A major area of research in microbial characterization has been the development of molecular methods for genotyping organisms. Genotypic methods can be highly specific and sensitive and are largely independent of the physiological or growth state of the organism.

Ribotyping, a molecular method based on the analysis of restriction fragment length polymorphisms (RFLPs) of ribosomal RNA genes, has been widely adopted to distinguish a variety of bacteria (1-5). For the past five years ATCC has been using the RiboPrinter® Microbial Characterization system (Qualicon Inc., Wilmington, DE) as a primary method for characterizing strains during the quality control process.

Ribotyping is a molecular methodology that performs a restriction digest (using EcoRI or other restriction enzymes) of the chromosomal DNA, separates the restriction fragments by gel electrophoresis, and simultaneously blots the DNA fragments to a membrane which is used for Southern blot analysis. Restriction digest fragments are hybridized to a bacterial probe that is based on the conserved regions of the genes for the ribosomal DNA operon. The result is a DNA fingerprint which is strain specific. Each fingerprint is stored in a database so it can be accessed for future comparisons and identifications.

We routinely use the RiboPrinter system in several steps of our quality control and authentication process for bacterial cultures. Ribotyping is per-...
Streptomyces coelicolor

The genome of Streptomyces coelicolor has recently been sequenced as a representative of the huge family of streptomycetes. Results have shown that this organism has the largest genome found in a bacterium (Nature 417: 141-147, 2002; Microbiology 145: 2183-2202, 1999). ATCC has the culture and the purified genomic DNA from a prototrophic derivative of the A3(2) strain called M145.

Multiwell Plate Seals

Protect your plates from evaporation and contamination with our exclusive multiwell plate seals. The durable foil seals are marked with column and row locations for each well, allowing you to find the well you need in one glance. Other features:

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- Forms a tight seal; will not curl.
- Withstands high and low temperatures (-80 to 150°C), high humidity, oils, and isopropanol.

Two configurations are available.

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Patent pending.

Mycoplasma Supplement

Mycoplasma Growth Supplement, also known as CMRL-1066 Cell Culture Medium, is a well-defined media supplement used in the cultivation of many mycoplasma strains. Add the powder during medium preparation or add the 10× liquid to sterile media. You’ll save time by adding several ingredients in one convenient step.

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Order online or call 800-638-6597.
Infectious salmon anemia is a viral disease responsible for severe economic losses in the Atlantic salmon aquaculture industry. Epizootics have been reported in Norway since 1984 (1) and more recently in the United States (2).

Prior to 1995, routine in vitro isolation and propagation of the infectious salmon anemia virus (ISAV) was not possible because existing continuous fish cell lines did not appear to support replication of the virus. This hampered characterization of the virus and consequently the development of diagnostic methods. In response many laboratories began isolating primary cell cultures from various organs of Atlantic salmon.

From these primary cell cultures, some long-term and continuous cell lines capable of supporting propagation and replication of ISAV were developed, including the SHK-1 line of Dannevig et al. (3), the TO line of Wergeland and Jakobsen (4), and the ASK cell line of Devold et al (5). Previously established cell lines were also found to support the growth of the virus, such as the CHSE-214 cell line from Chinook salmon embryo (6-8) and the AS cell line derived from the visceral organs of Atlantic salmon (9,10). However, difficulties such as low yields and variable results have been reported (11, 12). The TO line performed well in virus testing and appears to be a stable cell line, but patent considerations restrict its availability.

**Cell line assessment**

Scientists at the United States Geological Survey, Biological Resources Division, Western Fisheries Research Center in Seattle, Washington, compared the CHSE-214, SHK-1, and ASK cell lines to determine which was most suitable for ISAV propagation and titer determination.

Two strains of ISAV were used to test the cell lines. Strain CCBB was isolated by Micro Technologies, Inc. (Richmond, ME) from an outbreak in Atlantic salmon in Back Bay, New Brunswick, Canada. ISAV strain Bremnes was isolated from an outbreak in Bremnes, Norway by the University of Bergen, Department of Fisheries and Marine Biology, Section for Fish Health, Norway.

The CCBB strain was known to cause cytopathic effect (CPE) in the CHSE-214 cell line. However, we tested both this virus and the Bremnes strain and neither produced CPE in a pilot study using the CHSE-214 cell line.

