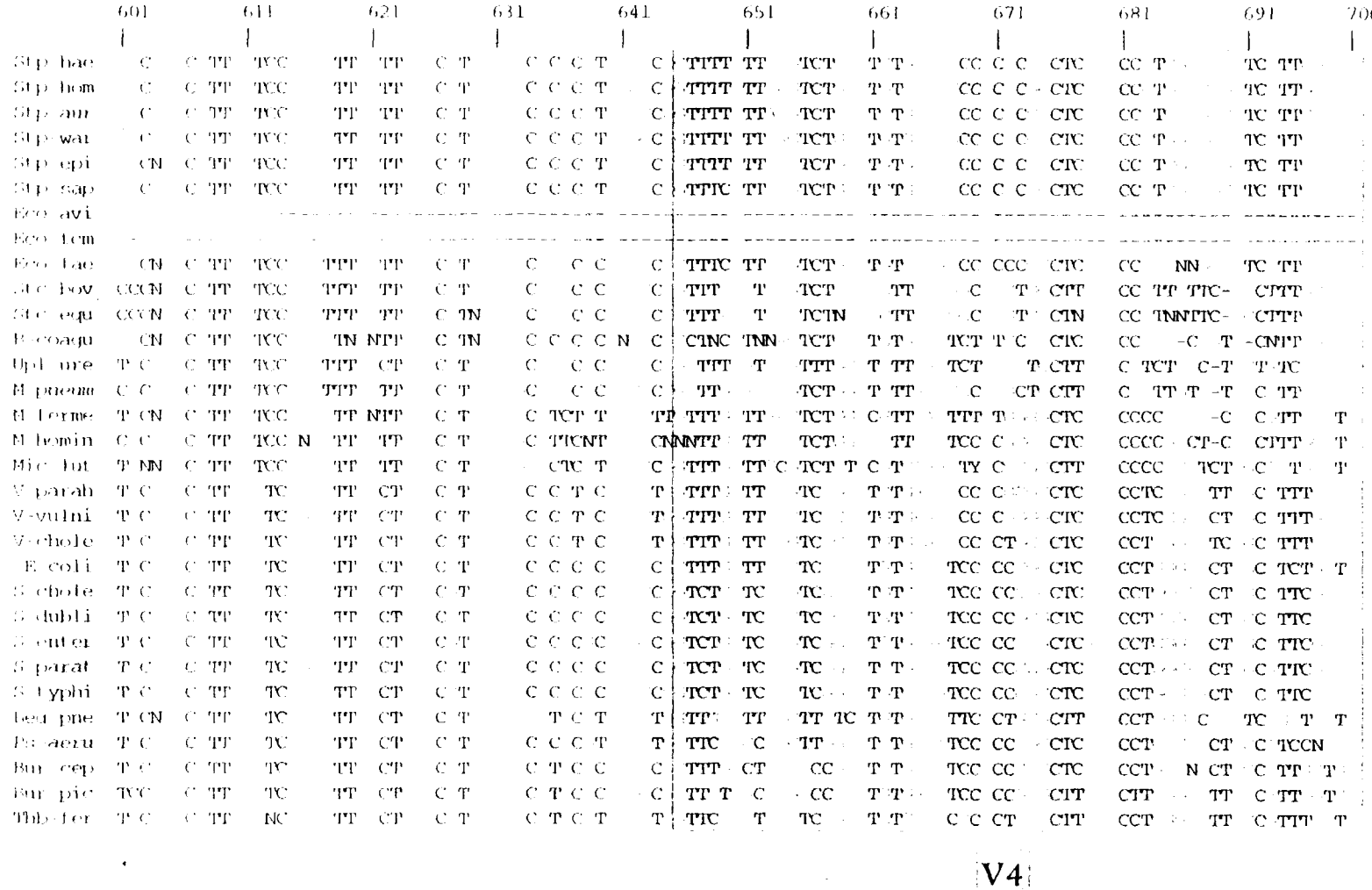
Prokaryotic ssu-rRNA Alignment

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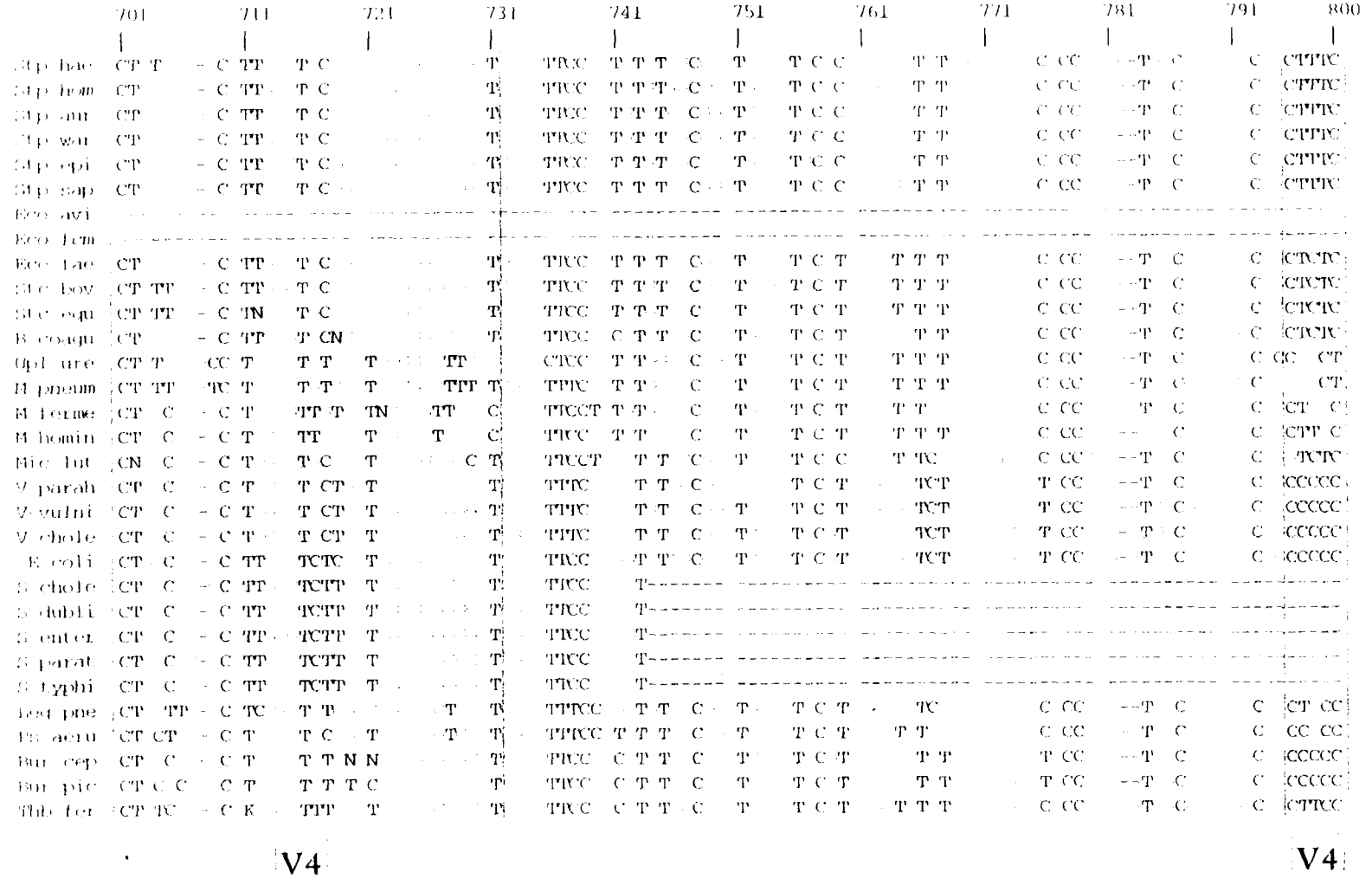
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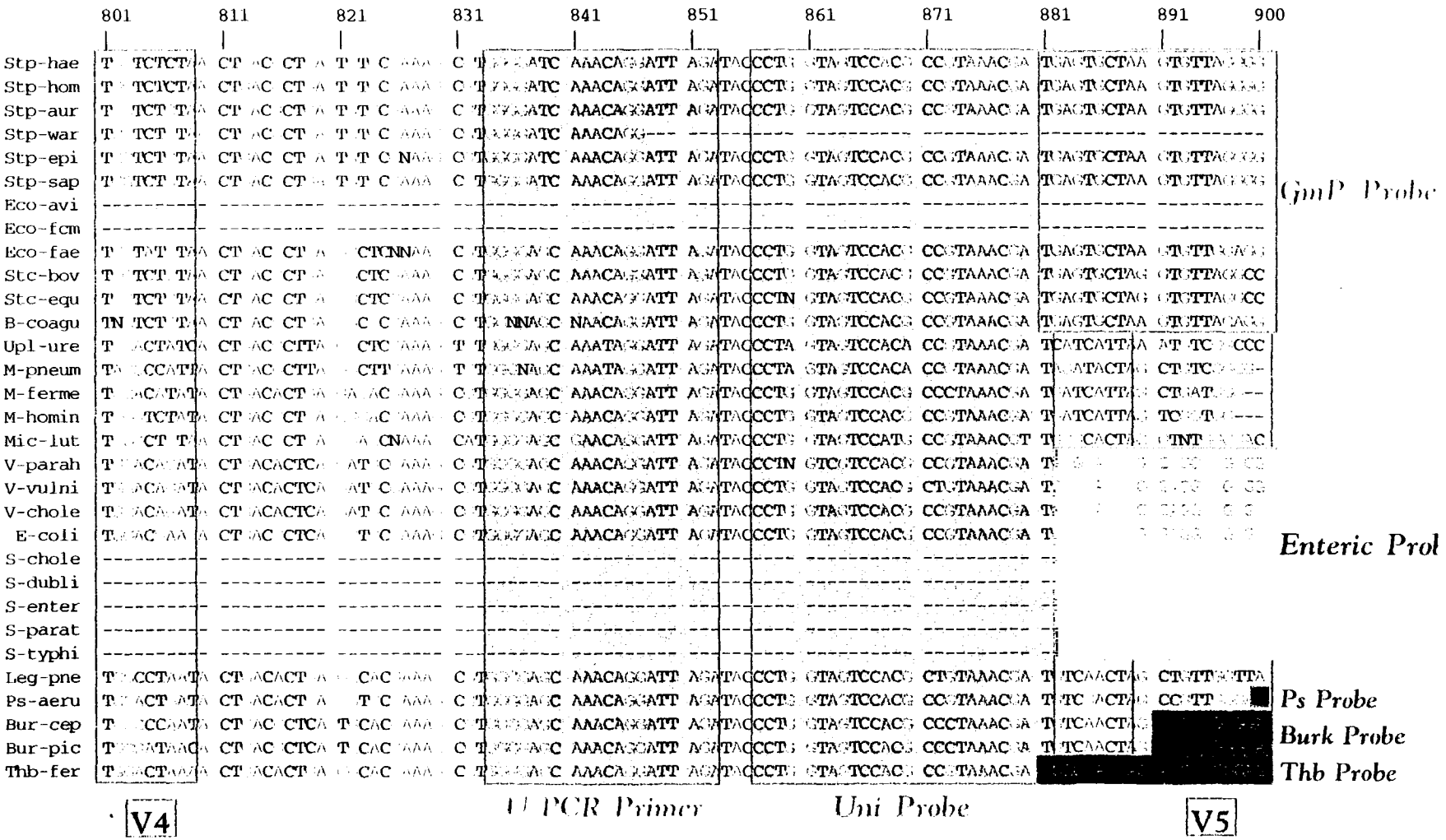
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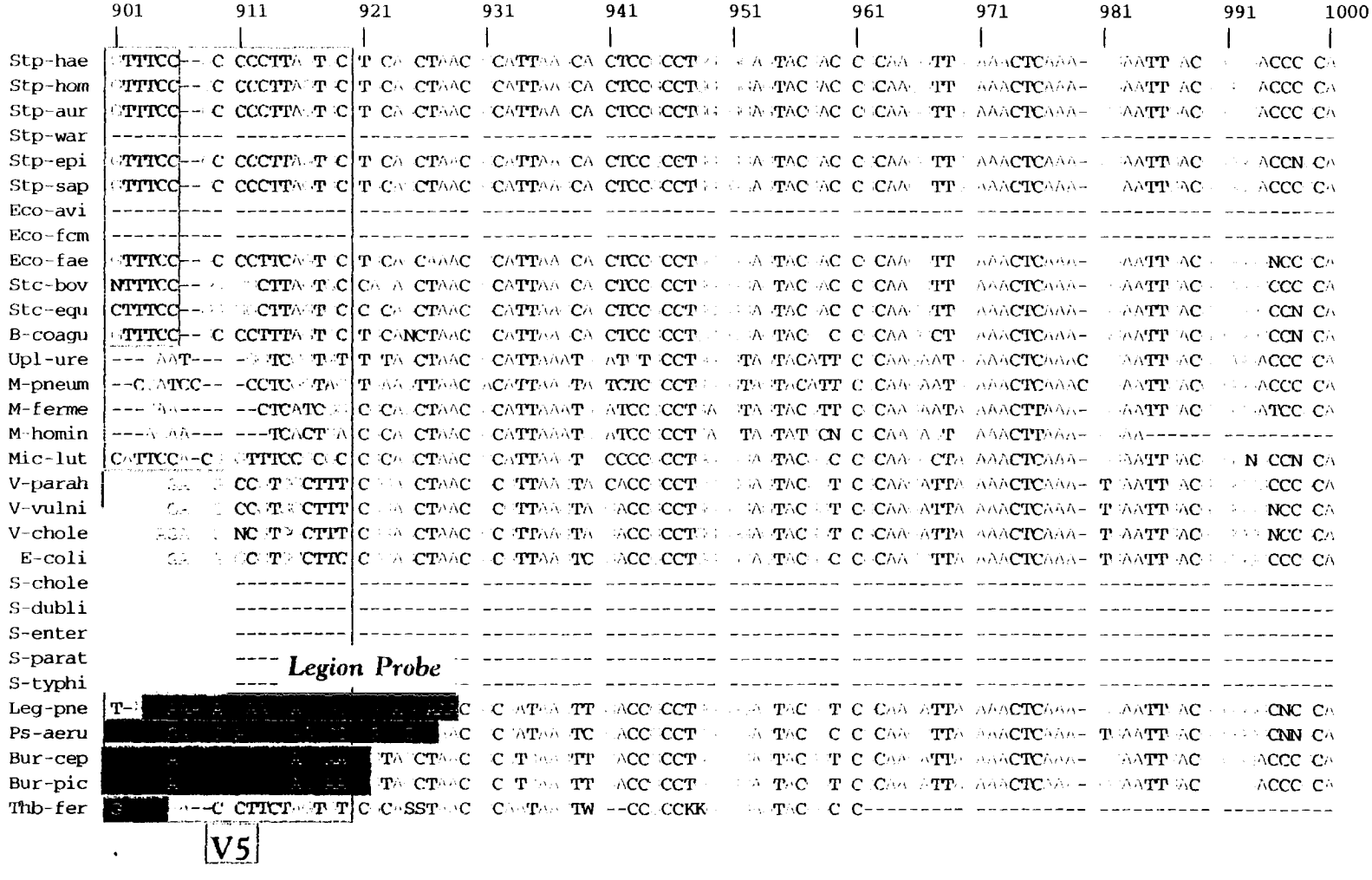
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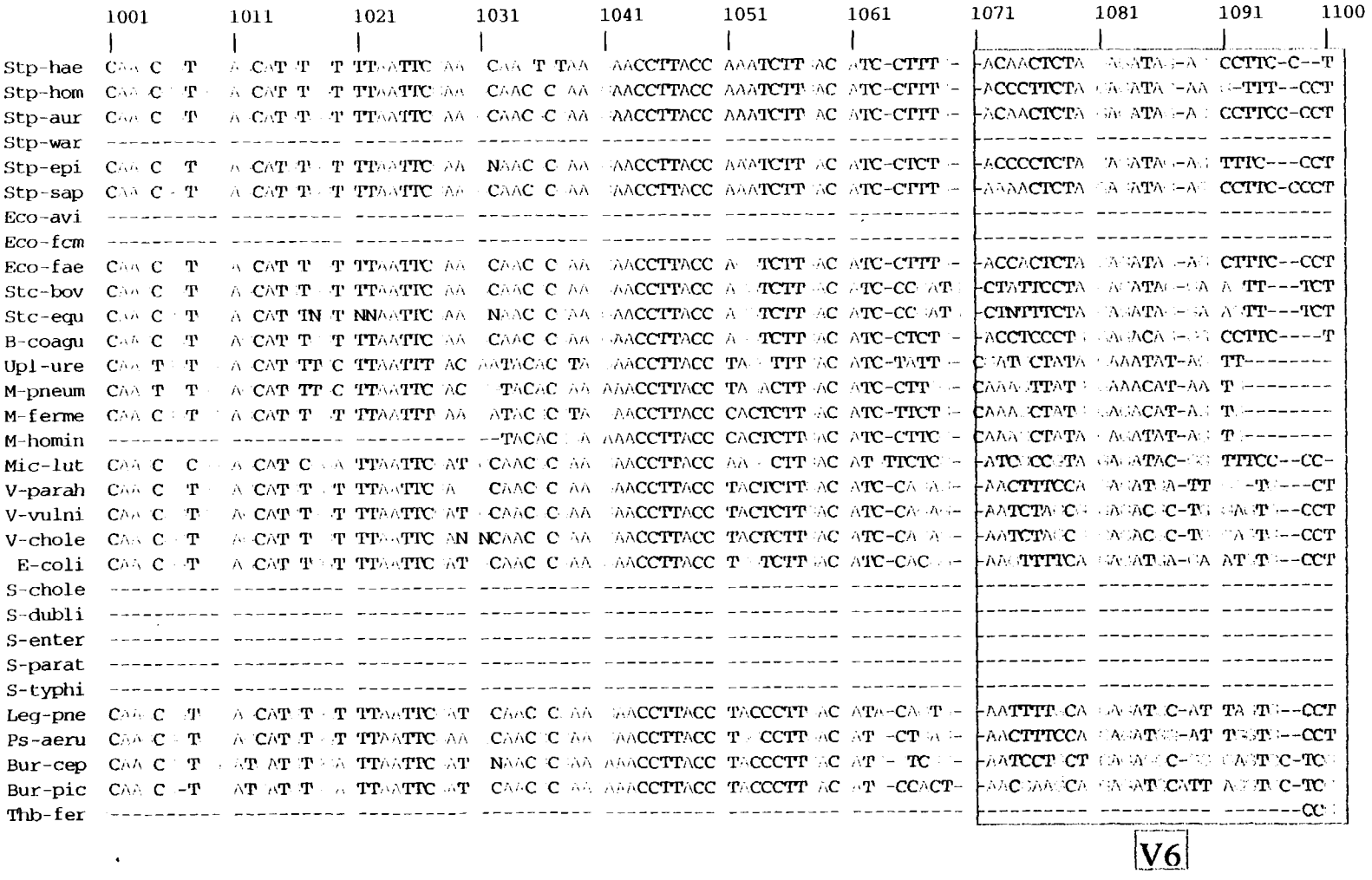
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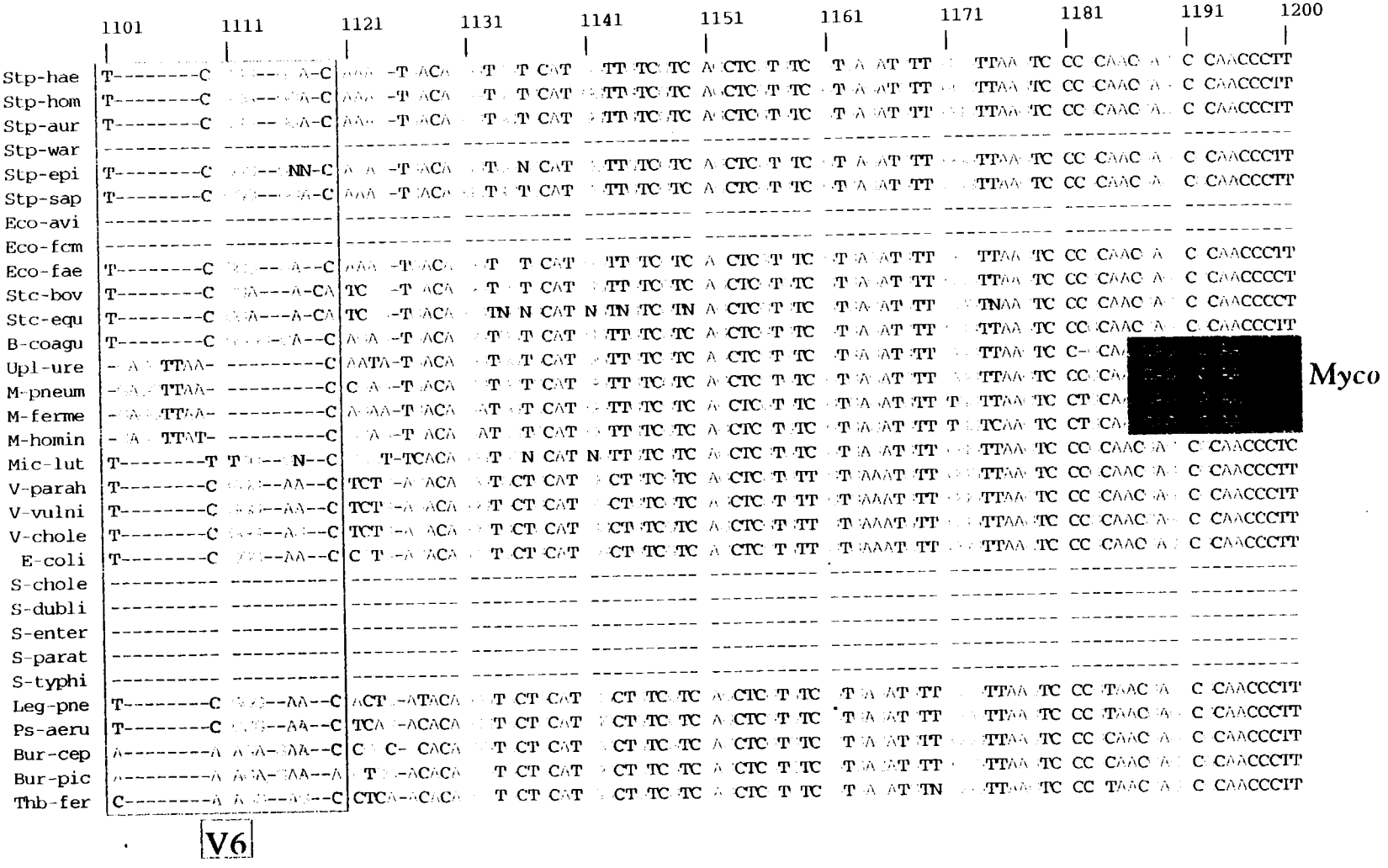
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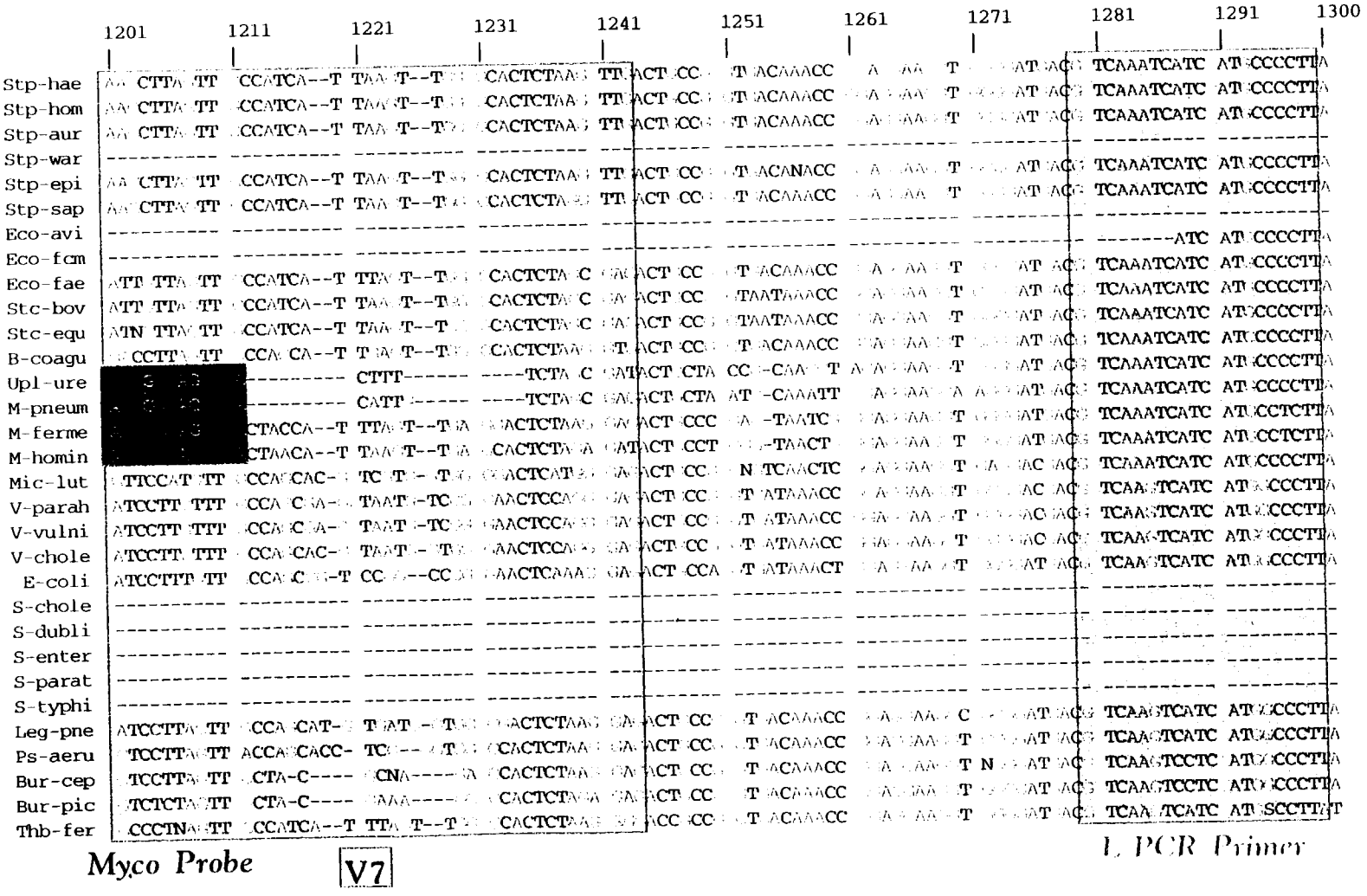
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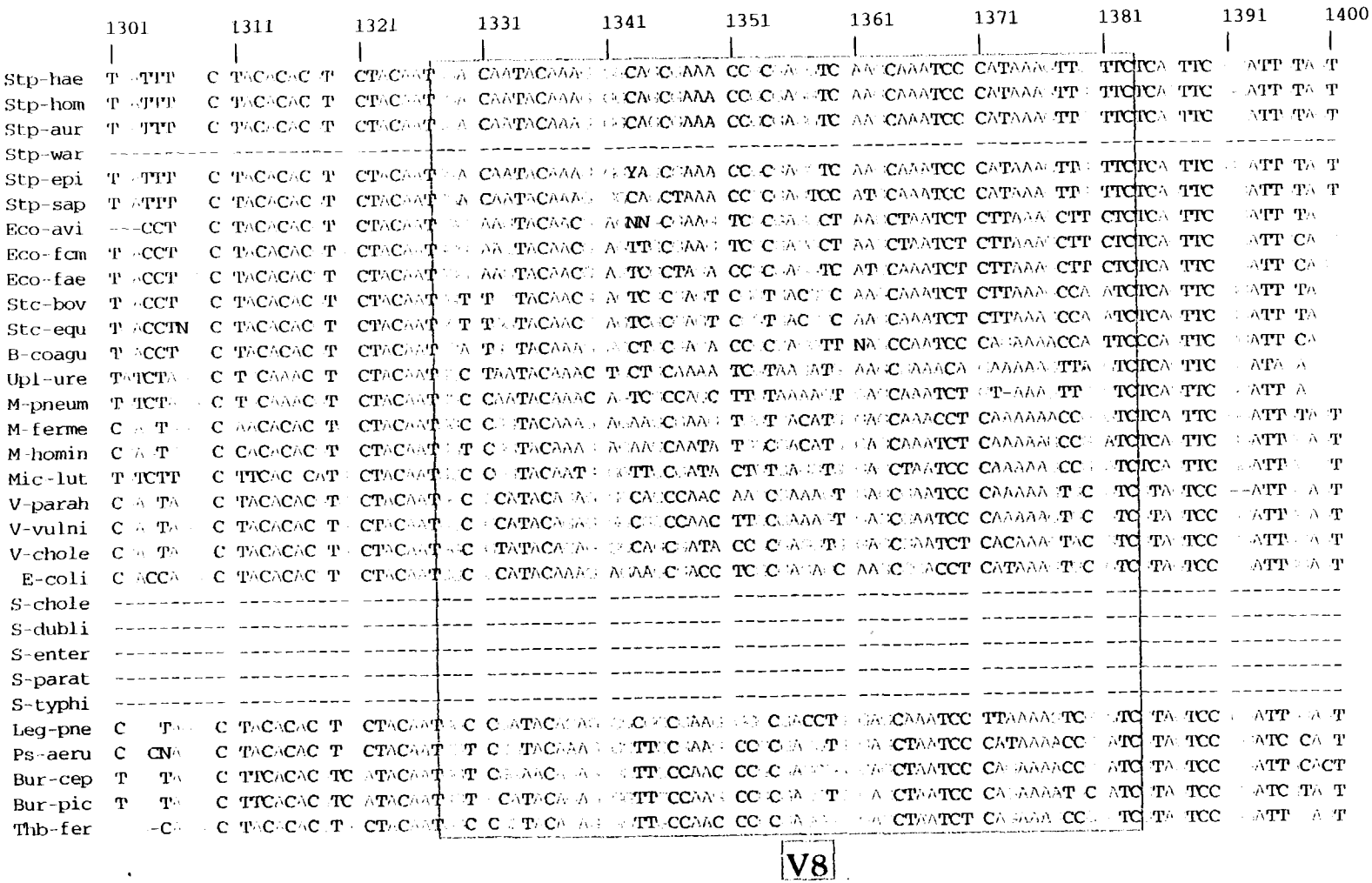
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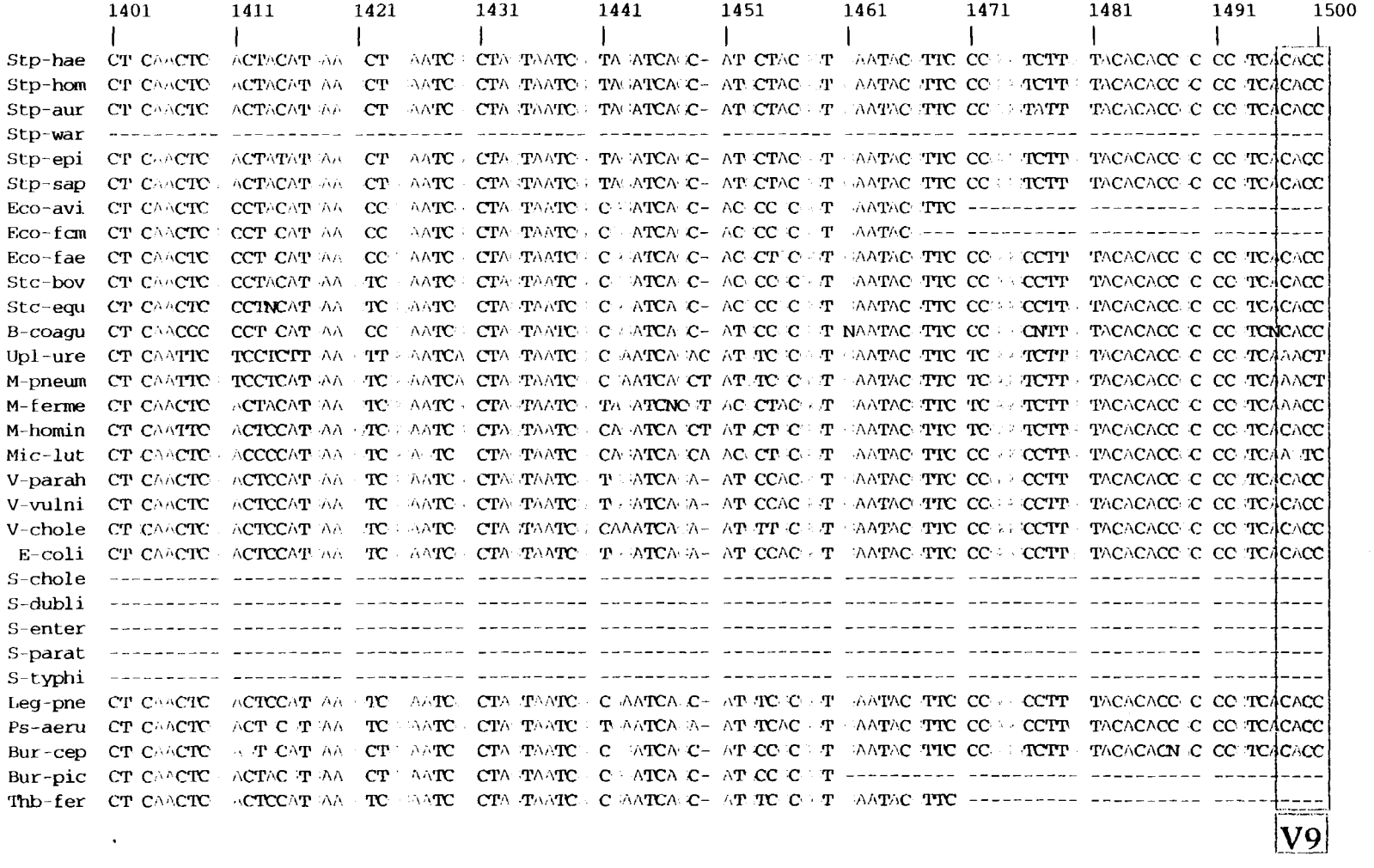
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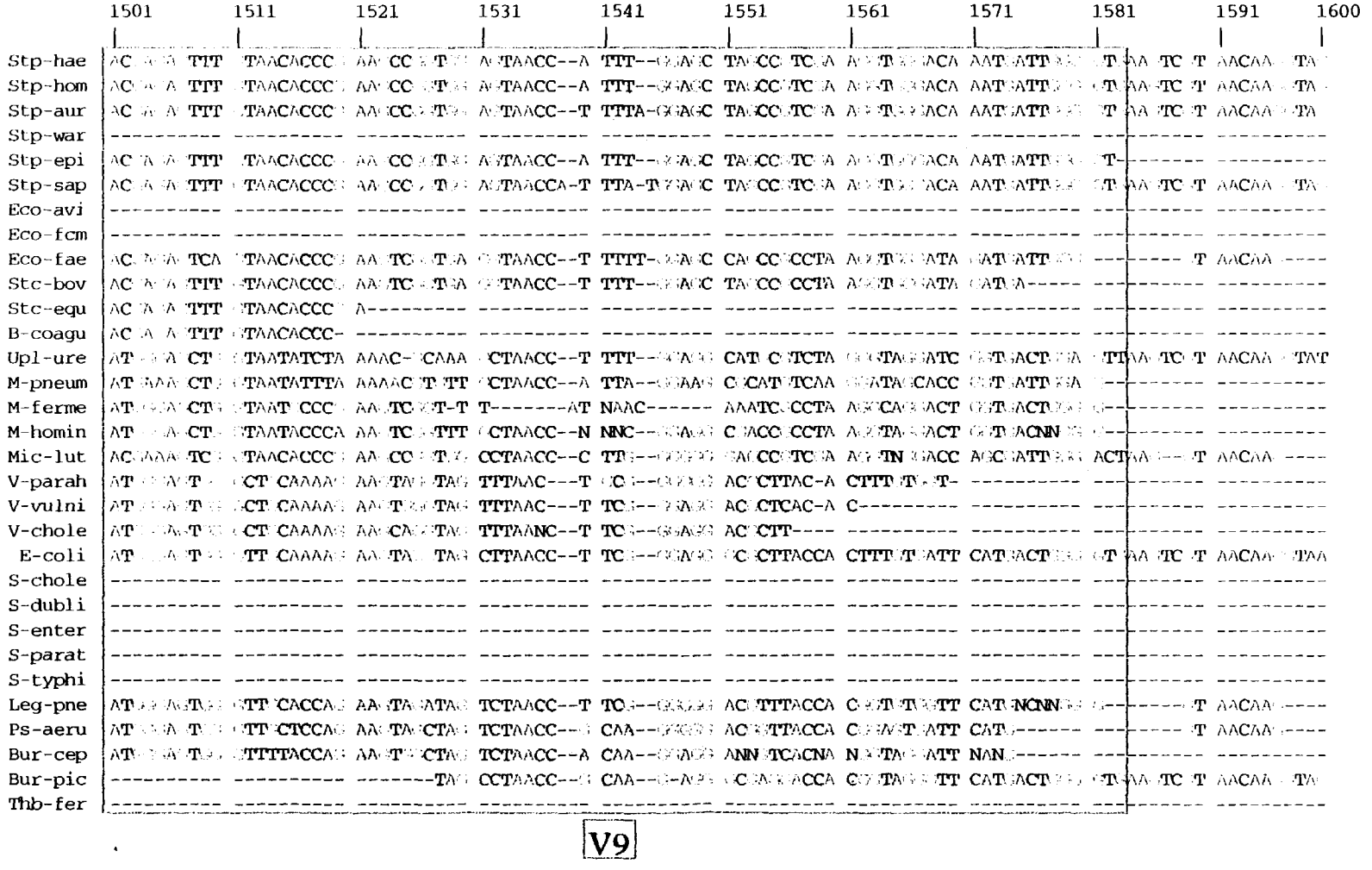
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M-homin
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V-parah
V-vulni
V-chole
E-coli
S-chole
S-dubli
S-enter
S-parat
S-typhi
Leg-pne
Ps-aeru
Bur-cep
Bur-pic
Thb-fer
V9

```
          1601        1611        1621              1638
          |           |           |                 |
Stp-hae   CC TATC  A A  T C  CT   ATCACCTC CTTTCT--
Stp-hom   CC TATC  A A  T C  CT   ATCACCTC CTTTCT--
Stp-aur   CC TATC  A A  T C  CT   ATCACCTC CTTTCT--
Stp-war   ---------- ---------- ---------- --------
Stp-epi   ---------- ---------- ---------- --------
Stp-sap   CC TATC  A A  T C  CT   ATCACCTC CTTTCT--
Eco-avi   ---------- ---------- ---------- --------
Eco-fcm   ---------- ---------- ---------- --------
Eco-fae   -- TATC  A A--------- - ATCACCTC CTTTCT--
Stc-bov   ---------- ---------- - ATCACCTC CTTTCT--
Stc-equ   ---------- ---------- ---------- --------
B-coagu   ---------- ---------- ---------- --------
Upl-ure   CCCTAC A A AC T   AT   ATCACCTC CTTTCTTC
M-pneum   ---------- ---------- ---------- --------
M-ferme   ---------- ---------- ---------- --------
M-homin   ---------- ---------- ---------- --------
Mic-lut   -- TACC  A A --------- - ATCACCTC CTTTCN--
V-parah   ---------- ---------- ---------- --------
V-vulni   ---------- ---------- ---------- --------
V-chole   ---------- ---------- ---------- --------
 E-coli   CC TA  A ACCT C  TT   ATCACCTC CTTA----
S-chole   ---------- ---------- ---------- --------
S-dubli   ---------- ---------- ---------- --------
S-enter   ---------- ---------- ---------- --------
S-parat   ---------- ---------- ---------- --------
S-typhi   ---------- ---------- ---------- --------
Leg-pne   ---------- ---------- - ATCACCTC CTTA----
Ps-aeru   --------- A ACCT ----- ---------- --------
Bur-cep   ---------- ---------- ---------- --------
Bur-pic   CC TATC  A A  T CN CT   ATCACCTC --------
Thb-fer   ---------- ---------- ---------- --------
```

