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PRESERVATION AND DISTRIBUTION OF FUNGAL CULTURES

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Maintaining and preserving fungal cultures are essential elements of systematics and biodiversity studies. Because fungi are such a diverse group, several methods of cultivation and preservation are required to ensure the viability and morphological, physiological, and genetic integrity of the cultures over time. The cost and convenience of each method, however, also must be considered. We encourage the reader to investigate the excellent papers on fungal preservation by Fennell (1960), Smith and Onions (1994), Smith (1991), and Simione and Brown (1991).

The primary methods of culture preservation are continuous growth, drying, and freezing. Continuous growth methods, in which cultures are grown on agar, typically are used for short-term storage. Such cultures are stored at temperatures of from 5°–20°C, or they may be frozen to increase the interval between subcultures. The methods are simple and inexpensive because specialized equipment is not required. Drying is the most useful method of preservation for cultures that produce spores or other resting structures. Silica gel, glass beads, and soil are substrata commonly used in drying. Fungi have been stored successfully on silica gel for up to 11 years (Smith and Onions 1983). Drying methods are technically simple and also do not require expensive equipment. Freezing methods, including cryopreservation, are versatile and widely applicable. Most fungi can be preserved, with or without cryoprotectants, in liquid nitrogen or in standard home freezers. With freeze-drying, or lyophilization, the fungal cultures are frozen and subsequently dried under vacuum. The method is highly successful with cultures that produce mitospores. Freeze-drying and freezing below –135°C are excellent methods for permanent preservation, and we highly recommend them. However, both methods require specialized and expensive equipment, as described in the next section (see "Liquid Nitrogen" and "Lyophilization" under "Long-term Preservation," later in this chapter).

The choice of preservation method depends on the species of concern, the resources available, and the goal

of the project. Some low-cost methods of preservation, such as storage in distilled water and the silica gel method, are good, but none is considered permanent. The maximum duration of storage varies with each method and with the species being preserved, but it generally is 10 years or less. Whenever possible, fungal strains should be preserved with one of the permanent methods (lyophilization, cryopreservation) described later in this chapter (see "Long-term Preservation"). Permanent preservation is essential for strains with critically important characteristics and for type specimens. Cultures that are permanently preserved in metabolically inactive states now can serve as type specimens, according to Article 8.4 of the International Code of Botanical Nomenclature (Greuter et al. 2000).

MAINTENANCE AND PRESERVATION OF CULTURES

SHORT-TERM PRESERVATION

Short-term preservation involves maintenance of cultures for up to 1 year. Most fungal cultures can be maintained for that period by serial transfer. The method is simple, inexpensive, and widely used. Although time consuming and labor intensive, periodic transfer is a good option for small collections with cultures in constant use for short periods (less than 1 year). The method also has several disadvantages, however. Cultures must be checked frequently for contamination by mites or other microorganisms and for drying. In addition, the morphology and physiology of a cultured fungus may change over time. In particular, the ability to sporulate or to infect a host may be lost after repeated transfers. Because of those disadvantages, the technique is generally inappropriate for long-term (more than 1 year) preservation of cultures.

Inoculum is transferred from an actively growing fungus culture to test tubes (screw cap or plugged with cotton or foam) or Petri dishes (wrapped with Parafilm to reduce drying) containing an agar medium of choice. Alternating nutrient-rich with nutrient-poor media at each transfer helps to maintain healthy cultures. Some fungi, such as endophytic and entomopathogenic species, have specific media requirements (Bacon 1990; Singleton et al. 1992; Humber 1994). After a culture is established, it is kept at room temperature or at 4°C. Cultures must be checked periodically for contamination and desiccation. Fungi such as oomycetes and some basidiomycetes (e.g., *Boletus, Coprinus, Cortinarius,* and *Mycena)* should be transferred monthly if kept at 16°C

(von Arx and Schipper 1978). Most filamentous fungi can survive at least 1–2 years at 4°C. Vigorous, sporulating cultures also can be sealed tightly and stored in a freezer at −20°C (Carmichael 1956, 1962) or stored at −70°C (Pasarell and McGinnis 1992) to enhance survival and increase the interval between required transfers (see "Freezing," later in this chapter).