We thus compared the ability of SHK-1 cells and ASK cells to support growth of the virus.

**Advantages of the ASK cell line**

Our results indicate that the ASK cell line is superior to SHK-1 when ISAV research studies or routine diagnosis requires viral isolation. Although end-point titers were comparable in SHK-1 and ASK cells, the ISAV induces rapid detectable CPE in the ASK cell line (Figure 1) consisting of complete lysis and detachment from the substrate within 14 days, while the CPE in SHK-1 cells is not definitive. In the first 14 days postinoculation, some SHK cells detach from the substrate (Figure 2). Up to 25 days postinoculation, we observed morphological changes in the SHK cells, such as vacuolization, that could be attributed to factors other than viral replication. Accurate measurement of viral titers from SHK-1 cells was therefore difficult to determine due to a lack of distinct CPE, and end-point titers were calculated for both lines in the ASK cells.

The ASK cell line is epithelial-like and has retained its morphology with each subculture at the Western Fisheries Research Center. The SHK-1 cell line has been partially characterized and the cells were found to be macrophage-like (3). However, morphologically the cell line appears to be a mixed population consisting primarily of fibroblast-like cells, but also containing a small population of leukocyte-like cells. The number of leukocyte-like cells in a culture declines with each passage. To avoid complete loss of this subpopulation whose role in ISAV replication is poorly understood, fresh cells from frozen stocks at a low passage must replace higher passage cultures at regular intervals. This mixed population could also make the standardization of ISAV detection protocols across different laboratories more challenging.
formed when the culture arrives at ATCC and the results then become part of the laboratory record. DNA fingerprints from future seed stock and replenished materials are compared with this original to ensure that the culture has not changed during the propagation and preservation processes (Figure 1). Over the last few years we have tested hundreds of genera and species using this automated genotyping method and the results indicate that the system is reliable and reproducible.

Ribotyping is very useful for authenticating fastidious organisms, which are often difficult to characterize by traditional physiological methods. Ribotyping patterns are generated from both environmental strains and clinical isolates and the records augment our characterization profiles. Since we accession many newly discovered and published bacteria, we do not expect the system to produce identification because the new names are not yet in the existing database. Rather we examine and archive the DNA fingerprint patterns to verify authenticity.

Characterization and relatedness

Recently we used the RiboPrinter system to survey members of the Legionellaceae family (6). The majority of Legionella species are currently typed by traditional serological methods. Our study, which included 110 strains comprising 48 genera and 70 serogroups, showed that the ribotyping system works well in distinguishing members of this genus. Strains within the same species displayed consistent patterns even when isolated from a variety of sources and geographic locations, yet species were clearly differentiated. We also provided examples where this system could be used to identify new isolates by comparing their patterns to those generated from known Legionella species.

In another research application we used the RiboPrinter to genotype a group of isolates from salt marsh sediments that belonged to the classes Flavobacteria and Sphingobacteria in the phylum Bacteroidetes. These bacteria are now being recognized as some of the most common members of both marine and soil habitats, yet little is known about their population structure. Riboprint analysis showed that these strains exhibited significant genotypic diversity despite a more limited phenotypic diversity (7).

Studying morphological variants

Ribotyping is also very useful for resolving problems of colony variation within a strain. For example, members of the genus Bacillus are infamous for producing a variety of colonies types (i.e., wrinkled vs. smooth). Ribotyping can confirm that the differing colonies originated from the same organism and are not contaminants (Figure 2).

Another example of the utility of this system is demonstrated in the analysis of a recent ATCC accession, Rhodospirillum indiensis BAA-36. This organism undergoes dramatic changes in cell morphology during aerobic growth, from spiral-shaped rods to curved short rods to packets of cocci, giving the appearance that the culture was contaminated (8). However, a repeated growth experiment coupled to analysis of the different growth stages by riboprinting proved that the different cell shapes were indeed the same organism (Figure 3).

The RiboPrinter system allows the flexibility in using other restriction enzymes besides the standard EcoRI. Some genera yield better patterns with PvuI or PstI. Other restriction enzymes can also be substituted in the process.