Figure 7.2. Alignment of eukaryotic ssu rRNA sequences.

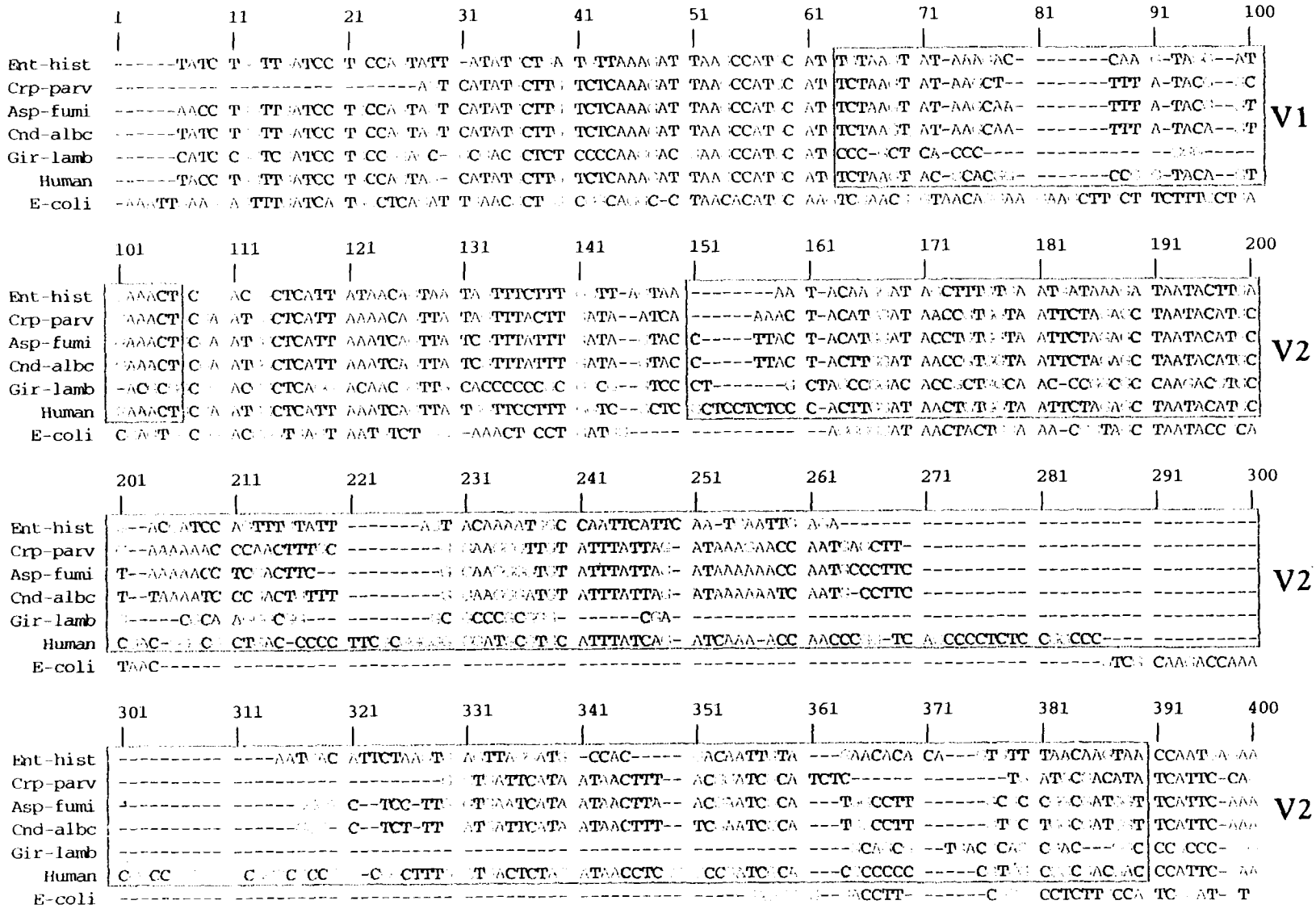
Alignments of the ssu rRNA sequences of the eukaryotic microorganisms listed in table 7.1 are shown. Gaps in an alignment position are indicated by dashes. Numbering across the top of the alignment include gapped positions.

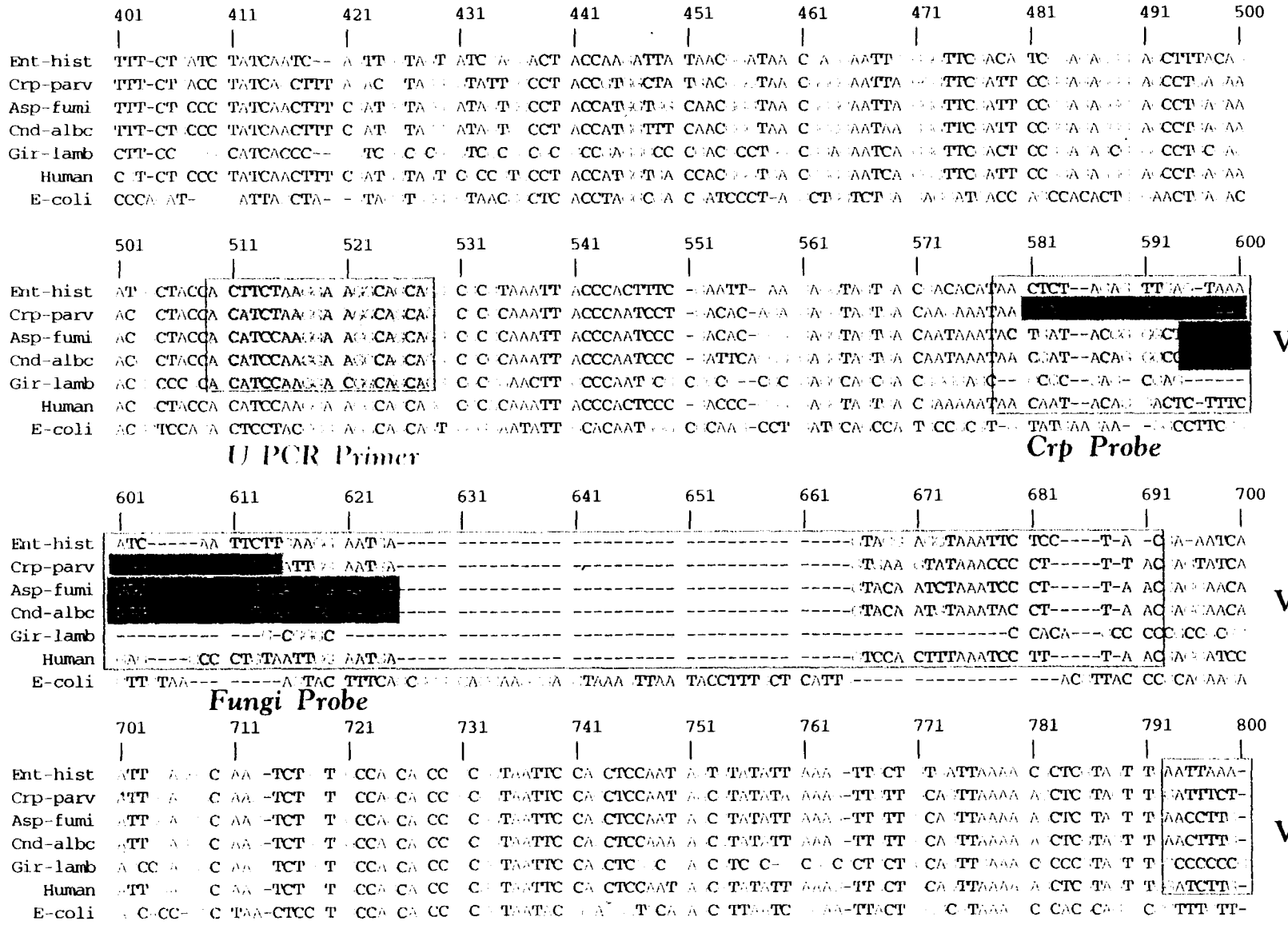
The variable regions of the ssu rRNAs are shaded in gray, and labeled in red. These correspond to the variable regions discussed in Neefs, et al., 1991.

The position of the upper and lower PCR primers are shaded in light blue. The position of the TaqMan probes listed in table 7.2 are also indicated. The numbering has been altered due to the inclusion of gaps in the numbering of the alignments.