LONG-TERM PRESERVATION

Sclerotization

Some fungi develop sclerotia or other long-term survival propagules in culture as well as in nature; preserving such structures, usually at 3°–5°C, is a good way to preserve fungal strains. Sclerotia and spherules of various myxomycetes have been germinated successfully after 1–3 years of storage. Many soil fungi, such as *Magnaporthe, Phymatotrichum,* and *Cylindrocladium* species, produce sclerotia or microsclerotia that remain viable for 2–5 years (Singleton et al. 1992).

Instructions for inducing formation of spherules, sclerotia, and microsclerotia are available in Daniel and Baldwin (1964) and Singleton and colleagues (1992). Sometimes rice straw or toothpicks are used as substratum to promote sclerotia production in culture. Jump (1954) described a simple method for inducing sclerotium formation in *Physarum* species. A piece of sterile cellophane cut to the dimensions of a Petri dish is placed over a dish containing 1% water agar (Appendix 11). An actively growing plasmodium is then transferred to the cellophane and allowed to grow overnight. The cellophane is removed from the agar; placed in a sterile, dry Petri dish; covered; and allowed to dry for 24 hours. The Petri dish lid is then removed to allow the sclerotia to air-dry until brittle. The cellophane is cut into small pieces, each of which is stored in its own screw-cap vial. Alternatively, the sclerotia are removed from the cellophane and stored in a vial.

Oil Overlay

A low-cost and low-maintenance method for preserving cultures growing on agar slants is oil overlay. Cultures can be kept for several years or, in exceptional cases, up to 32 years at room temperature or 15°–20°C. This method is appropriate for mycelial or nonsporulating cultures that are not amenable to freezing or freeze-drying. As an added benefit, oil also reduces mite infestations. Although many basidiomycetes can be maintained this way, the growth rates of the cultures slow as storage times increase (Johnson and Martin 1992; Burdsall and Dorworth 1994). The major disadvantage

of the oil overlay technique is that the fungi continue to grow, and thus, selection for mutants that can grow under adverse conditions may occur.

High-quality mineral oil or liquid paraffin is sterilized by autoclaving at 15-lb (6.8-kg) pressure for 2 hours. Entrapped moisture is removed by heating the liquid in a drying oven at 170°C for 1−2 hours (optional). Fungal cultures grown on agar slants are covered with about 10 mm of oil or paraffin. The entire agar surface and fungal culture should be submerged completely in the oil. The tubes are kept in an upright position at room temperature (15°-20°C; 12°C for *Pythium* species and *Phytophthora* species; G. Adams, personal communication). The oil level in the tubes or vials must be checked periodically, and more oil should be added, if necessary. To retrieve a culture from mineral oil, a small amount of the fungal colony is removed and placed on appropriate media after as much oil as possible has been drained. Lifting the Petri dish on one side to form a slight angle often helps the oil drain. It may be necessary to subculture the colony several times to get a vigorous oil-free culture.

Immersion in Distilled Water

Another inexpensive and low-maintenance method for storing fungal cultures is to immerse them in distilled water. Apparently, the water suppresses morphological changes in most fungi. The method has been used successfully to preserve oomycetes (Clark and Dick 1974; Smith and Onions 1983), basidiomycetes (Ellis 1979; Richter and Bruhn 1989; Burdsall and Dorworth 1994; Croan et al. 1999), ectomycorrhizal fungi (Marx and Daniel 1976), ascomycetes (Johnson and Martin 1992), hyphomycetes (Ellis 1979), plant pathogenic fungi (Boesewinkel 1976), aerobic actinomycetes (van Gelderen de Komaid 1988), and human pathogens and yeasts (McGinnis et al. 1974). Most basidiomycetes survived for at least 2 years at 5°C (Marx and Daniel 1976; Richter and Bruhn 1989); viability decreased after 5−10 years of storage (Burdsall and Dorworth 1994). Although Ellis (1979) reported that most of the basidiomycetes he tested survived for 20 months when stored at 25°C, Johnson and Martin (1992) recovered only 26% of the basidiomycete strains stored at 20°C. Ascomycetes, however, including their mitosporic forms, survived up to 10 years when stored at 20°C (Johnson and Martin 1992).