Careful, thorough characterization is a mainstay of ATCC’s collection of prokaryotes, and ribotyping is a method that works with a broad range of organisms. Other advantages include the ease of operation and data analysis. The system requires only a single colony as inoculum and there are no restrictions on media and growth conditions. The adaptability and flexibility of the system make it a valuable part of ATCC’s overall quality control program.
Authentication 101: Testing of Prokaryotes at ATCC

In this issue of ATCC Connection we describe just one of many procedures we use to characterize microorganisms. Given the great diversity of prokaryotes at ATCC it is a constant challenge to streamline the authentication process so that microbes can be characterized with the greatest efficiency. By utilizing several diverse identification strategies at the phenotypic and genotypic levels we have developed protocols that ensure thorough characterization of every strain.

The first step is to check the growth, purity, and cell and colony morphologies of all cultures that arrive at ATCC for deposit. Cultures then undergo biochemical testing if appropriate. For many of the more common bacteria we have developed a standard set of growth and biochemical tests that are based on well-known traits of these organisms. These biochemical tests, which include the use of API strips (bioMérieux, Inc.) and other commercial rapid tests, have been developed for different bacterial groups. We have refined these schema over the years to minimize the number of tests yet still provide robust identification. Even with these refinements there are 23 different characterization schemes encompassing nearly 350 individual tests that are performed on a routine basis.

The biochemical tests are instrumental for authenticating many important phenotypic properties of ATCC microbes. They are time-consuming, however, and the repertoire of biochemical tests is very limited for some organisms. Therefore, whenever possible we take a polyphasic approach to authentication that elucidates both phenotypic and genotypic traits of the organism. We are constantly evaluating and utilizing new technologies that balance selectivity, throughput, cost, and effort to ensure the best quality for our authentication procedures.

Over the next several issues we will describe other automated and molecular-based methods used at ATCC to characterize bacteria. To see an overview of the whole QC process, go to the bacteria search page on our Web site and look for the link under the query window.
blasts is described here. Use it as a starting point for designing your own protocol. All procedures should be carried out using aseptic technique.

1. Starting with approximately 4 cm² of skin sample including dermis and epidermis, rinse twice with at least 30 ml of medium containing 1× Antibiotic Mix and 2.5 µg/ml Amphotericin B (catalog no. 30-2301). Remove any fat layer from the skin and dissect the material into small pieces using mincing scissors.

2. Place the small tissue pieces in a spinner flask with sterile forceps and add 25 ml 0.025% trypsin-0.53 mM EDTA (catalog no. 30-2101), 25 ml collagenase (1800 U/ml), and 200 ml Iscove’s Modified Dulbecco’s Medium (IMDM; catalog no. 30-2005) containing 1× Antibiotic Mix and 2.5 µg/ml Amphotericin B. Incubate and spin in a spinner flask at about 60 rpm overnight (20 to 22 hours) at 37°C.

3. The next day neutralize the trypsin by adding fetal bovine serum (FBS; catalog no. 30-2020) to a final concentration of 25%.

4. Collect the cells by centrifuging at approximately 300 × g for 20 min. The pellet may be very loose so take care when removing the supernatant fluid.

5. Resuspend the cells in 25 ml of growth medium containing 20% FBS, 0.5× Antibiotic Mix and 1.25 µg/ml Amphotericin B. Count the cells.

6. For cells from a young donor (< 20 years old), seed cells at 2 × 10⁶ to 3 × 10⁶ in a 75-cm² flask. For older donor cells use 5 × 10⁶ to 6 × 10⁶ cells. IMDM with 20% FBS is used.

7. Allow the cells to grow for 5 to 7 days at 37ºC with 5% CO₂. Monitor the cells every other day but do not disturb them too much; do not change the medium. When cells appear to be growing replace the medium with fresh IMDM containing 20% FBS and 1× penicillin-streptomycin solution (catalog no. 30-2300). Do not maintain the cells with Antibiotic Mix in the medium. If the cells are not growing, continue incubation and discard if they are not growing after three weeks.

8. Before preparing frozen stock from these cells, we recommend that you remove all antibiotics, including penicillin-streptomycin, for at least 2 weeks. Antibiotics can be toxic to some cells and may mask the growth of low-level bacterial contaminants.