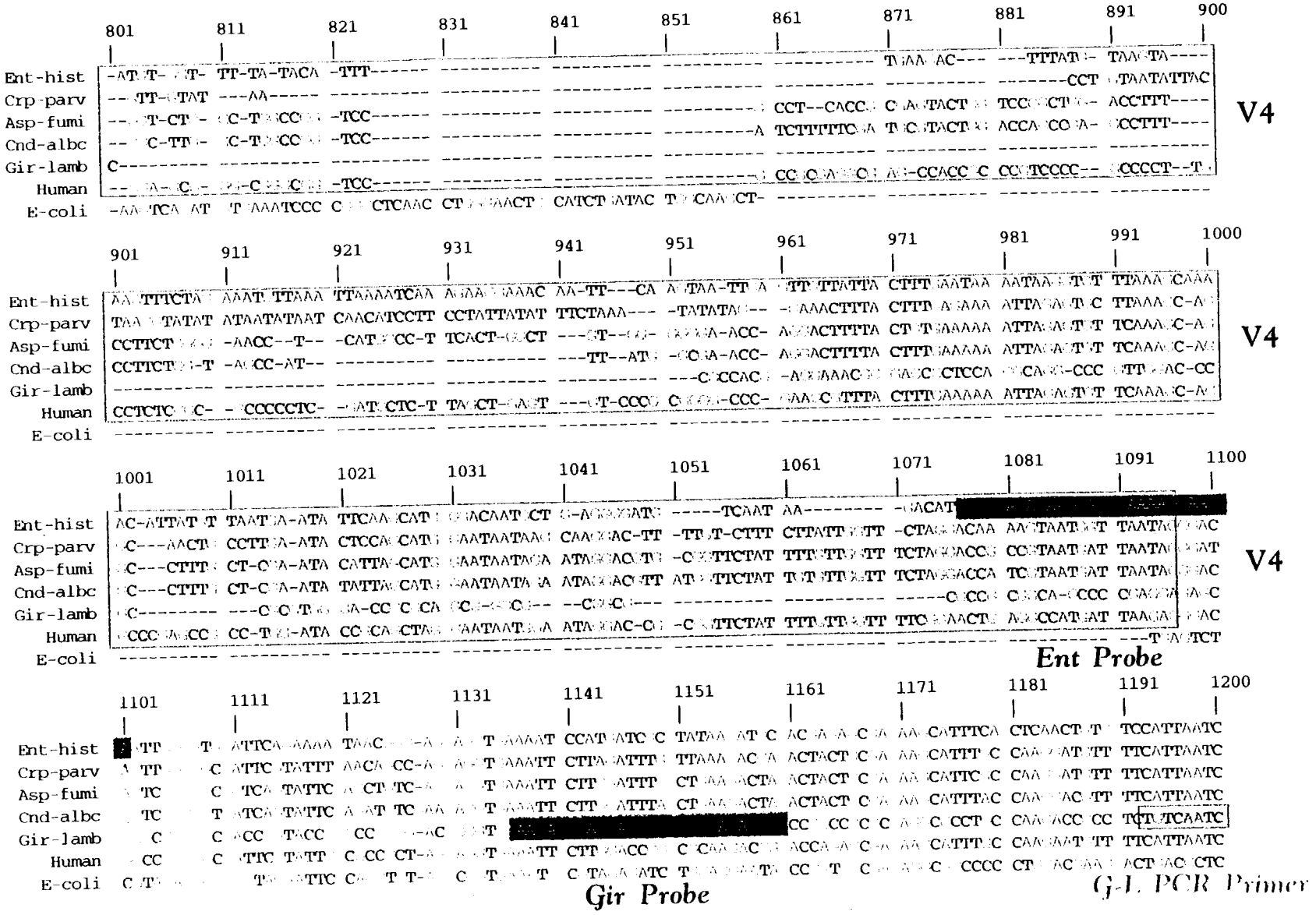
Data used in preparing this figure were derived from the Ribosomal Database Project (RDP) accessed at the University of Illinois in Urbana, Illinois via FTP (Maidak, et al., 1994). Some of these sequences were taken from release 4.1 of the RDP, October, 1994.

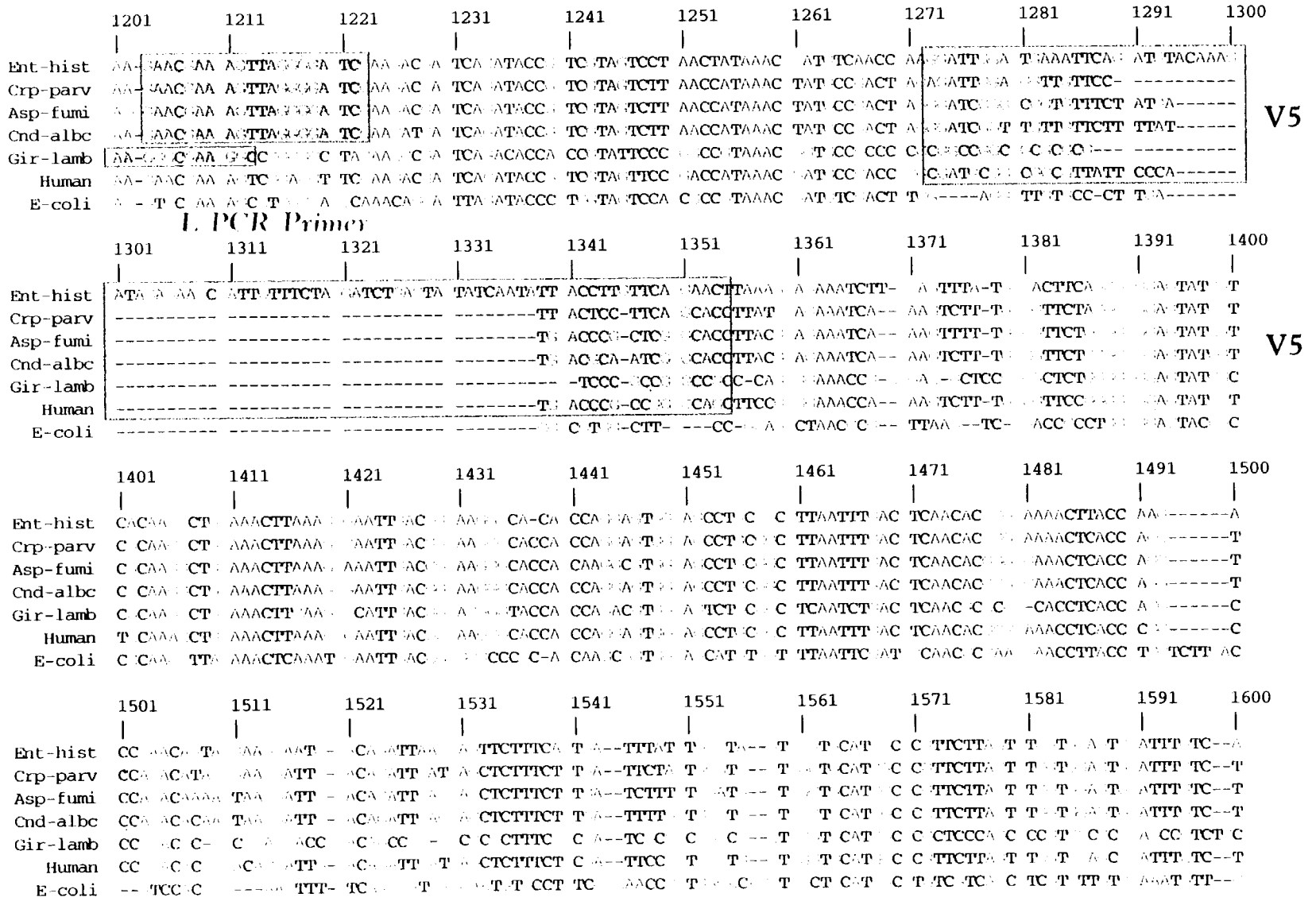
Eukaryotic ssu-rRNA Alignment

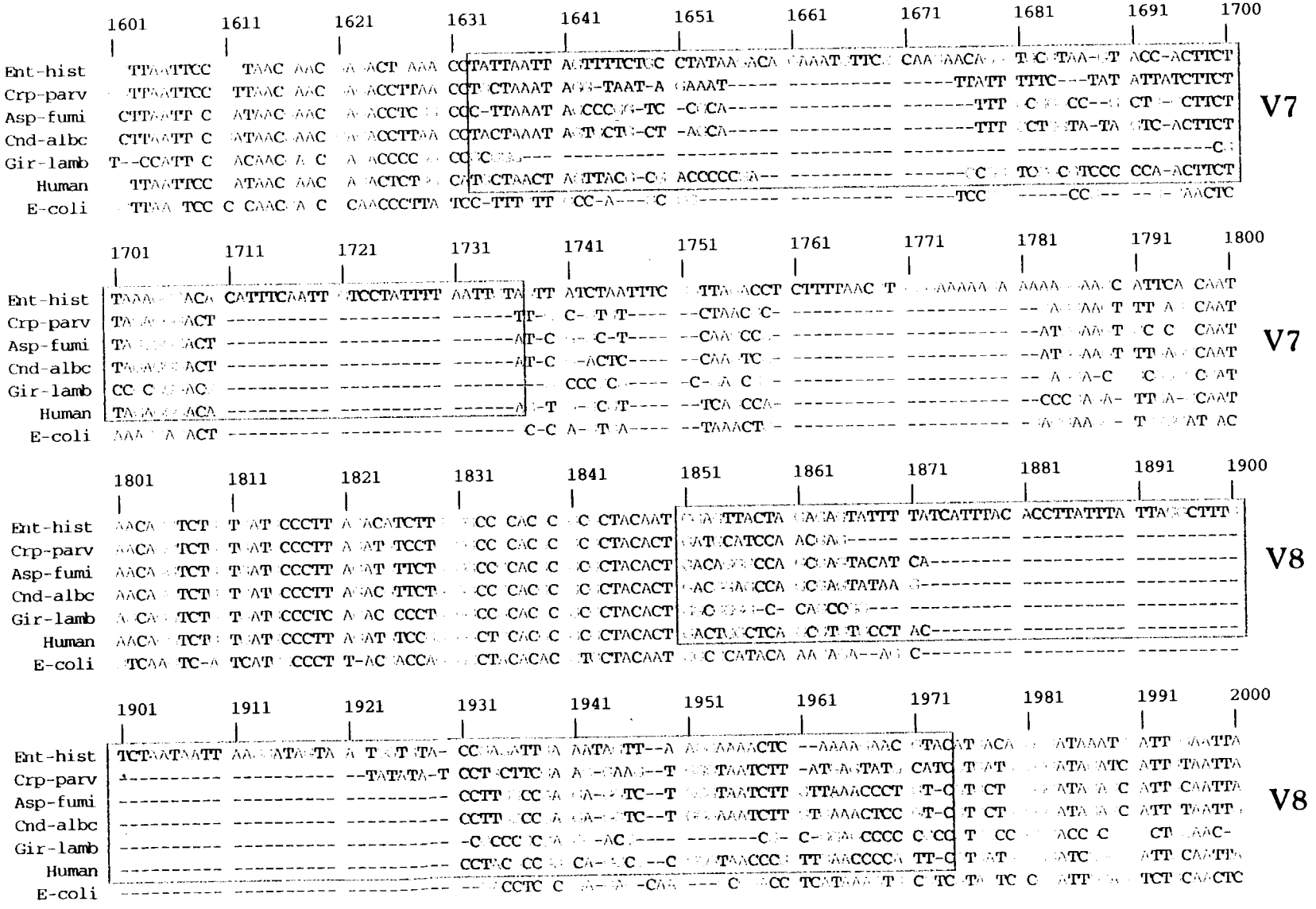



401 411 421 431 441 451 461 471 481 491 500
Ent-hist
Crp-parv
Asp-fumi
Cnd-albc
Gir-lamb
Human
E-coli
501 511 521 531 541 551 561 571 581 591 600
V3
U PCR Primer
Crp Probe
601 611 621 631 641 651 661 671 681 691 700
V3
Fungi Probe
701 711 721 731 741 751 761 771 781 791 800
V4

Eukaryotic ssu-rRNA Alignment




1201
1211
1221
1231
1241
1251
1261
1271
1281
1291
1300
Ent-hist
Crp-parv
Asp-fumi
Cnd-albc
Gir-lamb
Human
E-coli
V5
1. PCR Primer
1301
1311
1321
1331
1341
1351
1361
1371
1381
1391
1400
Ent-hist
Crp-parv
Asp-fumi
Cnd-albc
Gir-lamb
Human
E-coli
V5
1401
1411
1421
1431
1441
1451
1461
1471
1481
1491
1500
Ent-hist
Crp-parv
Asp-fumi
Cnd-albc
Gir-lamb
Human
E-coli
1501
1511
1521
1531
1541
1551
1561
1571
1581
1591
1600
Ent-hist
Crp-parv
Asp-fumi
Cnd-albc
Gir-lamb
Human
E-coli


1601 1611 1621 1631 1641 1651 1661 1671 1681 1691 1700
1701 1711 1721 1731 1741 1751 1761 1771 1781 1791 1800
1801 1811 1821 1831 1841 1851 1861 1871 1881 1891 1900
1901 1911 1921 1931 1941 1951 1961 1971 1981 1991 2000
Ent-hist
Crp-parv
Asp-fumi
Cnd-albc
Gir-lamb
Human
E-coli
V7
V7
V8
V8

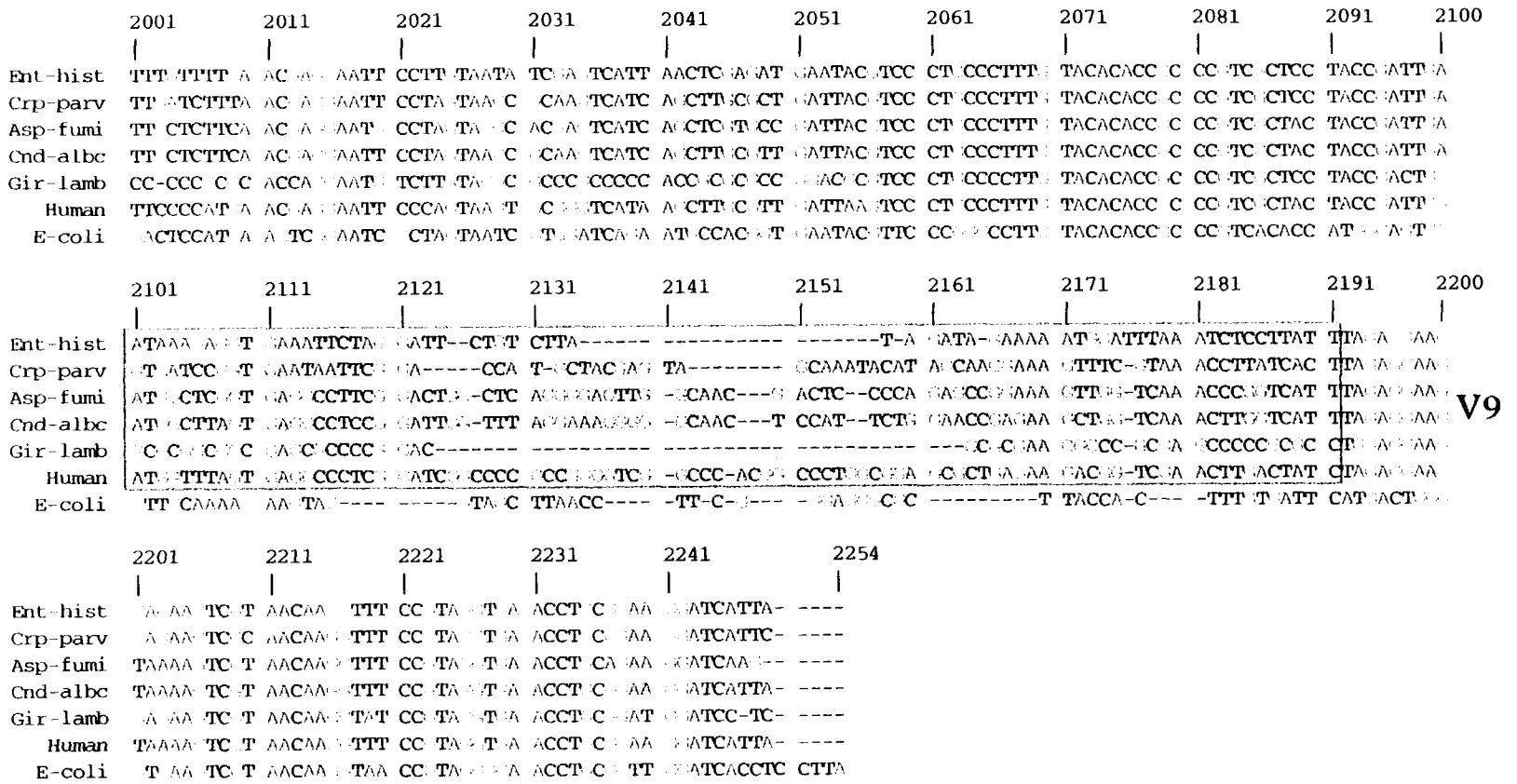

2001 2011 2021 2031 2041 2051 2061 2071 2081 2091 2100
Ent-hist
Crp-parv
Asp-fumi
Cnd-albc
Gir-lamb
Human
E-coli
2101 2111 2121 2131 2141 2151 2161 2171 2181 2191 2200
Ent-hist
Crp-parv
Asp-fumi
Cnd-albc
Gir-lamb
Human
E-coli
V9
2201 2211 2221 2231 2241 2254
Ent-hist
Crp-parv
Asp-fumi
Cnd-albc
Gir-lamb
Human
E-coli

Figure 7.3. Secondary structure of the *E. coli* ssu rRNA.

A predicted structure of the small subunit of the E. coli 16S ribosomal RNA is shown indicating possible folding and intramolecular base pairing of the ribosomal RNA bases. Variable regions are labeled in red.

Data used in preparing this figure were derived from the Ribosomal Database Project (RDP) accessed at the University of Illinois in Urbana, Illinois via FTP (Maidak, et al., 1994). This structure is taken from the current release of the RDP–release 5, May, 1995.

Figure 7.3. Secondary Structure: small subunit ribosomal RNA
Escherichia coli

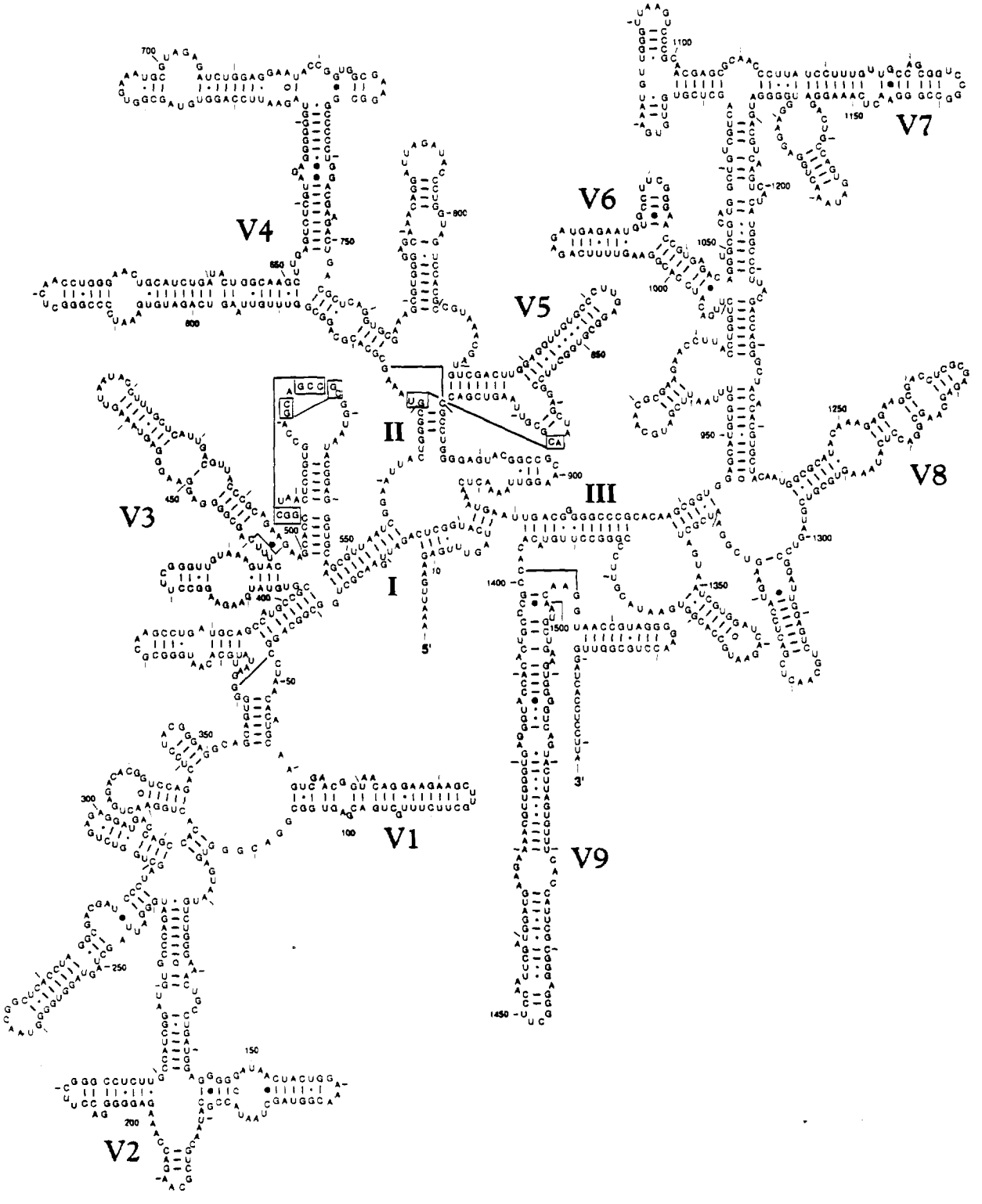


Figure 7.4. Sequence conservation between prokaryotic ssu rRNAs.

Conservation of sequence information at each alignment position for three groups of aligned prokaryotic ssu rRNA sequence is shown. The X-axis indicates alignment position, and the Y-axis plots a measure of evolutionary conservation. A higher number on the Y-axis represents a greater number of sequence changes over the course of the evolutionary history of these sequences—a greater variability and lower conservation of sequence information.

The position of the ssu rRNA variable regions (see figure 7.3) are shown in red bars under each panel.

The three panels represent the analysis of three separate data sets. The top panel shows sequence variability among all of the prokaryotic sequences listed in table 7.1. The middle panel analyzes only gram-negative organisms, while the lower panel shows the data for the four mycoplasma species.

Figure 7.4. Sequence Conservation between small subunit ribosomal RNAs

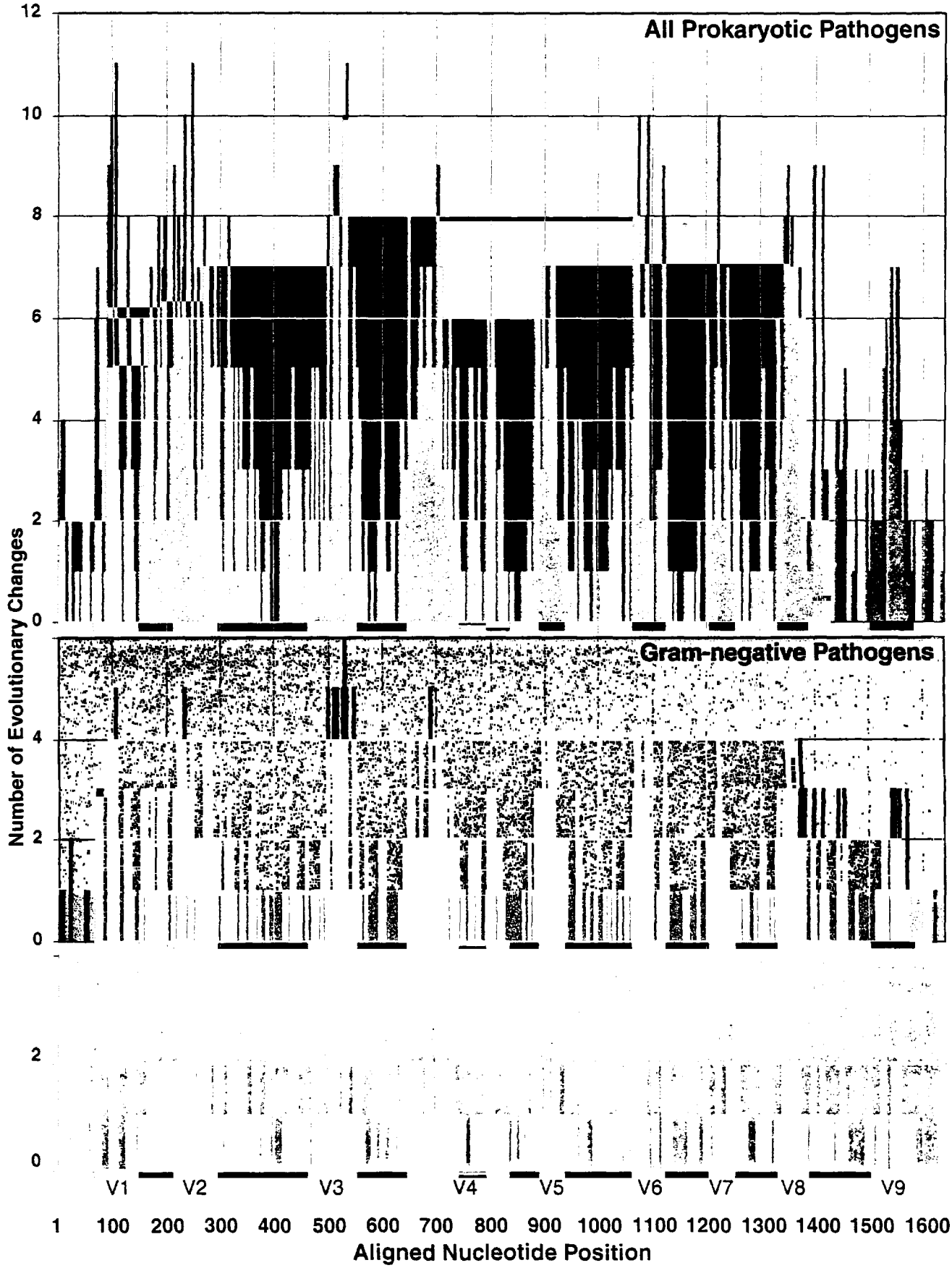


Figure 7.5. Prokaryotic ssu rRNA sequence evolutionary relationships.

The evolutionary relationships predicted for the ssu rRNA sequences of the group of prokaryotic sequences listed in table 7.1 are shown. The horizontal branch lengths of the tree are proportional to the evolutionary distance between each sequence as calculated by the Maximum Likelihood method (Felsenstein, 1994; Olsen et al., 1994).

TaqMan probes that are predicted to identify specific organisms or groups of organisms are indicated in red type within boxed regions representing the organisms that should be identified by that particular probe.

Data used in preparing this figure were derived from the Ribosomal Database Project (RDP) accessed at the University of Illinois in Urbana, Illinois via FTP (Maidak, et al., 1994). This structure is taken from the current release of the RDP–release 5, May, 1995.

Figure 7.5. Prokaryotic ssu rRNA Evolutionary Relationships

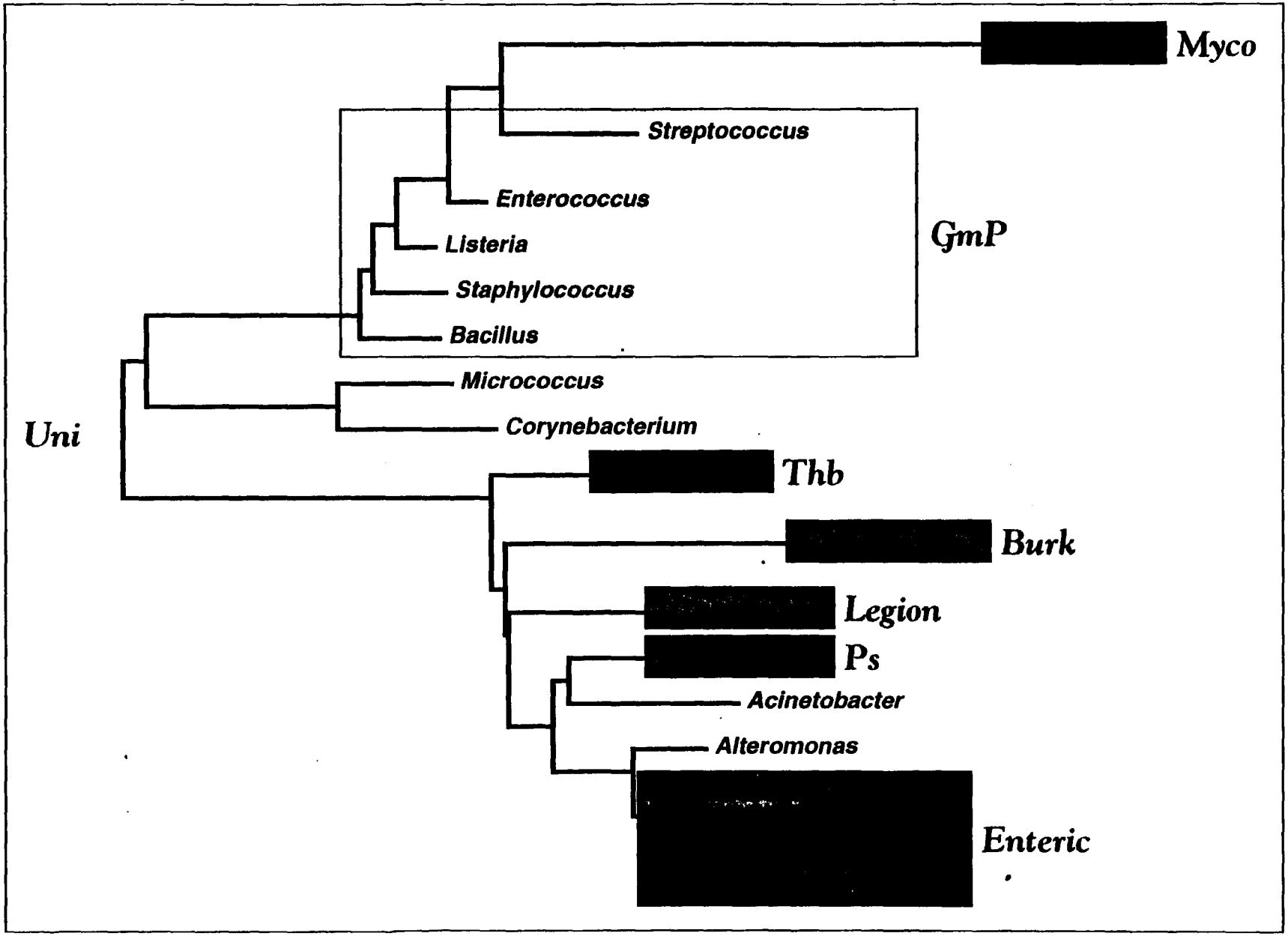


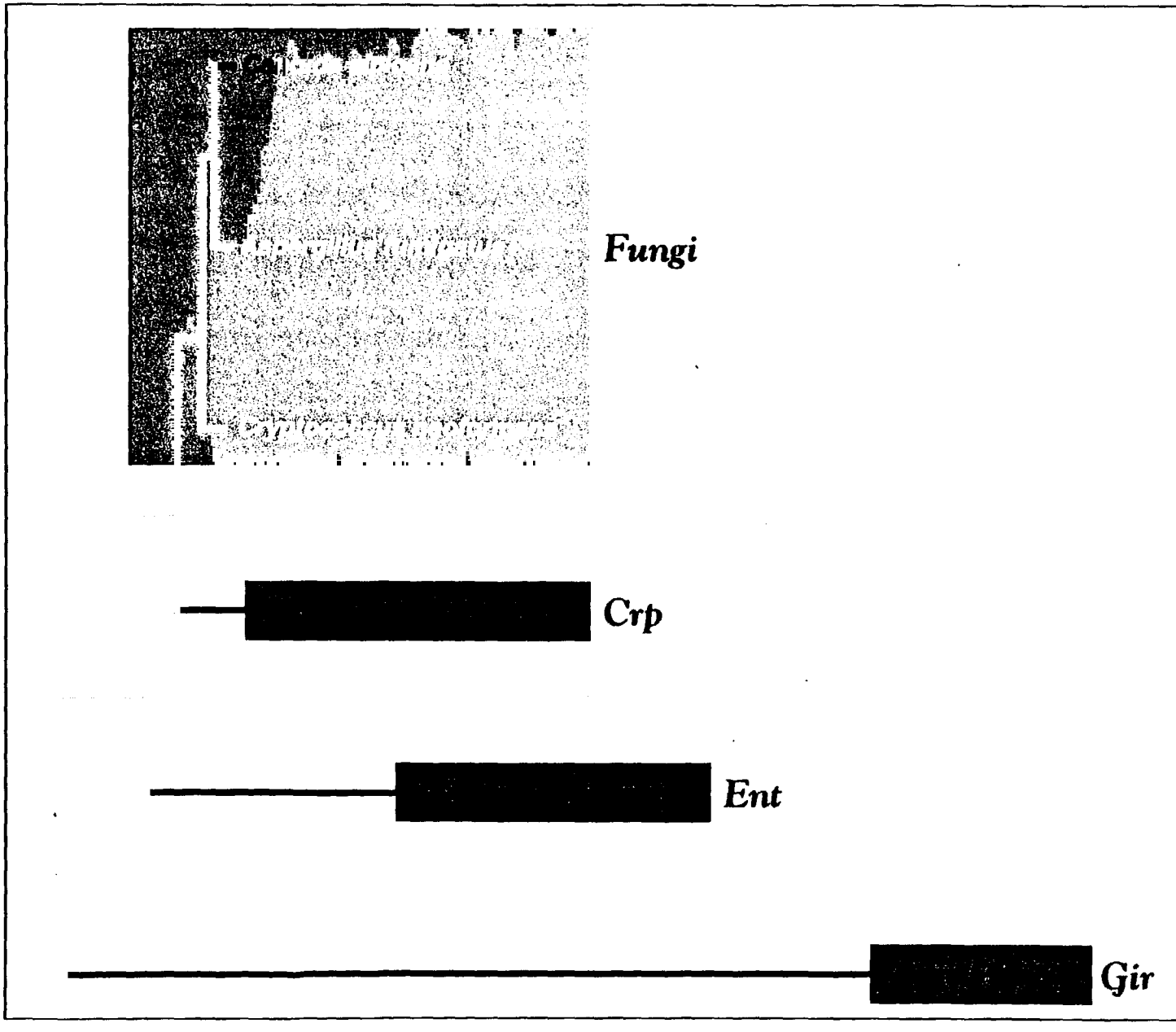
Figure 7.6. Eukaryotic ssu rRNA sequence evolutionary relationships.

The evolutionary relationships predicted for the ssu rRNA sequences of the group of eukaryotic sequences listed in table 7.1 are shown. The horizontal branch lengths of the tree are proportional to the evolutionary distance between each sequence as calculated by the Maximum Likelihood method (Felsenstein, 1994; Olsen et al., 1994).

TaqMan probes that are predicted to identify specific organisms or groups of organisms are indicated in red type within boxed regions representing the organisms that should be identified by that particular probe.

Data used in preparing this figure were derived from the Ribosomal Database Project (RDP) accessed at the University of Illinois in Urbana, Illinois via FTP (Maidak, et al., 1994). This structure is taken from the current release of the RDP–release 5, May, 1995.

Figure 7.6. Eukaryotic ssu rRNA Evolutionary Relationships



Section 8.
Conclusions and Recommendations

The monitoring of spacecraft life support systems for the presence of health threatening microorganisms is paramount for crew well being and successful completion of missions. Current NASA plans to monitor environmental samples call for the use of conventional microbiology techniques which are slow, insensitive, and labor intensive. The growing awareness at NASA that better methods of monitoring microorganisms in the crew environment on long space missions is evidenced by the emphasis given to development of microbiology sensor technology in the 1995 NASA Research Announcement for Ground-Based and Small Payloads Research in Space Life Sciences (NRA 95-OLMSA-01). During this project we have evaluated the field of gene-based diagnostic research and as a result can offer a number of recommendations as to how NASA might best proceed in developing suitable microbial monitoring systems.

Our government has already invested in developing gene-based diagnostic methods via Small Business Innovation Research grants to companies like Genometrix, Inc. If these ventures are successful in developing a microchip for quantitative detection of molecules using probes bound to CCDs and luminescent reporter groups, it will be a revolutionary advance in our capacity to detect microorganisms in environmental and clinical samples.[1] However it is impossible to know if this revolution will ever come about.

A technology much more likely to produce a functioning monitor in the next 3 to 5 years relies on the union of the molecular biology techniques of DNA probe hybridization and PCR. We believe that although the evolution of PCR-based systems for the detection, identification, and quantification of microorganisms will take place with or without NASA's involvement, an instrument that can meet the specialized needs of the space program may not be a product of that evolution. NASA's active involvement in supporting this research would accelerate the evolution of a PCR-based microbial monitor, as well as assure creation of instrumentation and chemistry

that would meet NASA requirements. This technology is theoretically capable of assaying samples in as little as two hours with specificity and sensitivity unmatched by any other method. This probe-hybridization/PCR has recently come of age in a technology called TaqMan™, invented by Perkin Elmer. Instrumentation using TaqMan concepts is evolving towards devices that can meet NASA's needs of size, low power use, and simplicity of operation.

To develop a working microbial monitor using TaqMan PCR that will meet NASA's needs for insuring water quality, work will need to proceed in concert in three different research areas. (1) A small, fully automated instrument with low power needs will need to be developed. M. Allen Northrup, the LLNL researcher who has developed the hand held PCR instrument described in Section 4 and Appendix C has estimated he could develop such an instrument in 2-3 years for $250,000. Northrup felt this could be done so cheaply because he could "piggyback" the NASA research along with similar work he is doing for NIH and other government agencies to develop a PCR-based microbial monitor. (2) Importantly, the chemistry and molecular biology needed to utilize a probe-hybridization/PCR instrument must evolve in parallel. Otherwise, it would require an additional two years to develop and optimize the PCR primers and TaqMan probes as well as determine quantitative relationships between the intensity of the TaqMan signal and the number of microorganisms in a sample. There are a number of other issues with the monitor's chemistry and molecular biology that must be resolved as well, as documented in earlier sections. (3) Finally, a system of water sample collection and contaminant concentration must be developed, and integrated with the actual monitor.

There are a scientists who have expressed doubts about the suitability of PCR based methods for microbial detection. Detractors of PCR based methods are largely basing their arguments against PCR on earlier versions of the technology.[2,3] The use of carry over contamination eliminators and TaqMan are solutions to most of the complaints of the technique's detractors. The fundamental problem of PCR based methods will be their inability to discriminate between viable and non viable microorganisms. At least

for NASA's need to analyze recycled water, that deficiency of PCR-based methods may be inconsequential. In a study that examined the capacity of bacterial DNA to be PCR amplified after the bacteria had been killed by chloroform treatment, although small PCR targets (108 bp) were still amplified, large targets (650 bp) were not.[4] The MSFC water reclamation includes iodine treatment, which is a halogen like chlorine, as part of its antimicrobial processing of wastewaters.

Importantly, development of microbial monitoring technology for analysis of recycled water in spacecraft will have utility far beyond that application. Not only can the monitor assay water, with development of suitable sample collection methods air and surface microbiology could also be monitored using the same instrument; although because these environments would harbor a different set of pathogens, the list of microorganisms to be detected would be amended. With new organisms to detect, new PCR primers and TaqMan probes would also need to be developed.

There would be spin-off uses for the technology as well. The world needs better ways of monitoring clinical, environmental, and industrial samples for microbial contaminants. The aspiration of designers of gene-based microbial diagnostics technology is small, inexpensive, fully automated devices that could rapidly describe the microbial population of any sample of interest. Such an instrument would have applications in every hospital, clinic, water processing plant, and chemical lab in the US. It might even be in every home so that people could test their food for pathogenic bacteria, or know if a child had a strep throat or Lyme disease before you went to see a physician.

References

1. Eggers, M., M. Hogan, R. K. Reich, J. Lamture, D. Ehrlich, M. Hollis, B. Kosicki, T. Powdrill, K. Beattie, S. Smith, R. Varma, R. Gangadharan, A. Mallik, B. Burke, and D. Wallace. 1994. A microchip for quantitative detection of molecules utilizing luminescent and radioisotope reporter groups. Biotechniques. **17**: 516-23.

Appendices

Appendix A

The University of Alabama at Birmingham
Department of Biology
205/934-8308
Telex: 888826 UAB BHM

March 6, 1995

To: John Glass
From: J.J. Gauthier

Subject: Organisms to be monitored during the space program

Please find attached 1) organisms listed in Standard Method, 18th ed. for monitoring of water, 2) organisms isolated from treated water during the Water Recovery Test, and 3) a brief list of incidences of infectious disease associated with space exploration, 4) my list of recommended organisms for monitoring. The general list and each section is prioritized.

Long-term habitation of a microgravity environment results in reduced functioning of the immune system. Consequently, most infections during space missions will probably come from normal human flora exchanged between crew members or from opportunistic pathogens ubiquitous in the environment. It is impossible to keep either normal flora or ubiquitous microorganisms out of the Space Station. "Everything is everywhere" (Winogradsky). Infection leading to disease could result from contact with high numbers of organisms in a specific locality. Identification of the presence of organisms without quantitative determinations will therefore be of little value.

In addition to measuring levels of organisms that potentially cause disease, it will also be necessary to consider organisms that could form biofilms in the water treatment system. High levels of these organisms could eventually lead to system failure. Heat and low levels of iodine are somewhat effective in reducing the levels of organisms, but it is impossible to sterilize the entire water treatment system. It is therefore recommended that biofouling organisms in the water treatment system be identified and included in the list of organisms to be monitored. Potential candidates are Thiobacillus and Pseudomonas.

Although Legionella and Mycoplasma have not been detected in short-term WRT experiments conducted by NASA, it is likely that they will appear during long-term operation of the water treatment system and could pose a serious threat to the health of the crew.

I recommend the following groups to be considered for monitoring:

General	Specific	
Legionella	Legionella pneumophila	
Mycoplasma		
Streptococcus	S. faecalis	S. gallinarum
	S. faecium	S. equinus
	S. avium	S. bovis
Staphylococcus	Staphylococcus epidermidis	
	Staphylococcus hominis	
	Staphylococcus aureus	
	Staphylococcus haemolyticus	
	Staphylococcus warneri	
	Staphylococcus saprophyticus	
Pseudomonas	Pseudomonas aeruginosa	
	Pseudomonas pickettii	
	Pseudomonas cepacia	
Gram negative rods	Klebsiella pneumoniae	
	Salmonella	
	Shigella	
	Escherichia coli	
	Vibrio cholerae	
	Vibrio parahaemolyicus	
	Vibrio vulnificus	
Other organisms	Mycoplasma	
	Bacillus coagulans	
	Micrococcus luteus	
	Mycobacterium	
	Candida albicans	
	Naegleria	
	Acanthamoeba	
	Aspergillus fumigatus	
	Histoplasma capsulatum	

Thiobacillus	Thiobacillus ferrooxidans
Protozoa	Giardia lamblia Entamoeba histolytica Cryptosporidium
Viruses	Coxsackie A and B Adenovirus types 3 and 4 Hepatitis A Norwalk

Standard Methods

Greensberg, A.E., L.S. Clesceri and A.D. Eaton. 1992. **Standard Methods for the Examination of Water and Wastewater.** 18th ed. American Public Health Association, American Water Works Association and Water Environment Federation. Published by American Public Health Association, 1015 Fifteenth Street, NW, Washington, DC 20005

9213 RECREATIONAL WATERS p. 9-26

Includes swimming pools, whirlpools and naturally occurring fresh and marine surface waters.

Indicator organisms:

Bacteria (primary list):
- Pseudomonas aeruginosa
- fecal streptococci (Landsfield's Group D)
  - S. faecalis
  - S. faecium
  - S. avium
  - S. bovis
  - S. equinus
  - S. gallinarum
- Staphylococcus aureus
- Salmonella/Shigella
- Legionella
- Escherichia coli

Other organisms (secondary list):
- Mycobacterium
- Candida albicans
- Naegleria
- Acanthamoeba

Viruses
- Coxsackie A and B
- Adenovirus types 3 and 4
- Hepatitis A
- Norwalk

Standard Methods (cont)

Water-associated pathogens p. 9-86

- Campylobacter jujuni (p. 9-93)
- Candida albicans
- Enteroviruses
- Klebsiella pneumoniae
- Pseudomonas aeruginosa
- Salmonella (p. 9-87)
- Shigella (p. 9-92)
- Vibrio cholerae (p. 9-94)
- Vibrio parahaemolyicus
- Vibrio vulnificus
- Yersinia enterocolitica (p. 9-100)

9240 IRON AND SULFUR BACTERIA p. 9-73

These organisms are associated with fouling (slime production) in wastewater treatment processes.

Culturable organisms

- Sphaerotilus natans
- Thiobacillus ferrooxidans
- Beggiatoa

9610 FUNGI p. 9-117

Found in potable water:

- Aspergillus fumigatus
- Histoplasma capsulatum
- Candida albicans

9711 PATHOGENIC PROTOZOA p. 9-124

Associated with drinking water and wastewater:

- Giardia lamblia
- Entamoeba histolytica
- Cryptosporidium

Summary of Data from Water Recovery Test Technical Reports

From May, 1990 to October, 1992, NASA conducted a series of water recovery tests of the water recycle system to be used on the Space Station. A model of the Space Station (End-Use Equipment Facility) was constructed in Building 4755 at Marshall Space Flight Center. Volunteers exercised, showered and carried out other activities and the water was treated by various subsystem configurations. Water samples were collected at various points in the treatment system and the microbial flora enumerated and identified at Boeing and at UAB.

The data show that high numbers of organisms were present in humidity condensate (e.g., 10^6 cfu/100mL) collected during exercise and in combined wastewater from shower, clotheswashing and other activities (e.g. 10^8 cfu/100mL). The water recovery tests showed that various subsystem configurations were generally effective in reducing these levels to <1cfu/100mL.

However, various bacteria were isolated in low numbers from the treated water. These organisms may have been present as the result of inability to sterilize the piping and subsystem components of the water recovery system, inability of the system to remove 100% of the organisms, or they may have been the result of contamination during sample collection or processing. At the low levels that were found during the water recovery test, these organisms do not pose a health threat. However, if they are indeed present in the system water and are capable of growth if nutrients become available, they are a potential health concern. It may therefore be desirable to monitor the levels of these organisms in treated Space Station water.

Attached is a list of organisms isolated from clean ports during the Water Recovery Test.

A total of 269 isolates were identified, representing 71 different species. The isolates from the Hygiene Storage Tanks were:

Organism	No of isolations/ 138 total
Pseudomonas sg VE-2	(3)
Unident Gm + coccus	(5)
Staphylococcus saprophyticus	(7)
Staphylococcus aureus	(9)
Staphylococcus hominis	(22)
Staphylococcus epidermidis	(49)

The isolates from the Potable Storage Tanks were:

Organism	No of isolations/ 131 total
Bacillus coagulans	(10)
Pseudomonas pickettii	(11)
Micrococcus luteus	(14
Staphylococcus haemolyticus	(14)
Staphylococcus warneri	(14)

The difference if flora isolated from the two sources (HT vs PT) suggests against external contamination.

References:

Roman, M.C. and S.A. Minton. 1992. Microbiology Report for Phase III Stage A Water Recovery Test. NASA Technical Memorandum TM-103584.

Roman, M.C. and S.A. Minton. 1993. Microbiology Report for Stage 4/5 Water Recovery Test. NASA Technical Memorandum TM-108405.

Roman, M.C. and S.A. Minton. 1992. Microbiology Report for Stage 7 and Stage 8 Water Recovery Tests. NASA Technical Memorandum TM-108443.

Microorganisms Associated with Health Problems in Previous Space Flights

The presence of microorganisms associated with crew members and their environment on Space Station Freedom has the potential for health problems. Long periods of time spent in a microgravity environment leads to reduced immune function. Consequently, normal flora organisms, especially those exchanged between different individuals, may be potential pathogens.

Upper respiratory illnesses due to microorganisms, including influenza, viral gastroenteritis, rhinitis, pharyngitis and dermatologic problems, occurred during the early days of the space program. The following are representative examples of infectious disease problems encountered in the space program.

Oct 11 1968	Apollo 7	Crew experienced upper respiratory symptoms during flight
Mar 3, 1969	Apollo 9	Launch postponed 3 days due to viral infection
Nov 14, 1969	Apollo 12	Contact dermatitis from biosensor electrode
April 11, 1970	Apollo 13	Urinary tract infection associated with combined effects of cold, dehydration and prolonged wearing of urine collection device. (Unusually stressful mission)
May 13, 1982	Soyuz T5	Incidence of ureterolithiasis
Sept 17, 1985	Soyuz T14	Crew returned early after illness event

Reference:

Taylor, G.R. 1993. Overview of spaceflight immunology studies. Journal of Leukocyte Biology 54:179-188.