Concentrations recommended above are suitable for skin fibroblasts. Tissues from internal organs are less likely to be grossly contaminated. They may require simply rinsing with Antibiotic Mix and using only penicillin-streptomycin in the growth medium.

The estimated number of population doublings (PDL) necessary to generate enough material for preparing a batch of frozen stock will vary depending on the original tissue, the age of the donor, and the amount of material available from the initial dissection. The PDL at senescence depends on the age of the donor and other factors that may not be known. Generally the cell lines from newborn tissue will have the highest doubling potential. A wide range of ages for the donor cells has been employed using this procedure and age has not adversely affected our ability to establish cell lines.

A healthy, established culture of skin fibroblasts can be seen in Figure 1.
ATCC Antibiotic Mix and FACS Sorting

Flow cytometry is a powerful method of analyzing cell type populations and marker-expression distribution based on cell size, cell surface complexity, and immunophenotyping by using specific fluorescence-tagged antibodies or fluorescent proteins. In addition to analyzing cell populations, flow cytometry can also sort cell populations by fluorescence activated cell sorting, or FACS. This technique can sort specific rare cells (stem cells, tumor cells) from the larger population of cells based on their expression of a fluorescent-tagged marker. A FACSCalibur™ instrument equipped with dual laser source and CellQuest™ software (both from Becton, Dickinson and Co.) was used for the analysis of samples at ATCC.

At several points in the procedure cells are exposed to nonsterile conditions. Using ATCC’s Antibiotic Mix during this process minimizes microbial contamination and thus helps in establishing pure cultures from sorted cells. Here is the procedure followed at ATCC:

Before sorting, suspend the cells in appropriate medium containing 10× Antibiotic Mix and then pass through a 28-gauge needle five times to ensure a dispersion of single cells. Perform the sorting procedure using FACSCalibur and collect the sorted cells in medium containing 10× Antibiotic Mix.

After sorting, centrifuge the cells and resuspend them in medium containing 2× Antibiotic Mix. Culture the cells overnight. Change the medium daily on Day 2 though Day 4 by removing the medium, rinsing the adherent cells with sterile phosphate buffered saline, and cultivating in medium containing 2× Antibiotic Mix. On the fifth day, begin using 1× Antibiotic Mix and continue to change the medium every day for an additional seven days. At the end of 12 days add regular medium without antibiotics.

Anaz Toumadje, Ph.D., and Richard Farnsworth, Ph.D., ATCC

Cultivating ISAV

Results suggest that the ASK cell line may be superior to the SHK-1 cell line in terms of relative ease of use in the laboratory. ASK cells do not require conditioned medium, and although they appear to grow faster in a medium containing serum supplement, the cell line is not dependent on specialized serum for growth. On the other hand, the SHK-1 cell line requires careful attention to culture conditions. It was necessary to acclimatize the cells using a conditioned medium, and serum supplement was added to avoid poor growth upon transfer to standard medium. The SHK-1 cells also appear to be sensitive to cell density and must be subcultured at close to 100% confluency for best results. In contrast, the ASK cultures are more tolerant to subculture once the cells have passed 100% confluency, although like other cell lines, subculturing at very low cell densities can result in loss of a culture.

Most importantly, the presence of clear and distinct CPE as well as high viral titers in ASK cells provide the fish health specialist with an economical method to quantify viral levels. We believe that these characteristics of the ASK cell line also make it appropriate for studying the epidemiology and pathogenesis of ISAV.

Acknowledgment

I thank James R. Winton, of the U.S. Geological Survey in Seattle, WA, and Deborah Bouchard, of Micro Technologies, Inc., of Richmond, ME, for their work on this research.

References


Catalog No.   Unit   Price

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Tech Qs

Why do some IMAGE clones have more than one ATCC number?

IMAGE clones are sent to ATCC in large batches (hundreds of thousands at a time) along with the accompanying data. Each individual clone within every deposit must be given a new ATCC number to track its data within our system.

Many of the IMAGE clones have been deposited with ATCC more than once and therefore will have two or more ATCC catalog numbers. If you find multiple entries for the same IMAGE number while searching for clones on our Web site we recommend you read each description carefully and choose the clone that has the correct IMAGE Clone ID and the highest ATCC number, which would be the most recent deposit.