James Barbaree, Ph. D.
Appendix B

Potential Pathogens - Recycled Water in the Space Station

BACTERIA

GRAM POSITIVE COCCI

Staphylococcus species
- Common pathogens:
  - *S. aureus*
  - *S. epidermidis*
  - *S. saprophyticus*
- Uncommon pathogens:
  - *S. haemolyticus*
  - *S. hominis*
  - *S. warneri*
  - *S. saccharolyticus*
  - *S. cohnii*
  - *S. simulans*
  - *S. lugdunensis*
  - *S. schleiferi*
  - *S. capitis*

Micrococcus krisinae

Streptococcus species:
- Lancefield groups:
  - *S. pyogenes* (A)
  - *S. agalactiae* (B)
  - Group G
  - Group D
    - *S. bovis*
    - *Enterococcus* species:
      - *E. avium*
      - *E. raffinosus*
      - *E. faecalis*
      - *E. faecium*
      - *E. casseliflavus*
      - *E. solitarius*
      - *E. durans*
      - *E. dispar*
      - *E. mundtii*
      - *E. hirae*
  - Viridans *Streptococcus* species:
    - *S. mutans group*
    - *S. salivarius gp.*
    - *S. sanguis gp.*

S. mitis gp.
S. anginosus gp.
Streptococcus pneumoniae
Aerococcus viridans
Gemella
G. hemolysans
G. morbillorum
Pediococcus species:
P. acidilactici
P. pentosaceus
Lactococcus species:
L. citreum
L. lactis
L. mesenteroides
L. pseudomesenteroides

GRAM NEGATIVE COCCI

Neisseria meningitidis
N. lactamica
N. cinerea
N. polysaccharea
N. flavescens
N. subflava
N. sicca
N. mucosa
N. elongata
Moraxella catarrhalis

GRAM NEGATIVE RODS

Members of the Enterobacteriaceae:
Budvicia aquatica
Cedecea species:
C. davisae
C. lapagei
C. neteri
C. sp. 3
C. sp. 5
Citrobacter species
C. freundii
C. diversus
C. amalonaticus
Edwardsiella species:
E. tarda
E. hoshinae

Enterobacter species:
- *E. aerogenes*
- *E. cloacae*
- *E. agglomerans* (now *Pantoea agglomerans)*
- *E. gergoviae*
- *E. sakazakii*
- *E. taylorae*
- *E. amnigenus*
- *E. intermedium*

Escherichia species:
- *E. coli*
- *E. fergusonii*
- *E. hermannii*
- *E. vulneris*

Shigella species:
- *O groups A, B, C*
- *S. sonnei*

Ewingella americana

Hafnia alvei

Klebsiella species
- *K. pneumoniae*
- *K. oxytoca*
- *K. ornithinolytica*
- *K. planticola*
- *K. ozaenae*
- *K. rhinoscleromatis*

Klyvera species:
- *K. ascorbata*
- *K. cryocrescens*

Koserella trabulsii

Leclercia adecarboxylata

Leminorella species:
- *L. grimontii*
- *L. richardii*

Moellerella wisconsensis

Morganella morganii

Proteus species:
- *P. mirabilis*
- *P. vulgaris*
- *P. penneri*

Providencia species:
- *P. rettgeri*
- *P. stuartii*
- *P. alcalifaciens*

P. rustigianii
Rahnella aquatilis
Salmonella species: (CDC-6 group ID classification)
Salmonella subgroup 1 strains:
S. typhi
S. choleraesuis
S. paratyphi A
S. gallinarum
S. pullorum
Salmonella subgroup 2 strains
Salmonella subgroup 3a & 3b strains (Arizona)
Salmonella subgroup 4 strains
Salmonella subgroup 5 strains
Salmonella subgroup 6 strains
Serratia species:
S. marcescens
S. liquefaciens group
S. rubidaea
S. odorifera biogroups 1 & 2
S. plymuthica
S. ficaria
S. fonticola
Tatumella ptyseos
Xenorhabdus luminescens DNA group 5
Yersinia species:
Y. enterocolitica
Y. frederiksenii
Y. intermedia
Y. kristensenii
Y. rhodei
Y. pseuotuberculosis
Y. bercoveri
Y. mollaretii
Y. ruckeri
Enteric Group 58
Enteric Group 59
Enteric Group 60
Enteric Group 68
Enteric Group 90

Haemophilus species:
H. influenzae
H. aegyptius
H. haemolyticus

H. parainfluenzae
H. parahaemolyticus
H. paraphrohaemolyticus
H. aphrophilus
H. paraphrophilus
H. segnis

Legionella pneumophila

Legionella species other (PCR oligonucleotides available and allow these 2 Legionella distinctions, i.e., L. pneumophila and Legionella - non-pneumophila)

Pseudomonas species:
P. aeruginosa
P. cepacia
P. fluorescens
P. putida
P. stutzeri
P. alcaligenes
P. diminuta
P. gladioli
P. mendocina
P. pertucinogena
P. pickettii
P. pseudoalcaligenes
P. thomasii
P. vesicularis
P. sp. CDC group 1
Pseudomonas-like group 2
UFP-1
UFp-2

Campylobacter species:
C. jejuni
C. C.hyointestinalis
C. lari
C. upsaliensis
C. concisus

Archobacter butzleri

Achromobacter groups B & E

Acinetobacter species
A. baumannii
A. calcoaceticus
A. haemolyticus
A. johnsonii
A. junii
A. lwoffi

Alcaligenes species:
- *A. faecalis*
- *A. odorans*
- *A. xylosoxidans ssp. denitrificans*

Chrysemonas luteola
Commamonas acidovorans
Commamonas testosteroni
Eikenella corrodens
Flavimonas oryzihabitans
Flavobacterium species:
- *F. meningosepticum*
- *F. indologenes*
- *F. odoratum*
- *F. thalophilum*

Kingella species:
- *K. denitrificans*
- *K. kingae*

Methylobacterium (Pseudomonas mesophilica)
Moraxella species
- *M. lacunata*
- *M. nonliquefaciens*
- *M. osloensis*

Shewanella putrefaciens
Sphingomonas paucimobilis
Sutonella indologenes
Xanthomonas maltophilia
CDC IVc-2 (included because it has been found in water)
Ochrobactrum
Oligella species:
- *O. urethralis*
- *O. ureolytica*

Actinobacillus actinomycetemcomitans
Pasteurella species:
- *P. multocida*
- *P. ureae*

Cardiobacterium hominis
Chromobacterium violaceum
Streptobacillus moniliformis
Vibrio species:
- *V. cholerae*
- *V. mimicus*
- (The remainder of the *Vibrio* species of consequence will not grow without NaCl)

Aeromonas species:
- *A. hydrophila*
- *A. caviae*
- *A. veronii biogp. sobria*
- *A. jandaei*
- *A. veronii biogp. veronii*
- *A. schubertii*
- *A. trota*

Plesiomonas shigelloides

GRAM POSITIVE RODS

Corynebacterium species:
- *C. ulcerans*
- *C. xerosis*
- *C. jeikeium*
- *C. urealyticum*
- *C. minutissimum*
- *C. matruchotii*

Arcanobacterium haemolyticum
Rothia dentocariosa
Kurthia species
Oerskovia species
Listeria monocytogenes
Erysipelothrix rhusiopathiae
Bacillus cereus
Mycobacterium species
- *M. avium-intracellulare complex*
- *M. fortuitum-chelonae*
- *M. genavense*
- *M. marinum*
- *M. scrofulaceum*
- *M. xenopi*

OTHER

Mycoplasma species:
- *M. fermentans*
- *M. hominis*
- *M. pneumoniae*

Ureaplasma urealyticum
Nocardia asteroides
Nocardia otitidiscaviarum

VIRUSES

Tables Attached)
Coxsackieviruses A & B
Echoviruses
Enteroviruses
Hepatitis Viruses A & E
Poliovirus (If vaccine not administered)
Gastroenteroviruses:
Norwalk or Norwalk-like
Rotavirus
Calicivirus
Astrovirus
Coronavirus

Fungi

Blastomyces dermatitidis
Paracoccidioides brasiliensis
Histoplasma capsulatum
Rhizopus arrhizus
Aspergillus fumigatus
Cryptococcus neoformans
Candida albicans

Other

Cryptosporidium
Giardia lamblia
Entamoeba histolytica
Naegleria fowleri
Acanthamoeba spp.

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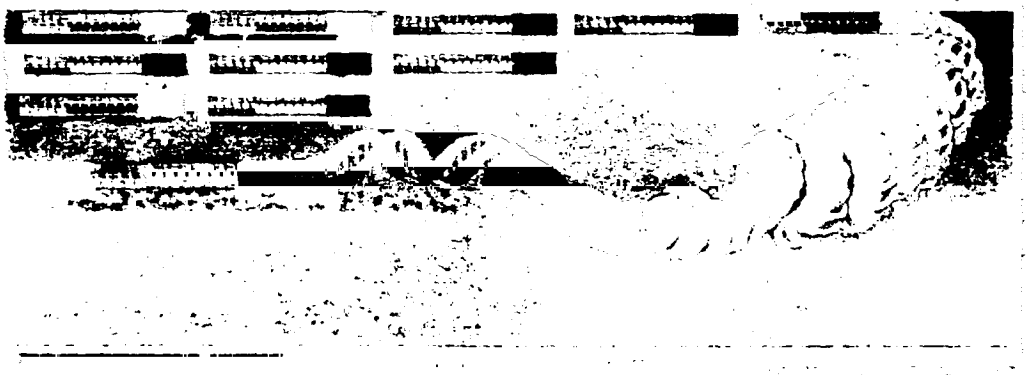
P.R. Murray, G.S. Kobayashi, M.A. Pfaller, and K.S. Rosenthal. 1994 "Medical Microbiology". Mosby, St. Louis, Mo.

Appendix C

Microfabricated DNA Analysis System

Semi-Annual Report: January 1995



Covering the period: August 1994 – January 1995

By
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Electronic Systems Technology Office
Advanced Research Projects Agency
3701 North Fairfax Drive
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ARPA

Microfabricated DNA Analysis System

Progress Report No. 95. 1
Period Covered: July, 1994 - January, 1995

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Robert Watson Sr.
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Executive Summary:

This report describes the third 6 months of effort under the 3-yr ARPA contract. It provides continuing results in the area of MEMS-based PCR reaction chamber design and testing. Much of the effort has been in the area of refining the hand-held thermal cycling instrument including low voltage/low power operation, temperature sensing/feedback, and performing verification experiments with state-of-the-art PCR. Detection of cystic fibrosis mutations on human DNA was performed with the new instrument and a simple reagent-based assay. We have shown promising results in various types of detection including real-time, kinetic monitoring of DNA production during the PCR process. MEMS-based electrochemiluminescence detection has continued as well. The significance of these results are that battery-operated, hand-held, PCR amplification and simple reagent-based, targeted detection of biologicals and diseases is possible for the first time.

Overview of second year goals:

In this third six month period we have continued the solidification of the infrastructure, personnel, and collaborators of this project. We are continuing our collaborative work with the University of California at Davis, the Armed Forces Institute of Pathology, and Roche Molecular Systems (RMS) of Alameda, CA. We have also continued our strong connection with LLNL's Biology and Biotechnology Research Program.

Technically, this second year of the project was to investigate and develop the detection and fluidic systems. In the previous report (94.2) we provided a description of the infrastructure, prototype testing laboratory; and results from modeling, reaction chamber designs, and preliminary ECL detection. In that report, three biological systems were used as verification experiments for the PCR microinstrument (two different HIV targets and ß-globin). A prototype of the microfabricated PCR reactor and a low-power, hand-held thermal cycling instrument was demonstrated. In the present report we describe: 1) continued improvements to the thermal cycling controller; 2) *in situ,* real-time, kinetic fluorescence detection of the reaction; 3) results from a complex PCR-based assay; and 4) detection of cystic fibrosis (CF) mutations with a immobilized DNA probe strip test. As well, we have performed evaluations of new materials for reaction compatibility and reaction chamber cleaning procedures to test for reusability.

Brief Overview of Recent Accomplishments

Reaction chamber designs and control of thermal cycling: We have continued to use thermocouple-based temperature sensing and feedback to ensure precise control of cycling temperatures. We have made several incremental improvements over the last report in the controller that include: integration of the thermocouple read/converter from a commercial device and voltage regulation onto our 3" by 5 " card. The improved controller has shown improvements in calibration and more precise control. These improvements have been partially responsible for our recent ability to obtain multiplex (simultaneous, multiple target) amplifications of eight separate targets on human DNA. These results are shown later. When compared to commercial instruments we have

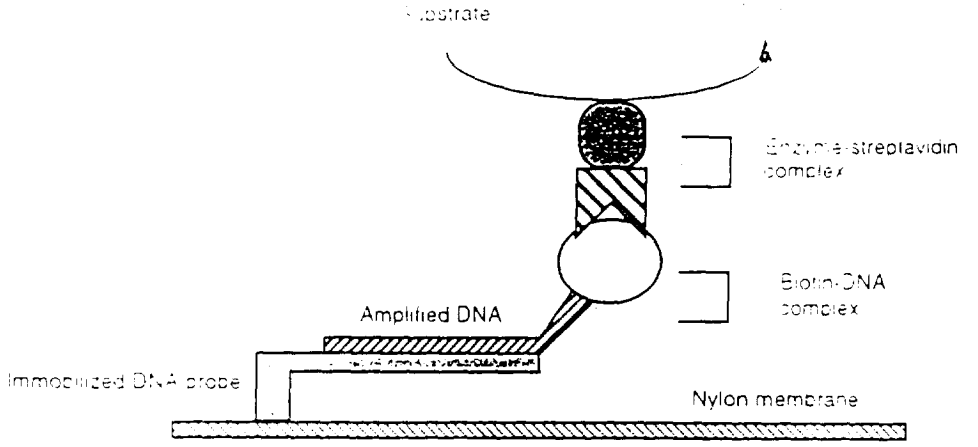


Figure 6. Immobilized DNA probe-based, "reverse dot" assay concept developed for CF testing by Roche.

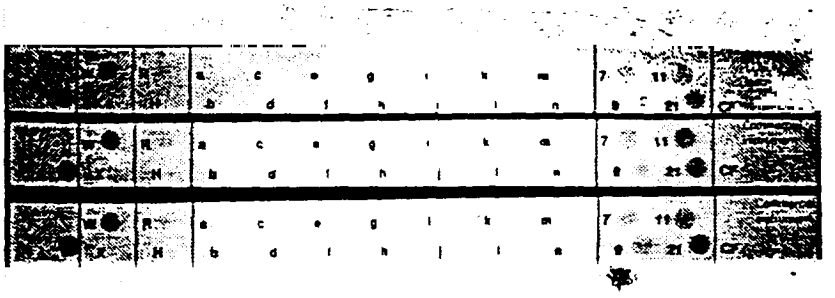


Figure 7. Photograph of reverse dot plot, DNA hybridization-based, detection of LLNL (top) and commercial instrument (bottom two) amplifications of CF mutations from human genomic DNA.

Real-time, kinetic detection of DNA production during PCR in the LLNL instrument compares favorably with published results obtained

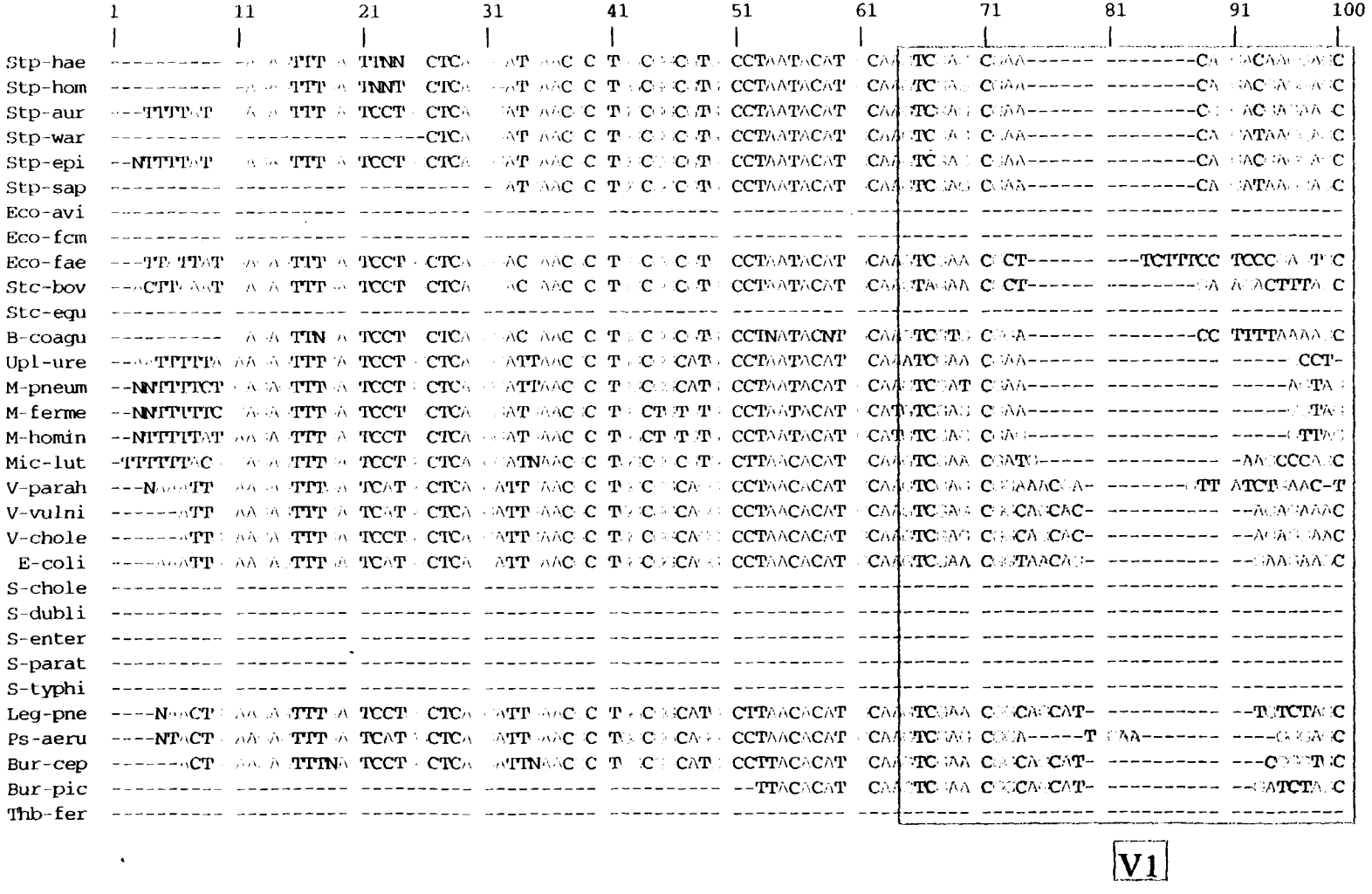
Figure 7.1. Alignment of prokaryotic ssu rRNA sequences.

Alignments of the ssu rRNA sequences of the prokaryotic microorganisms listed in table 7.1 are shown. Gaps in an alignment position are indicated by dashes. Numbering across the top of the alignment include gapped positions.

The variable regions of the ssu rRNAs are shaded in gray, and labeled in red. These correspond to the variable regions discussed in Neefs, et al., 1991.

The position of the upper and lower PCR primers are shaded in light blue. The position of the TaqMan probes listed in table 7.2 are also indicated. The numbering has been altered due to the inclusion of gaps in the numbering of the alignments.

Data used in preparing this figure were derived from the Ribosomal Database Project (RDP) accessed at the University of Illinois in Urbana, Illinois via FTP (Maidak, et al., 1994). Some of these sequences were taken from release 4.1 of the RDP, October, 1994.


1
11
21
31
41
51
61
71
81
91
100
Stp-hae
Stp-hom
Stp-aur
Stp-war
Stp-epi
Stp-sap
Eco-avi
Eco-fcm
Eco-fae
Stc-bov
Stc-equ
B-coagu
Upl-ure
M-pneum
M-ferme
M-homin
Mic-lut
V-parah
V-vulni
V-chole
E-coli
S-chole
S-dubli
S-enter
S-parat
S-typhi
Leg-pne
Ps-aeru
Bur-cep
Bur-pic
Thb-fer
V1

NAS8-38250

FINAL REPORT

PCR BASED MICROBIAL MONITOR FOR ANALYSIS OF RECYCLED WATER ABOARD THE ISSA: Issues and Prospects

Project Period:

October 1, 1994 - June 15, 1995

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Sponsoring Agency:

ION Electronics

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Date:

July 23, 1995

Summary

The monitoring of spacecraft life support systems for the presence of health threatening microorganisms is paramount for crew well being and successful completion of missions. Development of technology to monitor spacecraft recycled water based on detection and identification of the genetic material of contaminating microorganisms and viruses would be a substantial improvement over current NASA plans to monitor recycled water samples that call for the use of conventional microbiology techniques which are slow, insensitive, and labor intensive.

The union of the molecular biology techniques of DNA probe hybridization and polymerase chain reaction (PCR) offers a powerful method for the detection, identification, and quantification of microorganisms and viruses. This technology is theoretically capable of assaying samples in as little as two hours with specificity and sensitivity unmatched by any other method. A major advance in probe-hybridization/PCR has come about in a technology called TaqMan™, which was invented by Perkin Elmer. Instrumentation using TaqMan concepts is evolving towards devices that could meet NASA's needs of size, low power use, and simplicity of operation. The chemistry and molecular biology needed to utilize these probe-hybridization/PCR instruments must evolve in parallel with the hardware. The following issues of chemistry and biology must be addressed in developing a monitor:

- Early in the development of a PCR-based microbial monitor it will be necessary to decide how many and which organisms does the system need the capacity to detect. We propose a set of 17 different tests that would detect groups of bacteria and fungus, as well as specific eukaryotic parasites and viruses.
- In order to use the great sensitivity of PCR it will be necessary to concentrate water samples using filtration. If a lower limit of detection of 1 microorganism per 100 ml is required then the microbes in a 100 ml sample must be concentrated into a volume that can be added to a PCR assay.
- There are not likely to be contaminants in ISSA recycled water that would inhibit PCR resulting in false-negative results.

- The TaqMan PCR product detection system is the most promising method for developing a rapid, highly automated gene-based microbial monitoring system. The method is inherently quantitative. NASA and other government agencies have invested in other technologies that, although potentially could lead to revolutionary advances, are not likely to mature in the next 5 years into working systems.
- PCR-based methods cannot distinguish between DNA or RNA of a viable microorganism and that of a non-viable organism. This may or may not be an important issue with reclaimed water on the ISSA. The recycling system probably damages the capacity of the genetic material of any bacteria or viruses killed during processing to serve as a template in a PCR designed to amplify a large segment of DNA (>650 base pairs). If necessary vital dye staining could be used in addition to PCR, to enumerate the viable cells in a water sample.
- The quality control methods have been developed to insure that PCRs are working properly, and that reactions are not contaminated with PCR carryover products which could lead to the generation of false-positive results.
- The sequences of the small rRNA subunit gene for a large number of microorganisms are known, and they constitute the best database for rational development of the oligonucleotide reagents that give PCR its great specificity. From those gene sequences, sets of oligonucleotide primers for PCR and TaqMan detection that could be used in a NASA microbial monitor were constructed using computer based methods.

In addition to space utilization, a microbial monitor will have tremendous terrestrial applications. Analysis of patient samples for microbial pathogens, testing industrial effluent for biofouling bacteria, and detection of biological warfare agents on the battlefield are but a few of the diverse potential uses for this technology. Once fully developed, gene-based microbial monitors will become the fundamental tool in every lab that tests for microbial contaminants, and serve as a powerful weapon in mankind's war with the germ world.

Contents

Introduction

Safe water to drink and air to breathe are essential for human life. A critical aspect of air and water safety is the absence of pathogenic microorganisms; however the closed nature of spacecraft environments makes control of microbial contaminants all the more critical and difficult. That need is compounded by the attenuation of human immune system function due to long term exposure to microgravity.[1] To achieve control of microorganisms in spacecraft, NASA must develop environmental sensors capable of monitoring the microbial content of recycled air and water. Traditionally, analysis of environmental samples for microbial pathogens relied on culturing the organisms on suitable growth media or propagation of viruses in tissue culture cells. Such methods are costly, slow in that some species of bacteria may take as long as 2 weeks to culture, and in many cases ineffective. Perhaps 99% of all organisms in environmental samples may not be culturable.[2] Although the current plan for monitoring microbial contamination on ISSA will utilize culture methods, new technologies for microbial detection are under development that could let astronauts know in 2 hours instead of 1-14 days if there were dangerous pathogens in their air or water. The most promising of these technologies is based upon a technique called PCR, for polymerase chain reaction.

PCR is a powerful technique invented by Nobel laureate Kerry Mullis that allows enzymatic amplification of DNA segments *in vitro* through a succession of incubation steps at different temperatures.[3, 4, 5] Typically, the double-stranded DNA is heat-denatured, two oligonucleotide primers (the PCR primers) that are complementary to the 3' boundaries of the target DNA segment are annealed at low temperature, and then enzymatically extended by Taq DNA polymerase at an intermediate temperature. One set of these three steps is referred to as a cycle, and the instrument that repeatedly changes the temperature of a PCR sample is called a thermocycler. The PCR process is based on repetition of this cycle and amplify DNA segments, called amplicons, by 10^5 to 10^9 fold.

The technique is relatively new; however it is being used increasingly as a method of diagnosing and precisely identifying microbial contamination in environmental, clinical, and industrial samples. As with any new scientific technique, it is continually being refined and improved. This report is an evaluation of the state of PCR science as it applies to the needs of NASA to develop a microbiology monitor for use aboard spacecraft. We have evaluated the scientific literature; talked with scientists in academia, government, and industry; and using DNA informatics methods, designed a set of oligonucleotides that could be used to detect potential pathogens in recycled water.

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Section 1.
What Pathogenic Microorganisms Must a Spacecraft Microbial Water Quality Monitoring System Be Capable of Detecting?

NASA has spent ≈8 million dollars in the development and construction of a system to convert all of the waste water on the ISSA into potable water. In tests of NASA's water reclamation system at the Marshall Space Flight Center (MSFC) in Huntsville, Alabama, *Staphlococcus sp., and Pseudomonas picketti* were among the bacterial taxons identified from clean water ports.[1] Additionally, in a small scale PCR based analysis project DNAs *from Legionella sp., Salmonella sp.* and pathogenic *Escherichia coli* were amplified from clean water ports.[2] On the Russian space station Mir, cosmonauts had a high incidence of skin and gastro-intestinal infections. Clearly, current technology is incapable of completely controlling the occurrence of potential pathogens in space environments.

Currently, NASA plans to monitor ISSA air and potable water for microorganisms as described in the Table 1-1. Bacterial and fungal assays will be performed in flight by passing air or water through membrane filters and culturing filtered organisms on R2A and other media. Specific analysis for viruses and the listed air based organisms will be done on Earth.

Table 1-1. In flight microbiological limits for ISSA air and water.

Air Quality Requirements		Water Quality Requirements	
Total Bacteria	≤ 500 CFU/m³	Total Bacteria & Fungi	≤ 100 CFU/100 ml
Total Fungi	≤ 100 CFU/m³	Total Coliform Bacteria	0 CFU/100 ml
Branhamella catarrhalis	0 CFU/m³	Total Viruses	0 PFU/100 ml
Neisseria meningitidis	0 CFU/m³		
Salmonella spp.	0 CFU/m³		
Shigella spp.	0 CFU/m³		
Streptococcus pyogenes	0 CFU/m³		
Aspergillus fumigatus	0 CFU/m³		
Cryptococcus neoformans	0 CFU/m³		

Although the ISSA water quality requirements are tractable for culture based analysis, if it were technically feasible, spacecraft water should be tested for a more comprehensive list of potential pathogens. One of the important capabilities of PCR based methods for microbial analysis is the ability to identify defined targets. That specificity can theoretically be tailored to any taxonomic level, from species to kingdom. PCR conditions can be designed to specifically amplify almost any unique genetic element. Our consultants, Dr. James Barbaree and Dr. Joseph Gauthier, both experts in the area of water quality, constructed lists of potentially significant pathogens for which a comprehensive water quality monitor should test. Dr. Barbaree's list is comprehensive in its inclusion of all microorganisms that might be hazards in reclaimed water (Appendix B). Dr. Gauthier's list was much shorter and more directed towards the organisms likely to be encountered; however even it contained some organisms that probably would not be a risk in spacecraft water (Appendix A).

After evaluating the aforementioned two lists, several generations of tests on the ISSA water reclamation system at the MSFC, and consulting guidelines from the American Public Health Association[3], and U.S. Environmental Protection Agency[4], we compiled a consensus list of infectious agents and groups of agents that could be potential hazards in ISSA recycled water (Table 1-2). The most important microbial taxons are placed at the top of the list, i.e. all bacteria and fungi, *Legionella sp.*, enteric bacteria, and Gram positive bacteria. *Thiobacillus sp.* and *Pseudomonas sp.* (also an opportunistic pathogen) were included on the list because they are associated with fouling (biofilm production) in wastewater treatment processes, and thus indirectly could pose a health problem in spacecraft by damaging water processing systems.

Because long-term habitation of a microgravity environment results in diminution of immune system function, it is inevitable that most infections occurring in space will result from normal human flora being exchanged between crew members and from opportunistic environmental pathogens. It will be impossible to keep normal human

Table 1-2. Composite list of infectious agents that are potential hazards in ISSA recycled water for which a PCR based monitor should analyze.

Microorganism or Virus
1. Any Bacteria
2. Any Fungi
3. Legionella sp.
4. Enteric Bacteria
5. Gram Positive Bacteria
6. Pseudomonas aeruginosa
7. Pseudomonas sp.
8. Mycoplasma sp.
9. Acinetobacter sp.
10. Listeria sp.
11. Thiobacillus sp.
12. Cryptosporidium
13. Candida albicans
14. Cryptococcus sp.
15. Norwalk Virus
16. Hepatitis A Virus
17. Rotavirus

flora or ubiquitous microorganisms out of the ISSA. Infections, which may result in disease will probably come from contact with organisms not normally considered pathogens in a healthy adult population such as astronauts. There is a risk of making our list of probable pathogens too exclusive, accordingly we have included assays for microbes unlikely to cause problems such as *Mycoplasma* and *Acinetobacter*. Although spacecraft crews will undergo rigorous medical screening before launch to prevent potential carriers of microbial pathogens from infecting their colleagues in space, the same intense screening will not be applied to every technician who comes in contact with the spacecraft and its cargo as it is being prepared for launch. Many microorganisms and viruses can persist for long periods of time on surfaces, and an

infected launch site worker or insect vector could unwittingly contaminate a spacecraft days or weeks before launch with a pathogen such as a Gram positive bacillus, fungal spore, or enterovirus for which the astronauts are routinely screened.

In Section 7 of this report we present lists of PCR primers and probes designed to specifically detect a number of organisms not listed in Table 1-2. Because we envision PCR based microbial monitor technology will be used both in space and on Earth for water quality analysis, we included organisms in the primer design section which would need to be considered in terrestrial applications.

Although we list three viral pathogens, assays to detect viruses in ISSA water may be of little value for two reasons. First, because viruses are obligate parasites and can replicate only in host cells, no increase in viral titer can take place as a result of viral replication in the water. Any virions in the water system will have to have passed through the entire water purification process or have been deposited on the clean water side of the purification system. Viral titers should always be very low if not zero. Second, although PCR based methods can detect as little as a single nucleic acid template, sample concentration is necessary in order to effectively utilize PCR's great sensitivity[5] (see Section. 2). Currently available sample concentration techniques are based on filtration, and because viruses are so very small, current filtration methods are largely ineffective for collecting viruses. Environmental sampling methods have been reported that use filtration to concentrate viruses in sea water for detection by PCR;[6] however in the ultrapure low conductivity water generated by the ISSA water reclamation system, filter concentration of viruses would probably not be possible given the size and power consumption requirements of the ISSA. Nonetheless, one of the principle rationales for incorporating viral testing capability into any PCR based system would be for spin-off terrestrial uses where such a viral monitor could have numerous uses in both clinical and environmental settings.

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Section 2.
Current and Projected Methods for Pre-PCR Sample Concentration

Although PCR based methods are capable of detecting a single target organism or virion, it is essential that samples be concentrated in order to attain a high sensitivity per unit volume. NASA specifications call for detection of a single organism in 100 ml of water. However because PCR samples are typically 40 µl or less, without concentration the lower limit of detection is 25 PCR templates/ml because 1 template per 40 µl corresponds to 2500 templates per 100 ml sample. To attain a lower limit of detection of 1 microorganism in 100 ml it is necessary to concentrate any microorganisms in a 100 ml water samples so that they can all go into a 50 µl PCR reaction. This is a decrease in volume of at least 2500 fold.

Additionally, because we envision analyzing water for perhaps as many as 20 different microorganisms or groups of microorganisms it will be necessary to concentrate more than a single 100 ml sample of water if the 1 template per 100 ml lower limit of detection is to be achieved. For that sensitivity, each PCR sample will need a 100 ml water sample that had had any microorganisms present concentrated 2500 fold. The TaqMan™ technology for analysis of PCR products we propose NASA use (described in Section 4) can be configured to simultaneously test for 2 different templates in a single multiplex PCR reaction. Thus to assay for 20 different microbial taxons with the prescribed limit of detection, 10 multiplex PCRs would be needed and the potential PCR targets in 1 liter of water would need to concentrated into approximately 400 µl of water. Importantly, although 1 liter would be a large volume of water given the limitations of the ISSA, the sample concentration process need not consume more than 400 µl of that amount and event that water could be reclaimed after the PCR assays.

Two basic strategies have been used for concentration of microorganisms: centrifugation and filtration. Centrifugation is unlikely to be suitable because of the large sample volumes that would need to be concentrated as well as the power and

space requirements for a centrifuge that could pellet bacteria and viruses from one liter of water. Accordingly, filtration is a much more tractable option for the necessary sample concentration. Bacteria and eukaryotic parasites such as *Cryptosporidium* are large enough to be concentrated using filtration methods; however viruses are too small to be efficiently filtered using standard technologies and as a result are usually concentrated by centrifugation or vortex flow filtration.[1]

Using filtration, single cells of microorganisms in 100 ml water samples can be detected by PCR.[2,3] Samples were concentrated onto filters and the DNA of the microorganisms was released by freeze-thaw cycling prior to PCR. PCR can be performed without removing the filters. The choice of filtration media is critical. PCR amplification is unaffected by polyvinylidine fluoride filters and polytetrafluoroethylene filters, marketed by Millipore as Durapore® and Fluoropore® filters respectively. Cellulose acetate and nitrocellulose filters inhibit PCR amplification, presumably because DNA binds to the filter matrix.[2,3]

Filtration of water aboard the ISSA

Development of a system of filtration for use on the ISSA may prove to be problematic. Any filtration system must have a number of characteristics consistent with the NASA prescribed characteristics for a microbial monitor as well as for incorporation into a PCR based system:

- The filtration process must integrate with the ISSA water system and the PCR processor.
- The system must use minimal amounts of power, space, and water.
- If possible the filtration system should be fully automated, so that zero or minimal ISSA crew effort is expended to make it function.
- The filtration system will need to be kept sterile so that no microbial contamination from outside the water system becomes a source of false positive PCR results.

One liter water samples will need to be taken at some defined interval, perhaps daily, from the clean water side of the ISSA water system. Where should the water collection site be? Environmental detection of *Legionella* is usually done at all of the end use ports because those bacteria may exclusively colonize one site such as a shower head. On the ISSA it may be possible to collect water from every port; however that would require active crew involvement in the microbial monitoring process. Even if all the water was collected from a single port, probably the drinking water port, development of an instrument that would collect, filter, and recycle one liter of water on a daily basis and then transfer the filter to the PCR processor would be an elaborate and expensive project. Alternatives that require crew involvement could probably be developed using modifications of existing technology. For instance, an astronaut could collect the liter of water into a manifold that holds 10 filters. Thus 100 ml could be forced by compressed air or vacuumed through the filters (the ISSA does have a vacuum source for use in the hygene system), and then the water could be returned to the stainless steel bellows tanks or used directly. The filtration would constitute an additional purification step. Once the filtration was complete, an astronaut would then aseptically transfer the filters to the PCR sample tubes. Aseptic transfer so that no microbes form outside the water system contaminate the PCR samples could be difficult to accomplish; however methods and an appropriate apparatus should be possible to devise.

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Section 3.
Does the MSFC Water Reclamation System Introduce Chemicals into Water That Would Inhibit a PCR-Based Microbial Monitor?

Can PCR be done on water reclaimed by the system designed for use aboard the ISSA? Yes, in analyses performed on samples from the Stage 7 and Stage 8 test of the water reclamation system at MSFC, PCR was shown to be an effective and sensitive tool to monitor microbial contaminants.[1] Are there chemicals in the reclaimed water that inhibit PCR assays? That question must have the qualified answer of probably not, but we do not know for sure. The principle reason there are not likely to be any inhibitors of PCR is that as a result of the high efficiency of the water reclamation system, the recovered water is extremely clean. Chemical analysis of the MSFC reclaimed water for a great number of elements showed only iodine, which is used as a biocide, is present in greater than mg/L amounts (Table 3-1). We cannot be sure because although PCR analysis of the Stage 7 and 8 samples was successful, the scientist who performed those tests, Dr. Asim Bej of the University of Alabama at Birmingham, stated no effort was made to determine if there were PCR inhibitors in the water that would make the tests less sensitive.[2]

There are a number of chemicals that have been reported to inhibit the PCR enzymes; however none of the chemicals identified in the MSFC recycled water are present at concentrations known to inhibit PCR. There are no reports in the literature documenting the effect of iodine on PCR. The aforementioned work by Asim Bej on the Stage 7 and 8 samples of MSFC recycled water suggests iodine is inconsequential.[1] Another potential contaminant whose effect on PCR has not been reported is silver. The Russian space program employs silver as a biocide in its water reclamation system.[3] Solubilized metals can affect PCR. High levels of iron have been reported to inhibit Taq DNA polymerase, the PCR enzyme; however no other metals have been reported to affect PCR.[4]

Development of a PCR based microbial monitor for the ISSA should have as one its initial steps experiments to determine if the MSFC recycled water contains inhibitors of

PCR. Additionally, the affects on PCR of iodine concentrations greater than the 2.3 mg/L average value found in the MSFC recycled water, and silver in the concentration range found in Mir recycled water should be tested.

Table 3-1. Chemicals identified in the MSFC recycled water.[1]

Parameter	Units	Detected Average
(Z)-9-octadecan-1-ol	µg/L	6.6
1-methyl-2-piperdinone	µg/L	14
1-methyl-2-pyrrolidinone	µg/L	226
2-ethyl-12-hexanol	µg/L	8.9
toluene	µg/L	3
acetic acid	mg/L	0.21
ß-hydroxy butyric acid	mg/L	0.32
ethanol	mg/L	0.54
formaldehyde	mg/L	0.1
glycolic acid	mg/L	0.2
oxalic acid	mg/L	0.9
propionic acid	mg/L	0.32
aluminum	mg/L	0.6
barium	mg/L	0.01
calcium	mg/L	0.06
chloride	mg/L	0.08
fluoride	mg/L	0.06
iron	mg/L	0.01
manganese	mg/L	0.008
nickel	mg/L	0.03
nitrate	mg/L	0.16
phosphate	mg/L	0.47
potassium	mg/L	0.21
sodium	mg/L	0.63
sulfate	mg/L	0.22
residual iodine	mg/L	2.3
iodide	mg/L	0.64
conductivity	µohm/cm	5.6
pH	pH units	7 (4.4-8.5)
total organic carbon	mg/L	0.59

Analyzed for, but not detected		
Cadmium	Lead	Magnesium
Copper	Selenium	Silver
Molybdenum	Arsenic	Chromium
Zinc		

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Section 4.
Current and Projected Methods for Quantitative Analysis of Post-PCR Products

Gene-based microbial analysis: PCR

Since PCR's invention in 1985 as a method for the prenatal diagnosis of sickle cell anemia,[1] PCR has rapidly become the basic tool in all types of genetic diagnosis. For detection of low levels of microbial contamination in almost any kind of sample, PCR based methods are unsurpassed in speed, specificity, and sensitivity. PCR is based on the concept that repetition of a DNA extension reaction bounded by two synthetic oligonucleotide primers would generate a large quantity of any specified DNA sequence. Culture based microbial analysis relies on the reproduction of individual organisms until sufficient progeny exist to constitute a colony that can be easily detected, and identified based on a phenotype. Similarly, PCR based microbial monitoring replicates a specific segment of a target microbe's genome to a concentration sufficient for detection and characterization. As the number of colonies on a bacterial assay plate is a quantitative function of the number of that bacteria in a sample, so can the number of copies of a PCR amplified DNA sequence be a function of number of those sequences in the sample prior to PCR. It is important to note that because the efficiency of amplification varies among different templates and primer sets, so quantitative PCR assays must be evaluated independently.

In most current PCR applications, to analyze post-PCR products for amplified DNA sequences, called amplicons, there are two basic methods. Most simply, the PCR products are size fractionated by gel electrophoresis, stained with a fluorescent dye, and any amplicons present are visualized by exposing the gel to UV light. An alternative and vastly more sensitive method, often referred to as Southern blotting and hybridization, fixes any amplicons present to a substrate, usually after gel fractionation. The double stranded DNA amplicons are then denatured and the substrate, usually a nylon membrane, is incubated with a fluorescently or radioactively labeled oligonucleotide probe. The probe specifically hybridizes to a complementary sequence of any amplicons present and the amplicons are visualized by detecting the

bound probe using either radioactivity or fluorescence detection methods. Thus probe-hybridization/PCR offers increased sensitivity and specificity over direct analysis of PCR products; however the time (hours to days) and technical requirements of both methods of post-PCR product analysis make them unsuitable for NASA's needs.

Although these gel electrophoresis based methods for post-PCR analysis are in wide use in research and diagnostic labs, the techniques are too slow, and labor intensive for both NASA's needs, and to fulfill the promise of PCR as a rapid, highly automated diagnostic tool. For gene-based diagnostic technology to work as an effective microbial monitor the analysis of post-PCR products will have to advance beyond gel separation based methods. Otherwise alternative technologies such as described below, that do not rely on PCR, will need to be developed.

Alternative Gene-based diagnostic methods

DNA probe-hybridization techniques are under development that should lack some of the problems of speed and labor intensiveness characteristic of standard probe-hybridization/PCR. NASA has funded two of these efforts via Small Business Innovation Research contracts. Both methods rely on hybridization of fluorescently tagged oligonucleotide probes to bacterial ribosomal RNA (rRNA) molecules. BioTechnical Resources L.P.'s direct hybridization method can detect 10^4 bacteria in about 8 hours.[2] Although the method is simple and low-tech, its sensitivity is unsuitable for NASA's stated needs. Many probe-hybridization/PCR based methods can detect a single organism.[3] Genometrix Inc. is developing silicon microchips on which arrays of different oligonucleotides probes for rRNA sequences are bound at specific addresses. The rRNAs of any bacteria in a sample would specifically hybridize to their complementary probe on the microchip. Next, in a second hybridization step, labeled oligonucleotide probes would anneal to the bacterial rRNAs already bound to the microchip. A charged-coupled device (CCD) detector would then determine which locations on the chip had the tagged oligonucleotide attached. Genometrix predicts they will be able to detect 1000 rRNA molecules. No amplification is necessary because each bacterium contains 100-1000 ribosomes.[4] *Although this revolutionary*

direct hybridization technology is theoretically fast and sensitive enough to meet NASA's specifications for bacteria (although not viruses), it is unproven technology that may be many years from implementation. When this technology matures, it will have several major advantages over PCR based methods. Because it does not require amplification of a nucleic acid template, the risk of false positive results due to contamination is greatly reduced. Although this hybridization to a silicon chip technology would have limited sensitivity for viruses because each virion would have only one hybridization target, the method could be used in concert with PCR to allow sensitive detection of viruses.

Analysis of Post-PCR Products: Electrochemiluminescence

A system for analysis of PCR products has been reported that does not employ the standard methods of gel separation of products, or binding to the PCR products to filters followed by hybridization with radiolabeled or fluorescent probes. The method is based on the incorporation of a biotinylated oligonucleotide as a primer, with the inclusion of a labelled oligonucleotide. Oligonucleotides are labeled with an N-hydroxy succinimide ester of tris-bipyridine ruthenium (II) dihexafluorophosphate (Origen-label) by modifying the 3' and 5' ends of the oligonucleotide probes. The assay makes use of the inherent thermal stability and absence of polymerase activity on such probes to allow the PCR and probe hybridization to be completed automatically on the thermocycler. The assay is concluded by the addition of PCR samples to streptavidin beads on an electrochemiluminescence analyzer for binding and analysis.

Although electrochemiluminescence is an improvement in post-PCR analytic methods, in its current form the method is still cumbersome in that it requires addition of reagents after the PCR and the PCR products must be transferred from the cycler to a different instrument for analysis. This method, like a similar approach developed by Roche Molecular Systems for cystic fibrosis testing called "reverse dot,"[6] although amenable to quantitative analysis of PCR products, is insufficiently automated to afford the low technician effort NASA will need for monitor microorganisms in space vehicles. A

different system for combining PCR and post-PCR product analysis that we believe has the potential to meet NASA's needs for a microbial monitor is described below.

TaqMan™ PCR

This is a new method that combines probe-hybridization and PCR while eliminating the time consuming steps of electrophoresis and/or blotting of the post-PCR products. TaqMan employs a probe technology that utilizes the 5'-3' endonuclease activity of *Taq* DNA polymerase,[7] to allow direct detection of PCR amplicons by the release of a fluorescent reporter during the PCR (Figure 4.1).[10] The trademark TaqMan name is a

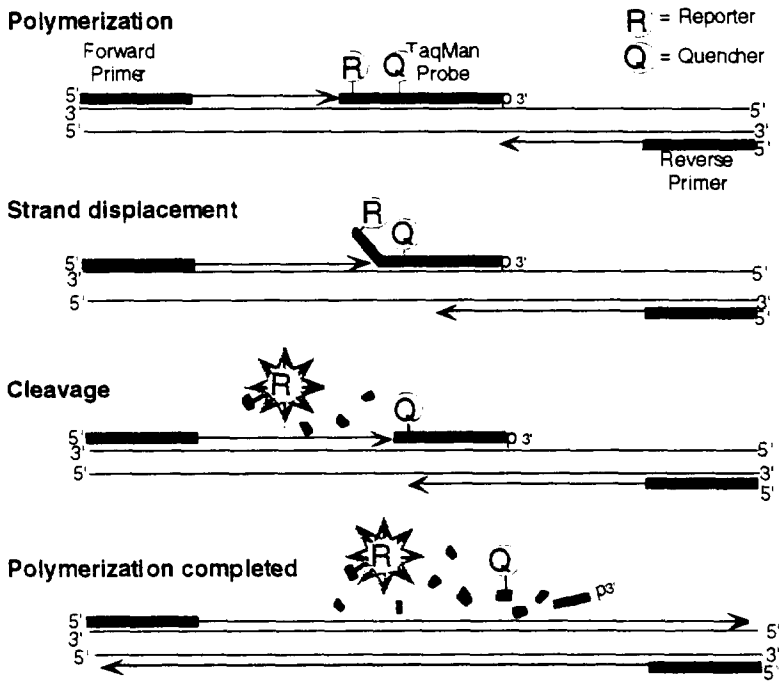

Figure 4-1. *Taq* DNA polymerase activity in TaqMan PCR. In a single cycle of PCR, the initial steps are template denaturation and annealing of that denatured DNA template with the forward and reverse primers, as well as the tagged TaqMan probe(both steps not depicted). After which, the enzyme's polymerization dependent 5'-3' endonuclease activity frees the reporter dye from the neighbor effects of the quencher dye, so it can produce a signal that is proportional to the PCR amplification. Cleavage of the TaqMan probe does not affect forward primer extension. (Modified from TaqMan™ Reagent Kit Protocol, Perkin Elmer/Applied Biosystems).[9]

oligonucleotide with a 5' reporter dye, an internal quencher dye, and a 3' blocking phosphate. The reporter dye, for which there are three different fluorescein options, is covalently bonded to the oligonucleotide's 5' end. A rhodamine quencher dye is similarly linked four to thirteen nucleotides 3' to the fluorescein reporter. To prevent the TaqMan probe from extending during PCR, there is a 3' phosphate instead of a 3' hydroxyl group. So long as the reporter and quencher are held in close proximity by the oligonucleotide, its fluorescence is quenched, principally by Förster-type energy transfer.[10] During PCR, if the TaqMan probe's target is present, the probe anneals between the two PCR primer sites. As *Taq* DNA polymerase extends from the PCR primer annealed to the same DNA strand as the probe, its 5'-3' endonuclease activity sequentially digests the probe's nucleotides. *Taq* DNA polymerase does not digest free probe (Figure 4.2). In every cycle, as the probe is displaced from the template, the PCR primer extends without interfering with the exponential accumulation of amplicon. Thus the reporter dye is liberated from the quencher and can now fluoresce when excited. Fluorescence increases in direct proportion to amplification of the PCR target. As with all probe-hybridization/PCR, the TaqMan's specificity is a result of the

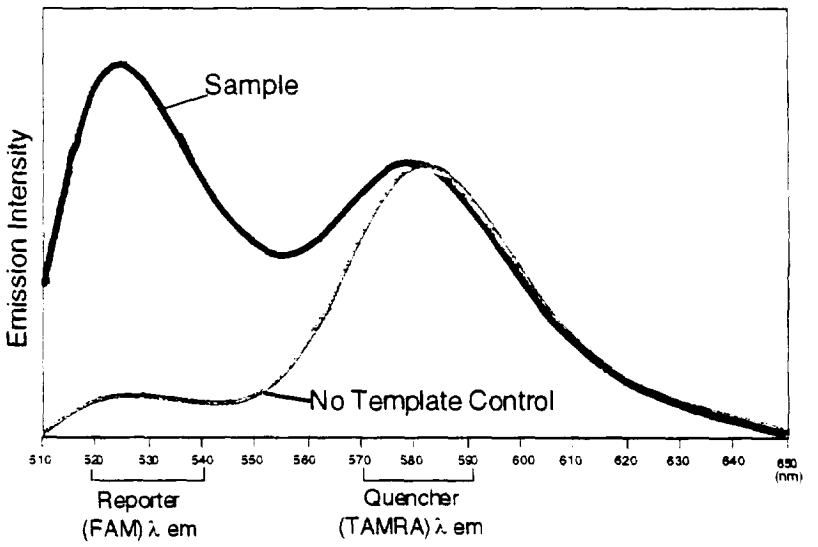

Figure 4-2. Two TaqMan emission scans post PCR, Sample and No Template. The reporter dye is 6-CA fluorescein (FAM) and the quencher dye is 6-Carboxytetrame rhodamine (TAMRA). (Adapted from the TaqMan™ Reagent Kit Protocol, Perkin Elmer/Applied Biosystems).[9]

requirement for primer and probe complementarity to the target DNA before any amplification and probe cleavage take place. Unlike other probe-hybridization/PCR methods, TaqMan PCR has no laborious post-PCR product analysis steps. *The entire reaction takes place in a single tube, and everything happens at once.* The samples and reagents are mixed, sealed in a reaction tubes, and then placed in a thermal cycler for amplification. To enhance specificity and minimize the risk of carry-over contamination the method employs the hot start method and UNG/dUTP.[11] In the system's present version at the conclusion of the PCR, aliquots of the amplified samples are transferred to microtiter plates for analysis in a luminescence spectrometer. Detection of all 96 wells takes only 7 minutes. The assay's results are expressed as the comparison of the increase in reporter dye fluorescence with that of a no template control. The ratio of reporter fluorescence to quencher fluorescence in the sample and no template control, ΔRQ, is proportional to the number of DNA templates in a sample.[12]

TaqMan is a great leap in PCR technology. It has to major improvements over gel-based post-PCR analytic methods, and both of these advances are essential to meeting NASA's needs for a microbial monitor for the ISSA.

- Samples are analyzed directly and in just a few seconds, as opposed to being transferred to a gel and electrophoretically analyzed.
- TaqMan is an inherently quantitative technique. Within a range of template concentrations, the TaqMan signal will be proportional to the amount of template present. Thus the number microorganisms in a sample can be quantitated.