An important distinction occurs when an IMAGE clone becomes part of the Mammalian Gene Collection, which is a special set of IMAGE clones that have been determined to contain the complete coding sequence of a gene. ATCC is assigning catalog numbers beginning with MGC- to these full-length clones.

Remember that currently our search engine performs a basic text search of catalog descriptions. The digits that you enter into your query may happen to match an identifier for a different clone. Please read the full record of any search results to make sure the IMAGE number is correct.

Why are nonessential amino acids included in cell culture medium?

All media formulations contain the ten essential amino acids as well as cysteine, glutamine, and tyrosine. The inclusion of the other nonessential amino acids—alanine, asparagine, aspartic acid, glycine, glutamic acid, proline, and serine—in some media formulations reduces the metabolic burden on cells, allowing for an increase in cellular proliferation.

I can’t find the B95-8 cell line in the catalog. What happened to it?

Many investigators look for B95-8 cells (CRL-1612) because this monkey cell line sheds the Epstein-Barr virus that is used in transforming B lymphocytes. However, the cell line is derived from a primate species that is listed as endangered by the U.S. Fish and Wildlife Service. The distribution of such cells is strictly controlled and requires a permit. To date, no customer has successfully obtained a permit to receive these cells.

An alternative for researchers is VR-1492, the supernatant from B95-8 cells containing the EB virus. This preparation can be used to transform human B lymphocytes according to the procedure of Caputo et al (J. Tissue Culture Methods 13: 39-44, 1991). This item is found in our animal virus collection.

Staff Research Notes

The protozoan inhabitants of Chesapeake Bay were the subject of two recent studies by ATCC scientists. While at Tennessee Technological University, Chanson Boman of ATCC’s Bioproduction Program characterized isolates of the parasitic dinoflagellate *Amoebophrya*. By amplifying rRNA sequences, Boman and colleagues found enough differences to provide evidence of several species (J. Eukaryot. Microbiol. 49(6):469-474, 2002), though the species group together into a single clade.

Thomas Nerad and Jeffrey Cole of Protistology worked with O. Roger Anderson of the Lamont-Doherty Earth Observatory to describe a new species of amoeba isolated from a salt marsh at Assateague Island, Virginia (J. Eukaryot. Microbiol. 50(1):57-60, 2003). *Platyamoeba nucleoliteralis* is distinguished from other species by the presence of a nucleus with a single parietal nucleolus and a floating form with long tapering pseudopods. The study is part of a systematic survey of salt marsh amoebae in the eastern United States.

Web tips

Searching for bacterial serotypes

In general, a specific serotype of a species can be found by using the advanced field search for bacteria (www.atcc.org/SearchCatalogs/A_Bacteria.cfm). Enter the complete species name in the Organism field and the serotype in the Antigenic Properties field. However, there are other tips which can help improve your search, especially with E. coli.

E. coli serotyping describes the antigenic properties of three different features of the cell surface: the lipopolysaccharide or O antigens, the capsular or K antigens, and the flagellar or H antigens. Although the serotype/serovar is usually listed together as O:K:H, our online catalog cannot perform a query using colons. Instead use the wildcard or * to separate the three antigens. For instance, strains listed as having the O15 and K14 antigens can be found by entering o15*k14* in the Antigenic Properties field. Always be sure to check your results when using a wild card. For instance, querying with *k1* will retrieve K14 as well as K1.

Because all of our serotyped E. coli strains reference an O serovar or the O group, you can generate a complete list of serotyped E. coli strains available from ATCC simply by entering coli in the Organism field and o* in the Antigenic Properties field.

A closer look

Photomicrographs of selected cell lines have been added to our online catalog. When you view the complete description of a cell line and see a microscope icon, click on it to view the photomicrograph. We’ve included images from low density and high density cultures. For examples see CCL-92 (3T3 Swiss albino mouse fibroblasts) or CRL-1476 (A-10 rat myoblasts).

SARS-related materials

We now have an online list of coronaviruses from various species that are available from ATCC. You can access this list from our home page. We also have the Vero E6 cell line, in which the SARS agent has been cultured (Ksiazek TG et al. New. Eng. J. Med. 348(20): 1947-1958, 2003). The ATCC catalog no. is CRL-1586.