In its present format, the TaqMan system requires that samples be manually transferred from a thermal cycler, where the PCR amplification is performed, to a fluorescent plate reader for analysis of the reactions. The next generation of TaqMan instrumentation, which Perkin Elmer/Applied Biosystems will begin field testing in the next year, can analyze samples directly in the PCR tube, thus eliminating the need for sample transfer. Additionally, because the next generation machine can analyze

samples in the reaction tubes, the progress of the PCRs can be monitored after each thermal cycle. This will improve the quantitative effectiveness of the instrument, because when a PCR template is present at high concentration during later cycles of a PCR, as reagents are consumed in the reaction, the efficiency of the PCR declines. Monitoring of the amplicon accumulation after each cycle permits template quantitation during the linear phase of the PCR.

The current TaqMan system being marketed by Perkin Elmer/Applied Biosystems consists of a thermal cycler, a fluorescent plate reader, and a dedicated computer. The next generation TaqMan instrument is even larger, and has significant power requirements. Because of the space and power limitations on ISSA the monitor must be small and energy efficient. Efforts at creating smaller instruments for gene-based diagnostics using microfabricated devices are ongoing in a number of laboratories.[4]

Microfabricated DNA Analysis System

A prototype miniaturized PCR thermal cycler was developed by researchers at Lawrence Livermore National Laboratory (LLNL) in conjunction with Roche Molecular Systems and Perkin Elmer/Applied Biosystems.[13, 14, 15(Appendix C)] Fabricated on a 3 inch by 5 inch Plexiglas platform, the unit consists of up to three PCR reaction chambers, a thermocouple converter chip reaction controller, and 4 nine-volt batteries to run the heaters and the control electronics. The reaction micro-chambers, made from an anisotropic etched silicon cavity with one or two medium low stress silicon nitride membrane windows, are typically 5 to 10 mm^2, 0.5 mm deep, and contain embedded polysilicon resistive heaters. The windows are designed for use in detection of PCR products. This device has been used to detect cystic fibrosis causing mutations on human DNA in a multiplex reaction simultaneously amplifying segments from eight different targets on the human genome. M. Allen Northrup, principal investigator of the LLNL group, envisions this technology evolving into a hand held PCR system that can take a sample, perform the PCR thermal cycling, and then analyze the sample by monitoring micro-electrochemiluminescence through the silicon nitride membrane windows in the reaction micro-chambers. His group has built a real-time fluorescence

monitoring system that uses laser excitation and CCD camera surveillance of the PCR progress. In collaboration with Dr. Rosemary Smith, of the University of California at Davis, the LLNL researchers are exploring the use of electrochemiluminescence with ruthenium labeled oligonucleotide probes[5] as a method to assay PCR amplification in the reaction tube. Ultimately, instruments consisting of large arrays of as many as 1000 individually controlled reaction chambers could be built. Northrup's January 1995 report to the Advanced Research Projects Agency (ARPA) is included with this report as Appendix C.

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Section 5.
Possible Methods of Avoiding False-Positive Results Due to the Detection of Dead Organisms or Free DNA.

Unlike culture based microbial diagnostic assays, which function by detecting an increase in the number of whole organisms or virions, PCR can amplify intact DNA from a living bacterium or infectious virion as effectively as from a dead microbe or even from solubilized DNA. Sixteen weeks after being killed by boiling, *E. coli* can be detected by PCR as effectively as before inactivation.[1] This limitation of gene based monitoring might be addressed in several different ways that could meet NASA's needs for monitoring water quality on spacecraft.

- Determine if PCR targets from nonviable microorganisms elute from the ISSA water reclamation system.
- Determine if microbial monitoring could be based on the observation of population growth changes in the ISSA water collection tanks.
- The PCR target could be short lived molecules of messenger RNA (mRNA) instead of DNA.
- Evaluate the use of vital dye staining, which would determine how many bacteria, fungi, or protozoans are respiring in a sample, in concert with PCR based assays.

Do nonviable organisms elute from the ISSA water reclamation system?

Although we know PCR is blind with respect to whether organisms are alive or dead, we do not know if or how long the DNA from organisms inactivated by the MSFC water reclamation apparatus can still be amplified by PCR. That water reclamation system's penultimate step in generating potable water is a catalytic oxidation system. Designed to completely oxidize any organic molecules that have made it past the upstream components of the water reclamation system (mixed bed resins provide growth media for many bacterial species), *the catalytic oxidation system should completely mineralize soluble nucleic acids.*[2] Nonetheless, previous PCR analyses of MSFC reclaimed water detected more species of bacteria than were found using culture based methods.[3] That suggests the PCR assays detected a great many nonviable cells

or DNA released from lysed cells; however, that result could be due to the greater sensitivity of PCR based assays relative to culture and the fact that many microorganisms when handled roughly are viable but not culturable (notably *Legionella sp.*[4]).

A recent test of the capacity of the MSFC water reclamation system to eliminate infectious viruses may have laid the groundwork to address the issue of nonviable microbes passing through the system as intact PCR targets. In January 1995, MSFC Chief Microbiologist, Ms. Monsi Roman, and Dr. Christon Hurst of the U. S. Environmental Protection Agency conducted a test in which they added a mixture of $\approx 10^8$ plaque forming units of four different bacteriophages into the water reclamation system intake. During 5 days of system operation, no infectious bacteriophage eluted from the system's clean water ports.[5] To date those samples have only been tested in infectivity assays. Ideally, PCR should be used to analyze those samples for bacteriophage DNA/RNA. Because the nucleotide sequences of all of the bacteriophage used have been published, it should be possible to develop effective PCRs to answer this question. If phage genomes are detected in the clean water in the absence of infectious particles then there is proof that nonviable organisms/viruses passing through the system can generate a false positive result for contamination. Thus any gene based assay system will be to some extent blind as to whether any virus detected is viable or nonviable. If no detectable bacteriophage is found in the clean water by PCR, one can still not rule out the possibility that the mixed bed resins in the system so retarded the virus that in the short test of 5 days, no bacteriophage had time to complete passage through the system. The experiment outlined below addresses that possibility.

Are intact target nucleic acid sequences are available for PCR amplification from or in nonviable cells and virions after passage through the MSFC water reclamation system's catalytic oxidation stage? Different bacterial, viral, protozoan, and fungal samples could be exposed to the system's multiple disinfection procedures, i.e. heat, 250°F for 20 minutes, and/or the 2 ppm iodine imparted to the water by the system's

microbial check valves.[3] One would need to investigate a variety of microbes because different species may respond differently to the inactivation treatments. This could be the result of differences in cell wall or capsid structure or it could be a function of the size of the PCR amplicon.[6] The genomic templates for large amplicons may be more susceptible to damage as a result of germicidal treatment than small templates due to the random nature of the effects of germicidal treatment. After either or both of those treatments the samples would be passed through the catalytic oxidation stage of the water reclamation system and the resulting water would be analyzed by both PCR and culture. Aliquots of the microbial samples should be analyzed by PCR before the heat and/or iodine treatments and between heating/iodination and catalytic oxidation. The PCR data from the three different stages of the water decontamination process would show the extent to which nonviable microbes can be detected by PCR after passage through the MSFC water reclamation system.

Can microbial growth be monitored as a way of bypassing false positive PCR results?

If nonviable microbes contribute significantly to the amount of DNA amplified by PCR of water samples from the MSFC water reclamation system, we would suggest attempting to use a PCR based microbial monitoring system for analysis of recycled water for pathogens to focus on changes in microbial concentration with time that are indicative of increasing populations in the processed water collection tanks. This analysis of microbial population growth approach should work despite the indefinite lifetime of nucleic acid sequences in nonviable cells as demonstrated by Josephson, *et al.*[1] Although there would be a continual influx of low levels of nonviable cells from the water reclamation system, use of the recycled water should result in a continual outflow of the nonviable cells. Thus the contribution of the dead organisms to the estimated microbial load of the collection tank should reach a steady state. Only the presence of microbial growth in the tank should disturb that equilibrium. Obviously, this approach would not work for analysis of viruses because they are obligate parasites and cannot replicate outside of their hosts.

mRNA instead of DNA as a PCR target

One of the main reasons PCR cannot distinguish between viable and non-viable organisms is the great stability of DNA. There is another potential gene target molecule that is much more fragile and short-lived called mRNA. The half life of *E. coli* mRNA is only 30 minutes in a living organism, and presumably much shorter in a dead organism. Similarly, soluble RNA is rapidly degraded in environmental waters and thus is In a pre-PCR step, mRNA can be enzymatically copied using reverse transcriptase.[7] The combination of reverse transcription and PCR, called RT-PCR, has been successfully used to detect mRNA in both eukaryotes and bacteria, and in fact is the only way to detect viruses with RNA genomes such as polio, rotaviruses, and Norwalk viruses. Detection of a short-lived molecular species that can only be made by viable microorganisms would theoretically be the same as detecting only viable organisms.

Unfortunately, at least for bacteria, this would be much more difficult than standard PCR. Although RT-PCR would be required for the detection of RNA viruses (influenza and Norwalk for example), the additional effort might not be feasible or practical bacteria. The half life of mRNA would need to be determined for each species analyzed, along with the average concentration of target mRNA in each cell. Additionally, it would be necessary to eliminate any potential DNA templates in a sample using DNA specific nucleases, and that step could prove to be very difficult.

Several research groups have investigated the possibility of using an RT-PCR approach to discriminate between viable and non-viable microorganisms, however no one has developed an assay that works yet. Dr. Ian Pepper, at the University of Arizona, and Dr. Asim Bej, at the University of Alabama at Birmingham, have both been able to detect bacterial mRNA; however neither see the technology as a method of detecting only viable organisms[1,8,9,10] Scientists at Perkin-Elmer's Applied Biosystems Division said they had experimented with RT-PCR as a tool to screen for

viable bacteria and had abandoned the effort because they felt it could never be made to work.

Vital dye staining as a complement to PCR for discrimination of viable organisms. It may be possible to use vital dyes to detect the presence of live bacteria and protozoa. Vital dye staining is an established technology that could be coupled with the TaqMan PCR (section 4). Thus one could estimate the total number of respiring microorganisms in a sample with the vital dyes, as well as speciate and enumerate the viable and the nonviable microorganisms present using TaqMan PCR. The TaqMan PCR detection system's LS-50B fluorescent plate reader would analyze both the vital dye samples and the TaqMan PCRs.

Viability staining could be achieved through the use of several vital dyes to determine which is most suited to these investigations. Potential dyes include the redox dye 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), and acridine orange to directly observe respiring microorganisms. In the case of INT the reducing power of the electron transport system converts INT into insoluble INT-formazan crystals that accumulate in metabolically active bacteria.[11] Microscopically the INT-formazan deposits are observed as red deposits under bright field microscopy. The INT method has been successfully combined with the acridine orange direct count method to simultaneously enumerate total and viable bacterial concentrations.[12] A method developed by Kogure, *et al.*,[13] also allows for the simultaneous enumeration of both total and viable cells. This method utilized nalidixic acid, a gyrase inhibitor, and yeast extract as a nutrient source. The nalidixic acid prevents cells from dividing while they continue to metabolize the yeast extract and enlarge, dead cells will be unable to utilize nutrients and remain "normal-size". However, there are a number of problems with this method. For example, not all cells are sensitive to the effects of nalidixic acid and not all cells are capable of utilizing yeast extract as a food source. In addition the metabolic rate of microbial pathogens varies which may cause some cells to swell to various sizes

making enumeration difficult. Finally, some bacteria such as *Legionella* are resistant to the effects of nalidixic acid, therefore the Kogure method is not a viable option.[13]

Recently a fluorescent redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), has successfully been used to directly visualize actively respiring bacteria. The oxidized CTC dye is almost colorless and nonfluorescent, however once the dye is reduced via the electron transport system, it becomes fluorescent, insoluble CTC-formazan compound that accumulates intracellularly.[14] Based on published studies with other microorganisms, the dye should provide valuable viability information that would complement the PCR data.

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Section 6.
Current and Projected PCR Quality Control Techniques.

A critical aspect of a PCR based microbial monitor will be a set of quality control measures. Methods must be in place that will insure the following:

- That the assay is functioning according to specifications.
- That reagents are prepared, aliquoted, and stored so that the microbial monitor can function effectively throughout long space missions.
- That samples are not contaminated with microbes from outside of the water reclamation system or with PCR amplicons from earlier reactions resulting in false positive results.

The first two items on this list should be easily attainable. Development of effective internal control reactions has been done for other microbial detection assays; adaptation of that technology to NASA needs should be straightforward. Methods have been reported that would permit long term storage of reagents that have been assayed and aliquoted so that only the sample and water would need to be added prior to assay. Unfortunately, the problem of false positive results due to contamination may prove to be one of the most difficult aspects of developing a PCR based microbial monitor. Diagnostic PCR labs strive to avoid contamination problems through devotion to fastidious technique and laboratory practice as well as through a number of structural and procedural safeguards (Table). Any PCR based instrument used to monitor microorganisms aboard the ISSA will need to incorporate these procedures into the systems design.

Table. Guidelines for the operation of a PCR laboratory.[adapted from 1]

- Establish separate pre- and post-PCR work areas with dedicated supplies and reagents.
- Carefully plan experiments: do not enter the pre-PCR area after handling amplicons or target DNA.
- Use plugged pipet tips or positive -displacement pipettes.
- Use aliquots of all reagents to limit handling.
- Incorporate enzymatic or chemical methods to control amplicon carryover.
- Always use a low-copy number (10-50 templates per PCR) of positive controls, a large number of negative controls, and reagent controls with every amplification.

Control reactions to confirm PCR effectiveness.

A positive control will be incorporated into every sample to insure the PCR worked properly. Reactions could fail because of contamination of the sample with inhibitors, degradation of one of the enzymes or other reagents, or problems with the instrument. An effective internal positive control that is designed to generate a fixed amount of PCR amplicon can provide a quantitative assurance that the PCR system and individual reaction are performing to design specifications.

Included in the reagents used for each PCR will be 10-50 copies of part of the human ß-actin gene, as well as primers and a TaqMan probe that will generate and allow monitoring of the synthesis of an amplicon from the human ß-actin gene.[2,3] Although several different genes are commonly used as an internal positive control molecules, Perkin Elmer Corporation developed the TaqMan system with the intent of using the ß-actin gene for that purpose. As mentioned previously, the TaqMan system can do multiplex PCR because there are three different reporter dyes for labeling probes. Thus every PCR tube will contain two TaqMan probes specific for different microorganisms or groups of microorganisms, plus a probe specific for the ß-actin amplicon. Each of the three probes will be labeled with a different reporter dye.[4]

In addition to the positive controls, every set of PCRs would also include a number of negative controls. Negative control reactions are necessary for confirmation that PCR amplicon carry over is not generating false positive results and to serve as a baseline value for the TaqMan system. With TaqMan each sample being assayed has two tubes containing only the reagents and no sample. Thus, if the ISSA microbial monitor assays for 20 different microorganisms or groups of microorganisms in 10 multiplex PCRs, then an additional 20 negative control PCRs will be required also.

Reagent storage

To simplify the microbial monitor, it will be critical that most reagents be prepared and aliquoted on earth and then stored, potentially for months or years, until needed. In its current configuration, the TaqMan system is designed to assay samples in a 96-well

tray format. Although a full 96-well tray would not be needed to analyze water samples for 20 different kind of PCR targets, NASA should design its PCR based microbial monitor to use a multi-well tray. Reagents could be pre-loaded into multi-well trays on earth so that enzymes, primers and dNTPs are segregated until the reaction is heated, thus preventing reagent degradation due to PCR reactant assembly and storage prior to thermal cycling. One method for accomplishing this is encapsulation of subsets of the PCR reagents in special agarose beads so that they can be stored for long periods of time.[5] G. K Smith, of the University of Houston, believed his microencapsulation methods could be refined to meet the PCR reagent storage needs of an ISSA microbial monitor.[6]

By pre-encapsulating aliquoted amounts of all the components of the PCR except the sample to be assayed , the quality control criteria for diagnostic PCR can largely be addressed. A method and instrumentation will need to be developed to transfer the samples to be analyzed from the filtration system (see Section 2) to a multi-well tray. Perhaps a 10-filter manifold (one filter for each multiplex PCR) could be used to insert filters directly into the multi-well tray containing the reagents prior to thermal cycling. For this to work, procedures would need to be included to release of the DNA or RNA form any microorganisms on the filters without damaging the PCR reagents. Two of the most simple methods for liberation of the nucleic acids from bacteria and viruses prior to PCR are boiling and repeated cycles of freezing and thawing.

Control of carry over contamination that could yield false positives.

The sensitivity advantage that PCR contributes to the detection of microorganisms can also potentially be a major disadvantage. Previously amplified DNA that is replication competent can be carried over and can serve as a template in later amplifications, resulting in false positives. The capacity of single molecule amplification requires special methods be used to insure accurate results. Several approaches utilizing either chemical or enzymatic methods to minimize PCR product carryover have been described.[7] Analysis and comparison of these methods indicates the most effective

method for spacecraft use uses uracil N-glycosylase (UNG) to degrade any contaminating PCR amplicons present in a reaction before the onset of PCR.

UNG is an *E. coli* enzyme that modifies DNA containing uracil so that it can later be degraded by heating. By substituting dUTP for dTTP in the PCR, the resulting amplicons are susceptible to UNG degradation.[8] A 2 minute incubation at 50° is sufficient to modify any contaminating amplicons as well as any mis-primed or non specific products produced prior to specific amplifications, but not degrade native nucleic acid templates. At the end of the 2 minute treatment a 10 minute incubation at 95° completes the degradation of uracil containing DNA, inactivates the UNG, and denatures the template DNA prior to thermal cycling. The procedure actually enhances the quality of the PCR by eliminating any misprimed reaction products that result from the primers annealing incorrectly to templates at low temperature during the mixing of reagents prior to thermal cycling.[9]

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Section 7.
Prediction and Analysis of PCR Primers and TaqMan Probes for the Detection of Microorganism Contaminants in Environmental Samples

Detection of microbiological organisms contaminating environmental samples using TaqMan PCR technology will require primer and probe oligonucleotides to be defined for each organism or group of organisms to be detected. The basis of primer and probe definition is through analysis of available genomic sequence data for the organisms in question. Following the initial step of constructing a list of organisms to be detected, genomic sequences for these organisms are obtained from sequence databases, and then analyzed using parameters appropriate for designing functional primers and probes. All of these steps are computer-based, and result in a library of primer and probe oligonucleotide sequences that have the potential of providing relatively specific and sensitive detection of the desired microorganisms. While use of computers for oligonucleotide design can greatly facilitate construction of an oligo library, these primers and probes will need to be tested empirically in the laboratory to ensure that they work "as advertised". If not, additional oligo sequences will need to be defined. A reiterative process of computer prediction and laboratory testing is the most efficient means available for deriving the basic library of oligonucleotides necessary for environmental monitoring.

Below we discuss some of the considerations that are involved in the process of primer and probe prediction. These include determination of sequences to be detected; computer analysis of these sequences prior to oligo prediction; and analysis of the resulting oligonucleotide library. These methods were then used to predict primer and probe combinations for both a prokaryotic and eukaryotic data set of potential microorganism contaminants.

Genomic Sequences to be Detected

Choice of the particular genomic sequence to be detected is the first critical step in the process of primer and probe design. A wrong choice can lead to high background levels–low specificity (e.g., detection of normal microbiological flora) and low

sensitivity (failure to detect the desired organism). It has been estimated that the determination of the total diversity of microorganisms in environmental samples using culturable plate counts greatly underestimates the true level of diversity by over 90% (Amann, et al., 1995). These authors propose that using methods based upon detecting the presence of ribosomal RNA genes, a much more accurate analysis of the true levels of microorganism diversity can be obtained. The same reasons that make ribosomal RNAs useful in a study of microbiological diversity make them a good candidate for detection in a PCR-based environmental monitor.

Ribosomal RNA Genes

Ribosomal genes are universally present in the cells of all living organisms since they are critical to the process of protein synthesis. Ribosomes consist of two subunits that contain a combination of protein and structural RNAs. The sequences of the large subunit ribosomal RNA and in particular the small subunit ribosomal RNA (ssu rRNA) have been determined for a large number of different prokaryotic and eukaryotic organisms. The availability of these sequences has allowed a significant amount of work to be done in analyzing the biological features and evolution of these sequences between different species (Hillis, et al., 1991; Neefs, et al., 1991). The properties listed below contribute to the usefulness of these genes for detection of environmental contaminants:

- Sequences are present in all living organisms
- Genes contain multiple genomic copies undergoing concerted evolution
- Sequences have undergone variable rates of evolutionary change
- Primers and probes can be defined for hierarchical detection of microorganisms
- Sequences and alignments for most organisms are currently available through the Ribosomal Database Project (RDP) (Maidak, et al., 1994) and Genbank (National Center for Biotechnology Information–National Institutes of Health)

Having available such a large database of genetic sequence information for such a broad range of organisms allows a thorough analysis of the potential specificity of any potential primer and probe combination. Oligonucleotides can be designed with low specificity, but high sensitivity allowing detection of a broad range of organisms using

a single "universal probe". Alternatively, primer and probe combinations can be designed that are very specific, detecting the presence of only one particular pathogen. This provides the capability to design hierarchical probes that initially screen for gross contamination by microorganisms using universal probes, and then, if such contamination is present, the sample can be screened for the presence of particular pathogens using very specific primer and probe combinations. This technique has already been demonstrated using probes derived from small ribosomal RNAs that are designed to detect pathogenic bacteria in cerebrospinal fluid (Greisen, et al., 1994).

The property of these RNAs that provides this capability to detect either broad groups or specific organisms is the variable rates of evolution that these sequences have undergone over time. Certain regions of the ribosomal RNA genes have remained relatively conserved among species (probably due to functional constraints), while other regions show high variability when sequences from different species are compared (Hillis, et al., 1991). These regions have been mapped and correspond to specific regions of the predicted secondary structures of these molecules (Neefs, et al., 1991) (See Figures 7.1-7.4 below). This variable rate of evolutionary change can be exploited for primer and probe design purposes. The highly conserved regions are used to construct universal, or genus-specific probes, while the variable regions provide the necessary specificity to construct species-specific probes (Greisen, et al., 1994; van Kuppeveld, et al., 1992).

Other genes for PCR-based detection

While small ribosomal RNA genes can be used to detect a broad range of organisms, it may be useful to design probes based upon other genomic sequences. Detection of particularly pathogenic organisms may be best accomplished by designing probes to detect the genes specifically involved in the pathogenic mechanisms of these organisms. Examples are the toxin genes in strains of *Shigella* and *E. coli* (Stacy-Phipps, et al., 1995; Read, et al., 1992; Yavzori, et al., 1994; Sethabutr, et al., 1993). These authors have used PCR primers and oligonucleotide probes to detect the

presence of a number of the different toxin genes that have been identified in various strains of these species.

Another reason for utilizing non-rRNA sequences for PCR-based detection schemes, is that the ribosomal RNAs of several species have either not been sequenced, or sequenced to a limited extent. Currently, rRNA sequences for several *Klebsiella*, *Shigella*, and *Salmonella* species among others are absent or incomplete. Inclusion of primers and probes for these species using the ssu rRNA scheme will be dependent on new sequence information as it becomes available. Detection of these organisms will generally need to be based upon other species-specific gene sequences that are in the database; though the evolutionary history of these organisms does predict that they should be detectable by at the very least, the universal primer and probe sets, and possibly by some of the more specific primer and probe combinations (e.g. *Shigella, Salmonella,* and possibly *Kelbsiella species* should be detectable by the Enteric probe described below due to the close relatedness of these organisms to *E. coli*).

Currently several organisms are detected in PCR-based assays using probes not based upon ribosomal RNAs. Two examples are detection of *Legionella pneumophila* (Paszko-Kolva, et al., 1995) and enterotoxigenic E. coli. (Stacy-Phipps, et al., 1995). When appropriate, comparisons will need to made empirically to test the specificity and sensitivity of detection using these currently defined primers to newly defined ssu rRNA-based primers and probes.

Finally, viruses, which have no ribosomal RNA genes since they utilize the host cell's protein synthesis machinery, need to have a separate library of primers and probes designed for their detection. Primers and probes have already been defined and tested for most of the viruses that would need to be in an environmental monitor. These include the enteroviruses (Straub, et al., 1994), adenoviruses (Rousell, et al., 1993), rotaviruses (Sethabutr, et al., 1992) and Norwalk virus (DeLeon, et al., 1992; Jiang, X. et al., 1992).

Primer and Probe Prediction

Using the list of organisms discussed in Section 1, the process of designing primers and probes proceeded as follows:

- Sequences were obtained from both the RDP and Genbank Databases
- Sequence alignments from the RDP were refined, and new sequences were added to the alignments
- Evolutionary relationships between the organisms were inferred based upon the aligned ssu rRNA sequences, and a rough evolutionary tree was constructed
- The organisms were grouped into a detection hierarchy
- Conserved and variable regions within the aligned genes were mapped
- Primer and probe sequences were determined based upon the sequence conservation necessary to detect the desired group of organisms
- These primer and probe combinations were analyzed by computer programs for the desired primer and probe characteristics consistent with optimum TaqMan-PCR detection

As a final critical step, these primers and probes must be tested in the laboratory to ensure that the computer-predicted characteristics actually result in a reliable detection system. This process is designed to provide the most efficient means of combining computer analysis and laboratory testing to establish a library of primers and probes. Each of these steps is described in more detail below, along with the results.

Desired Primer and Probe Characteristics

To design primers and probes that will be optimized for TaqMan-based PCR detection, it is necessary to follow a number of guidelines for probe design. These guidelines attempt to ensure that the desired sensitivity, specificity, primability, and overall usefulness of the oligonucleotides are optimized for the established reaction conditions. Some of the parameters that are known to be important in PCR primer design are as follows (McPherson, et al., 1992):

- Specificity for the desired target
- Appropriate melting temperature (formation of stable duplexes)

- Lack of internal secondary structure (dimers and hairpin loops)
- Lack of secondary structure formation with other primers and probes
- GC content between 40 and 60%
- Avoidance of long runs of a single base

and these additional parameters for TaqMan probe design (Livak, et al., 1995):

- No G at the 5' end
- Add a T at the 3' end if not normally present for attachment of the TAMRA quencher
- Located from 1 to 100 bases to the 3' end of the PCR primer
- Melting Temperature at least 5° C higher than the PCR primers

Computer analysis was used to screen potential PCR primer pairs and TaqMan probes to ensure compliance with the above criteria.

Data Analysis

Data collection

As indicated above, the basic genomic sequence information necessary for this project is available through databases that provide public Internet access to the desired sequence data. The Genbank database is the main US repository for sequence data. It is maintained by the National Center for Biotechnology Information (NCBI) under the auspices of the National Library of Medicine, a part of the National Institutes for Health. We maintain tools for searching and retrieval of sequences from this database, as well as maintaining a local copy of the complete database for internal use. In addition, the Ribosomal Database Project (RDP) at the University of Illinois (Maidak et al., 1994) maintains a subset of this database pertaining to ribosomal RNA sequences. This database includes pre-aligned sequences and predictions of evolutionary relationships that greatly facilitate using this information for primer and probe prediction. Genbank and RDP data were obtained through anonymous FTP.

Sequence Analysis

General sequence analysis tools are provided by a comprehensive package of sequence analysis programs published by the Genetics Computer Group (GCG) of Madison, Wisconsin (Devereux, 1994). This package has tools that allow simple pattern recognition, multiple sequence alignment, evolutionary analysis, and most other programs necessary for sequence analysis. This package provided the basic core of analysis tools used in this project.

Evolutionary analysis

In addition to the GCG programs, several other programs were used for evolutionary analysis of aligned sequences. These include Clustal (Higgens, 1991); Phylip (Felsenstein, 1994); and Phylogenetic Analysis Using Parsimony (PAUP) (Swofford, 1993). Evolutionary analysis of the sequence information was an important step in determining which groupings of microorganisms can be effectively detected with a single primer and probe combination.

Primer/Probe analysis

Prediction and analysis of PCR primers and TaqMan probes was accomplished using the OLIGO program from National Biosciences, Inc. (Wojciech, 1994). This program predicts and analyzes oligonucleotides that satisfy the criteria outlined above for optimal PCR and probe characteristics.

Primer and Probe Prediction

Listing of organisms to be detected

The microorganisms listed in table 7.1 formed the basic data set from which a series of PCR primers and TaqMan probes were derived for environmental monitoring. This list of organisms does not include all of the organisms indicated in section 1 as being desirable for detection. This is due to the lack of ssu rRNA sequence information for some microorganisms. As additional sequence information becomes available, additional organisms can be analyzed using the procedures followed below. Never-the-less, contamination by many of the organisms not listed (such as *Klebsiella pneumoniae*, and *Shigella* species) should be detectable by the universal PCR

primers and TaqMan probes listed below. In addition, references were provided above for the detection of additional organisms, including viral contaminants, using PCR and probe-based methods not dependent on rRNAs.

Table 7.1. Microorganisms Analyzed

Prokaryotic Organism	Abbreviation
Acinetobacter	*
Alteromonas	*
Bacillus coagulans	B-coagu
Burkholderia cepacia	Bur-cep
Burkholderia pickettii	Bur-pick
Corynebacterium	
Enterococcus avium	Eco-avi
Entercoccus faecium	Eco-fcm
Enterococcus faecalis	Eco-fae
Escherichia coli	E-coli
Legionella pneumophila	Leg-pne
Listeria	*
Micrococcus luteus	Mic-Luteus
Mycoplasma fermentans	M-ferme
Mycoplasma hominis	M-homin
Mycoplasma pneumonia	M-Pneum
Pseudomonas aeruginosa	Ps-aeru
Salmonella cholera	S-chole
Salmonella dublin	S-dubli
Salmonella enteritidis	S-enter
Salmonella paratyphi	S-parat
Salmonella typhi	S-typhi
Staphylococcus aureus	Stp-aureus
Staphylococcus epidermidis	Stp-epider
Staphylococcus haemolyticus	Stp-haemo
Staphylococcus hominis	Stp-homin
Staphylococcus saprophyticus	Stp-saprop
Staphylococcus warneri	Stp-war
Streptococcus bovis	Stc-bovis
Streptococcus equinis	Stc-equins
Thiobacillus ferrooxidans	Thb-fer
Ureaplasma urealyticum	Upl-ure
Vibrio cholerae	V-chole
Vibrio parahaemolyticus	V-parah
Vibrio vulnificus	V-vulni

Eukaryotic Organism	Abbreviation
Aspergillus fumigatus	Asp-fuki
Candida albicans	Cnd-albc
Cryptosporidium parvum	Crp-parv
Cryptococcus neoformans	*
Entamoeba histolytica	Ent-hist
Girardia lamblia	Gir-lamb

* These organisms are not displayed in the sequence alignment or analyzed for Figure 7.4 (see below), but were analyzed for detectability using the primer and probes oligonucleotides indicated in Table 7.2.

Alignment

The sequences of the ssu rRNAs for these sequences were obtained from the RDP and Genbank databases. These sequences were reformatted as necessary for use in subsequent analyses. The RDP also provided sequence alignments and evolutionary trees for these RNAs. Where necessary, these alignments were refined, and additional sequences added that were not present in the RDP database. Programs in the GCG package were used for these purposes.

The alignment of the ssu rRNA sequences is shown in Figures 7.1 (prokaryotic) and 7.2 (eukaryotic). Gaps have been introduced into the sequences to account for evolutionary changes due to insertions and deletions into sequence lineages. Gaps are represented by dashes. Also shown are the positions of the variable regions that are interspersed with more conserved sequences as these RNAs evolved (see below). The positions of the predicted set of PCR primers and TaqMan probes is also shown (see below). The eukaryotic alignment includes Human and *E. coli* ssu rRNA sequences for reference purposes.

Ribosomal RNA Secondary Structure and Sequence Conservation

As discussed above, one of the features of ssu rRNAs that make them particularly suitable for environmental monitoring are the conserved and variable sequence features that are interspersed throughout these genes (Hillis, et al., 1991; Neefs, et al.,

1991). These RNAs must form secondary and tertiary structures to function as components of the protein-synthesizing ribosomes. Certain features of these RNAs must be maintained for functional purposes, while other features need not be strictly conserved, and can vary. This results in alternating patterns of conserved and variable domains seen when comparing ssu rRNA sequences from different species. Figure 7.3 shows the predicted secondary structure for the *E. coli* ssu rRNA, and the conserved and variable region domains. Conserved features can be utilized to derive universal PCR primers and TaqMan probes that will bind to, amplify, and detect ssu rRNAs from a wide variety of organisms, while additional TaqMan probes can be designed from the more variable regions that would be very specific and detect only one particular species.

Figure 7.4 is a graph showing the extent of evolutionary change for three separate groups of sequences. The top, blue shaded graph is for the alignment of all of the prokaryotic organisms indicated in table 7.1. The middle, pink shaded graph analyzes the gram-negative organisms from the above list, and finally, the bottom, yellow shaded graph shows the similarity among the *Mycoplasma* species. To generate this data, the aligned set of sequences were grouped according to their evolutionary relationships (see below), and then the program MacClade (Maddison and Maddison, 1992) was used to calculate the extent of evolutionary change at each position in the sequence alignment. The Y-axis is proportional to the number of sequence changes that have occurred at each alignment position as these sequences (organisms) have diverged over the course of evolutionary history. The greater the divergence, the greater the number of evolutionary changes, and the higher the value seen on the Y-axis. As can be seen, as the set of organisms analyzed is reduced to those that are more closely related, the extent of sequence identity and evolutionary conservation increases. Never-the-less, the variable rates of evolution can be clearly seen even among just the *mycoplasma* group by noting the interruption of highly identical (conserved) regions with extremely variable regions. This information provided the basis by which the location of potential PCR primers and TaqMan probes were determined that could be used to detect specific groupings of organisms.

Sequence Evolution

The sequence alignments in Figures 7.1 and 7.2 were used to construct the evolutionary trees in Figures 7.5 (prokaryotic) and 7.6 (eukaryotic). These trees show the evolutionary relationship between these organisms as calculated by Maximum Likelihood methods using Phylip (Felsenstein, 1994) and fastDNAml (Olsen, 1994). The trees displayed are based upon data obtained from the RDP (Maidak, et al., 1994). These relationships were confirmed using additional analysis methods based upon maximum parsimony using PAUP (Swofford, 1993), and neighbor-joining using Clustal (Higgens, 1991) and GCG (Devereux, 1994), These trees are shown only to indicate approximate evolutionary relationships between these organisms. No attempt was made to clearly define the branching order between closely related sequences (and thus define the common ancestry and evolutionary lineages of these organisms).

The length of the horizontal branches are proportional to the extent of sequence divergence among these sequences. Therefore, these figures show both the inferred evolutionary relationships and the extent of evolutionary change. For the purposes of environmental monitoring by PCR, we are only concerned with the sequence relationships and how these organisms can be grouped together. The prokaryotic evolutionary tree clearly shows the division between gram-negative and gram-positive organisms. Other relationships are as expected, and these groupings formed the basis of determining primer/probe combinations that could be used in a hierarchical detection scheme.

Primer and Probe Prediction

Using the data from the above analyses, a set of PCR primers and TaqMan probes were predicted that could be used in a PCR-based environmental monitor. These primers and probes were predicted with the aid of the OLIGO program (Rychlik, 1994) along with direct visualization of the alignment—looking for regions showing the appropriate conservation and/or divergence necessary for the indicated specificity. OLIGO was initially used to derive a set of compatible PCR primer pairs that meet all of the criteria indicated above. Each of these primer pairs were than located on the

sequence alignment and visually analyzed to determine primer pairs that would best satisfy the criteria of providing a set of universal primers for amplifying prokaryotic sequences, and another set for eukaryotic sequences. After these sets of universal PCR primer pairs were established, a combination of OLIGO and direct visualization was again used within the confines of the PCR-amplified product, to predict sets of TaqMan probes that again satisfy the criteria outlined above for optimal probe design. The primers and probes that resulted from this analysis meet the above criteria to the extent possible for optimal activity. Empirical testing will of course need to be performed to ensure the adequacy of these oligos for their intended purpose. This includes assaying for the desired sensitivity to amplify and detect the indicated organisms, and the desired specificity in only detecting the intended group of organisms.

The location of the PCR primers and the TaqMan probes are indicated on the sequence alignments in figures 7.1 and 7.2. The sequences of these primers and probes, their locations, and their predicted melting temperatures (T_m) are listed in table 7.2.

Table 7.2. PCR Primers and TaqMan Probes

Prokaryotic

Name	Sequence	Location	Description	T_m °C
PCR Primers				
U	GGGGAGCAAACAGGATTAGA	E-coli:773U20	Universal Upper	64.0
L	AAGGGCCATGATGACTTGAC	E-coli:1193L20	Universal Lower	64.1
Probes				
Uni	CCTGGTAGTCCACGCCGTAAACGAT	E-coli:796U25	Universal	76.8
GmP	TGAGTGCTAAGTGTTAGGGGGTTTCCt	Stp-aur:U828	Gram Positive	73.7
Enteric	TCGACTTGGAGGTTGTGCCCTTGAGt	E-coli:822U25	*E. coli, Vibrio, Salmonella sp.*	77.8
Legion	TGAAAATAATTAGTGGCGCAGCAAAt	Leg-Pne:842U25	*Legionella sp.*	72.9
Burk	TTGTTGGGGATTCATTTCCTTAGTAACt	Bur-Cep:824U27	*Burkholderia*	71.2
Ps	TCCTTGAGATCTTAGTGGCGCAGCT	Ps-Aeru:833U25	*Pseudomonas sp.*	75.2
Thb	TGGGTACTAGACGTTGGGAGGTTTAt	Thb-Fer:661U25	*Thiobacillus*	70.9
Myco	TAACTAACGAAAGGGGTTGCGCTCGt	Upl-Ure:1094L25	*Mycoplasma sp.*	77.2

Eukaryotic

Name	Sequence	Location	Description	T_m °C
PCR Primers				
U	ACATCTAAGGAAGGCAGCAG	Crp:371U20	Universal Upper	61.8
L	CGATCCCCTAACTTTCGTTC	Ent:952L20	Universal Lower	63.8
G-U	ACATCCAAGGACGGCAGCAG	Gir:322U20	*Girardia* Upper	70.3
G-L	GCCTTCGCCCTTGATTGACA	Gir:713L20	*Giardia* Lower	70.4
Probes				
Fungi	CTTTTGGGTCTCGTAATTGGAATGAt	Asp:489U25	*Aspergillus, Candida, Cryptococcus*	71.2
Crp	CAATACAGGGCCTAACGGTCTTGTAt	Crp:440U25	*Cryptosporidium*	71.4
Ent	TGTTCCTTTTAATCCTTCTCTCGAAt	Ent:827L25	*Entamoeba*	68.6
Gir	CGGTCTCGGCGGGATCATCCTGTTT	Gir:656L25	*Giardia*	82.1

Table 7.2. PCR Primers and TaqMan Probes. The composition of the predicted optimal PCR primers and TaqMan probes are listed for prokaryotic and eukaryotic monitoring. The oligo sequences are written 5' to 3' in the orientation necessary for synthesis. Therefore for upper strand oligos, the indicated sequence is the same as what would be seen in the sequence alignments (Figures 7.1 and 7.2), while for lower strand oligos, the sequence shown represents the reverse-complement of the sequence in the sequence alignments.

A lower case t at the 3' end of a probe sequence indicates the necessary addition of a non-templated T to the end of the probe to which the TAMRA quencher will be added. The fluorescent reporter dye should be added to the base at the 5' end of the probe sequences.

The oligo location indicates the organism from which the sequence information was derived, the number of the sequence base (this number excludes gaps introduced for alignment purposes) at the left-most position of the oligonucleotide as the sequence is viewed in the 5' to 3' direction of the rRNA. Therefore for oligos derived from the upper DNA strand (U in the location designation), this number represents the base at the 5' end of the oligonucleotide. For oligos derived from the lower DNA strand (L in the location designation), this number represents the base at the 3' end of the oligonucleotide. The L or U designation in the location is followed by a number indicating the length of the oligonucleotide.

The melting temperature—T_m of each oligo is predicted using the nearest-neighbor method as implemented by the program OLIGO. These are indicated for reference purposes and are useful in comparing the melting temperature properties of one oligo to another, but the actual melting temperatures will vary with reaction conditions, and will have to be determined empirically.

The PCR primers consist of a set of universal forward and reverse oligos that should be able to amplify DNA from any of the prokaryotic organisms, and another set for the

eukaryotic microorganisms with the exception of *Giardia.* An alternate set of PCR primers was necessary for *Giardia* due to the extensive divergence of it's ssu rRNA sequence from the other eukaryotic organisms.

The TaqMan probes consist of a number of different probes designed to detect particular groupings of organisms based upon similarities in specific regions of their ssu rRNA sequences. These groupings are shown in figures 7.5 and 7.6. along with the intended targets for each of the TaqMan probes. The prokaryotic probes are designed to detect either all of the organisms using a universal probe; a probe for gram-positive organisms; a probe for *mycoplasma* species; a probe to detect gram-negative enterics including *E. coli*, *Vibrio*, and possibly *Salmonella* species; a *Legionella*-specific probe; two probes specific for different species of *Burkholderia* and *Pseudomonas*; and a *Thiobacillus*-specific probe.

In addition to the organisms specifically analyzed in Figures 7.1 and 7.2, the universal probe should also detect most other organisms that might be of concern as environmental contaminants. The universal prokaryotic probe falls within an extremely conserved domain of the prokaryotic ssu rRNAs. All prokaryotic organisms examined, including many organisms not specifically mentioned here, should be detected by this probe. We specifically looked at the ability of the universal probe to detect several organisms that may prove to be rare environmental contaminants, but would be important, never-the-less, to be detected by an environmental monitor. These include *Listeria*, *Corynebacterium*, *Acinetobacter*, and *Alteromonas* species. All of these organisms should be detected by the universal probe. If deemed necessary, probes specific for the detection of these, and other possible environmental contaminants can be designed and tested, using the same procedures outlined in this report.

The *Legionella* probe should efficiently detect all types of *Legionella pneumophila*. Sequence analysis also indicates that it may also function as a universal *Legionella* probe detecting other Legionella species as well. Only empirical testing will ensure the applicability of this probe as a universal *Legionella* probe. Alternatively, universal

primer/probe combinations already described in the literature may be used as desired (Paszko-Kolva, et al., 1995).

The *Pseudomonas* probe should efficiently detect *Pseudomonas aeruginosa.* Sequence analysis also indicates that it might function as a universal *Pseudomonas* probe detecting other *Pseudomonas* species as well. The diversity of ssu rRNA sequences between different *Pseudomonas species* makes prediction of a universal *Pseudomonas* probe difficult. Only empirical testing will ensure the applicability of this probe as a universal *Pseudomonas* probe.

For the eukaryotic microorganisms, a universal fungi probe was designed to detect the presence of various Fungi including *Aspergillus, Candida*, and Cryptococcus species. Cryptococcus is not specifically listed in Table 7.3 or Figure 7.2, but analysis of Cryptococcus ssu rRNA sequences indicates that it should be detected using this probe. Specific probes were also designed to detect *Cryptosporidium*, *Entamoeba*, or *Giardia species* . It was not possible to design a universal eukaryotic probe due to the more extensive divergence of these ssu rRNA sequences in comparison to the prokaryotic sequences.

Primer and Probe Analysis

To ensure to the extent possible that the set of primers and probes predicted above satisfy the criteria for sensitivity and specificity of detection, a feature of the OLIGO program was used to quantify the ability of each of the oligos to hybridize to the different ssu rRNAs. OLIGO includes a priming efficiency (PE) statistic that attempts to infer the binding probability of a specific oligo to a specific sequence. The PE statistic includes analysis of base content, sequence mismatches, duplex stability, and terminal stability of the oligo. Table 7.3 lists the PE for all prokaryotic and eukaryotic primers and probes, along with the intended PCR product size and location for each of the ssu rRNA sequences.

Table 7.3: PCR Product; PCR Primer; and TaqMan Probe Statistics

Prokaryotic	*PCR Product*			*PCR Primers*		*TaqMan Probes*							
Organism	*Size*	*Start*	*End*	*U*	*L*	*Uni*	*GmP*	*Enteric*	*Legion*	*Burk*	*Ps*	*Thb*	*My*
		Max. P.E.:		*440*	*437*	*562*	*552*	*540*	*538*	*533*	*542*	*507*	*566*
B-coagu	439	774	1193	*311*	*290*	*562*	*448*	109	87	65	95	97	*389*
Bur-cep	435	767	1182	*440*	*368*	*465*	138	66	165	*533*	128	91	*403*
Bur-pic	435	725	1140	*440*	*368*	*465*	152	80	165	*533*	128	91	*384*
Eco-avi*													
Eco-fcm	440	792	1212	*440*	*290*	*562*	*420*	197	*212*	120	0	64	*413*
Eco-fae*													
E-coli	440	773	1193	*440*	*437*	*562*	123	*540*	170	81	130	13	*379*
Leg-pne	442	773	1195	*440*	*437*	*456*	140	85	*538*	71	105	86	*404*
Mic-Luteus	443	752	1175	*381*	*290*	*291*	174	97	114	67	158	115	*340*
M-ferme	426	768	1174	*440*	*245*	*249*	108	14	190	147	*282*	110	*414*
M-homin	377	766	1123	*440*	*245*	*562*	101	38	178	84	207	32	*442*
M-Pneum	418	771	1169	*338*	*290*	*410*	91	31	138	65	108	77	*466*
Ps-aeru	440	767	1187	*440*	*437*	*562*	144	205	*255*	96	*542*	63	*412*
Salmonella*													
Stp-aureus	442	781	1203	*374*	*290*	*562*	*542*	24	149	163	203	31	*371*
Stp-epider	440	782	1202	*374*	*290*	*562*	*542*	24	155	65	203	31	*371*
Stp-haemo	440	773	1193	*374*	*290*	*562*	*542*	142	149	65	203	31	*371*
Stp-homin	440	773	1193	*374*	*290*	*562*	*542*	24	149	65	203	31	*371*
Stp-saprop	442	754	1176	*374*	*290*	*562*	*542*	131	149	65	203	88	*371*
Stp-war*													
Stc-bovis	440	781	1201	*440*	*290*	*562*	*313*	192	167	137	108	184	*459*
Stc-equins	440	675	1095	*440*	*290*	*512*	*303*	93	184	139	*268*	190	*460*
Thb-fer**	308	614	902	*440*	*298*	*562*	150	98	176	44	0	*507*	*372*
Upl-ure	415	772	1167	*383*	*290*	*410*	0	11	126	38	143	158	*566*
V-chole	441	771	1192	*440*	*437*	*562*	122	*375*	181	17	21	13	*366*
V-parah	441	771	1192	*440*	*437*	*479*	122	*378*	181	33	103	58	*366*
V-vulni	441	771	1192	*440*	*437*	*456*	122	*393*	181	65	103	64	*366*

Eukaryotic	*PCR Product*			*PCR Primers*				*TaqMan Probes*			
Organism	*Size*	*Start*	*End*	*U*	*L*	*G-U*	*G-L*	*Fungi*	*Crp*	*Ent*	*Gir*
		Max. P.E.:		*420*	*443*	*465*	*476*	*508*	*523*	*490*	*594*
Asp-fumi	583	408	971	*383*	*443*	*388*	108	*508*	24	121	204
Cnd-albc	570	408	958	*387*	*443*	*388*	108	*439*	*295*	204	79
Crp-parv	557	371	908	*420*	*443*	*338*	108	*323*	*523*	153	92
Ent-hist	567	405	952	*383*	*443*	*304*	175	111	92	*490*	93
Gir-lamb	411	322	713	*339*	11	*465*	*476*	51	98	0	*595*
Human	591	458	1029	*387*	*278*	*388*	116	*320*	*260*	95	78
E-coli	443	338	761	*279*	100	*234*	112	82	7	42	103

* No sequence information available within the PCR primer region

** Limited sequence information within the PCR primer region

Table 7.3: PCR Product; PCR Primer; and TaqMan Probe Statistics. For each prokaryotic and eukaryotic organism listed in table 7.1, the size and the start and stop positions (numbered excluding alignment gaps) of the expected PCR product using either the universal prokaryotic or eukaryotic primers is shown. For *Girardia lamblia*, the Giardia-specific PCR primers are used.

For each of the different PCR primers and TaqMan probes, the priming efficiency (PE) value as calculated by the program OLIGO is shown for each organism. The higher the PE value, the greater the chance that the indicated oligo will hybridize to the indicated sequence. Values above 250 are highlighted in bold type.

These PE statistics provide a rough guide as to the potential sensitivity and specificity of each of the primers and probes. As indicated previously, all of these combinations will need to be tested empirically because the PE values may not necessarily represent the true ability of some of the probes to function as intended. For example, the *mycoplasma*-specific probe shows high PE values for all of the prokaryotic ssu rRNA sequences. Even though the *mycoplasma* sequences show the highest values, it might be assumed that this probe would act more as a universal probe rather than a *mycoplasma*-specific probe. In this instance the PE values may be misleading. For a TaqMan probe to function, it is important that the 5' end of the probe be efficiently base-paired to the sequence template to allow for the nuclease activity of the Taq polymerase to cleave the 5'-fluorescently-labled base of the probe away from the rest of the probe oligo and the TAMRA quencher on the 3' end. The *mycoplasma*-specific probe shows a fair degree of homology to non-*mycoplasma* sequences at the 3' end of the probe. Much less homology exists at the 5' end of this probe to non-*mycoplasma* sequences. Therefore, we would predict that in spite of the high PE values for non-*mycoplasma* sequences, this probe may still function specifically to detect only ssu rRNAs from *mycoplasma* species.

Limitations of Computer Prediction

All of the analyses performed for section 7 rely on translating molecular biological knowledge into computer programs that try to make biological predictions based upon our current understanding of biological processes. While these programs provide a useful basis to make the sorts of predictions seen above, the limitations of these predictions must always be considered. The process of primer and probe prediction is necessarily a reiterative one in which the initial computer-predicted oligos are tested in the laboratory by using them to detect samples of actual microorganisms under conditions that come as close as possible to those utilized by an environmental monitor. Following the initial round of laboratory testing, primer and probe sequences will need to be refined as necessary, and the testing repeated until the desired characteristics are obtained. This process should eventually lead to a functional and efficient monitor for the detection of microorganisms in environmental samples.

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