Cell Success

ATCC can help you succeed with your cell cultures. Our media and sera are put to the test every day because we use them in our own labs, and these products measure up to our high standards. We also have buffers, reagents, media supplements, stains, and test kits.

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ATCC has a rapidly expanding selection of DNA clones, including a complete set of IMAGE clones, plant clones, microbial clones, and full-length clones from the Mammalian Gene Collection. We're constantly adding new sets to our collection. You can order single clones, custom arrays, or replicated plate sets. Our full-service capabilities will get you what you need, when you need it, at a reasonable price. And growth of each clone is guaranteed.

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Streamline Gene Expression with the AdEasy® System

The AdEasy system, developed by Dr. Bert Vogelstein and associates at the Kimmel Cancer Center at Johns Hopkins, employs the recombination processes in E. coli to produce recombinant adenovirus quickly and efficiently (Proc. Natl. Acad. Sci. USA 95: 2509-2514, 1998). The gene of interest is cloned into a shuttle vector and then inserted into the adenovirus genome backbone in the E. coli cells. The recombinant plasmid is used to transfect a packaging cell line such as 293 cells, which are also available from ATCC (CRL-1573).

Using the AdEasy system for gene transfer has several advantages over other methods:

- Adenoviruses infect a broad range of cell lines, even those that are difficult to transfect with other methods.
- Active cell division is not required for transfection.
- High-level gene expression can be achieved.
- The multiple plaque assays traditionally involved in identifying and selecting recombinant adenoviruses are avoided, saving weeks of work.

The AdEasy basic kit contains the E. coli cells and plasmids necessary to produce recombinant virus. The supplement kit contains components to test the efficiency of the AdEasy system for making recombinant adenoviruses.

Details of this system and its use can be found on the Vogelstein/Kinzler Web site at www.coloncancer.org/adeasy.htm.

### Catalog No. | Unit | Price
--- | --- | ---
AdEasy Basic Kit | JHU-23 | $195
AdEasy Supplement Kit | JHU-24 | $195
293 Cells | CRL-1573 | $175

AdEasy kits are covered under U.S. Patent No. 5,922,576.

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The CMV promoter used in some of the constructs and the polyadenylation site were both from pEGFP-C1 and obtained from Clontech (Palo Alto, CA). The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242. Use of Clontech's Living Colors™ products containing DNA sequences coding for mutant Aequorea victoria green fluorescent protein (GFP) variants or proteins thereof requires a license from Aurora Biosciences Corporation under U.S. Patent Nos. 5,625,048, 5,777,049, 6,054,321, and 5,804,387 and other pending U.S. and foreign patent applications. In addition, certain Clontech products are made under U.S. Patent No. 5,804,387 licensed from Stanford University.

AdEasy is a registered trademark of the John Hopkins University.
Exhibit Schedule
Comments, suggestions, kudos? Come visit the ATCC booth at the following meetings and let us hear them. We enjoy talking to you about how we can help your research.

American Society for Microbiology
Washington, D.C.
May 18-23

Univ. of California Biotech Show
San Francisco
June 12

International Congress of Biochemistry and Molecular Biology
Toronto
July 20-24

ICAAC
Chicago
Sept. 14-17

Harvard Biomedical Research
Boston, MA
Sept. 17-18

NIH Research Fair
Bethesda, MD
Oct. 16-17

The University of Miami and ATCC are pleased to announce a conference entitled “Aquatic Animal Models of Human Disease.” This meeting is sponsored by the National Center for Research Resources of NIH and will be held at the ATCC facility in Manassas, VA, from September 29 through October 2, 2003.

Topics for scientific sessions will include the use of aquatic animal models for various studies:

- Comparative genomics
- Gene expression
- Transgenesis
- Carcinogenesis
- Toxicology
- Infectious disease
- Neurological disorders
- Aging

Workshops are planned on transgenesis and gene expression, as well as on current strategies for resource development and funding mechanisms. Submissions of abstracts for oral and poster presentations are invited with a deadline of July 1, 2003. Registration will be limited. For information, please see the conference Web site at http://pasteur.atcc.org/aquatic_conference/.