The procedures used for covering cultures on agar slants with oil also can be used when covering them with sterile distilled water. Alternatively, sterilized straws or Pasteur pipettes (large-diameter end) are used to cut disks from the growing colony edge. The disks are transferred to sterile cotton-plugged or screw-cap test tubes filled with several milliliters of water. To save space, small (1.8 ml), sterile, screw-cap cryovials are filled with several discs and topped with sterile distilled water. Test tubes (loosely capped and wrapped with Parafilm) are stored at room temperature; tightly capped tubes and vials are stored at 4°C. Disks are removed aseptically and transferred to fresh agar medium to retrieve cultures.

An alternative method for sporulating fungi (McGinnis et al. 1974) involves inoculating agar slants of preferred media with fungal cultures and then incubating them at 25°C for several weeks to induce sporulation. Sterile distilled water (6−7 ml) is added aseptically to the culture, and the surface of the culture is scraped gently with a pipette to produce a spore and mycelial slurry. This slurry is removed with the same pipette and placed in a sterile, 2-dram glass vial (or cryovial). The cap is tightened, and the vials are stored at 25°C. To retrieve a culture, 200−300 µl of the suspension is removed from the vial and placed on fresh medium.

Organic Substrata

Over the years, researchers have developed practical, effective, and ingenious methods of preserving fungi on various organic substrata such as wood chips, cereal grains, straw, filter paper, and insect and plant tissues. Many of the techniques were developed for pathogenic or other specific fungi and have not been rigorously tested with a range of fungi.

Wood. Wood-inhabiting fungi can be successfully stored on wood chips or toothpicks as long as the colony is growing vigorously (Nelson and Fay 1985; Delatour 1991; Singleton et al. 1992). Some wood-inhabiting basidiomycetes and ascomycetes can be stored on wood chips for up to 10 years. If the fungi do not vigorously colonize the wood chips, however, the method fails.

Small pieces of untreated beech wood (12-mm diameter × 6-mm thick) are added to 2% malt-extract broth (about 60 pieces of wood per 100 ml broth; Appendix 11) and sterilized for 20 minutes at 121°C (Delatour 1991). The mixture is sterilized again 24 hours later. About 15 wood chips are drained and placed on a colony of the fungus that is growing on malt extract agar (Appendix 11) in Petri dishes. The Petri dishes are sealed with Parafilm, and the fungus is allowed to colonize the wood chips. After 10−15 days, the inoculated wood chips are transferred to sterile test tubes (18 × 180 mm) containing 6−7 ml of 2%-malt agar. The tubes are plugged with cotton and incubated for about 1 week, after which time the cotton is replaced with sterile Parafilm and aluminum foil. The tubes are stored at 4°C. To retrieve a culture, a piece of wood chip is removed and placed on fresh agar medium. The tube is resealed and returned to the refrigerator.

Cereal Grains. Fungi such as *Sclerotinia, Magnaporthe, Leptosphaeria,* and *Rhizoctonia* species have been stored for up to 10 years on seeds of oats, barley, wheat, rye, millet, and sorghum (Singleton et al. 1992). To preserve isolates of *Rhizoctonia* species, barley, oat, or wheat grains (Sneh et al. 1991) are soaked overnight in water containing chloramphenicol (250 g/ml). The water is removed, and the grain is autoclaved for 1 hour at 12°C over 2 consecutive days. Screw-cap vials are filled with the grain and autoclaved. The vials are inoculated with transfers from the margins of actively growing cultures and incubated at 23°–27°C for 7–10 days. The cultures then are dried thoroughly in a desiccation chamber. The caps are tightened and wrapped with Parafilm, and the vials are stored at −25°C.

Agar Strips. Nuzum (1989) described a method of vacuum-drying fungal cultures on agar strips. *Pythium, Rhizoctonia,* and some basidiomycete species survived 18 months with this method, whereas ascomycetes and their mitosporic forms survived from 3–5 years. Fungal cultures are grown on appropriate media in Petri dishes. Strips 1-cm long are cut from the growing edge of the colony and placed in sterile Petri dishes. After 1 week at room temperature, the pieces of dried agar are transferred to sterile ampoules, vacuum-dried, and sealed. To revive cultures, agar strips are placed on fresh medium of choice.

Insect or Plant Tissue. The host tissue can be used as a substrate on which to maintain and store cultures of some pathogenic fungi. For example, roots of plants infected with *Pyrenochaeta* and *Thielaviopsis* can be dried and then frozen (Singleton et al. 1992). *Neozygites fresenii* cannot be cultured in vitro, but Steinkraus et al. (1993) developed a method of preserving viable conidia on frozen, infected aphid mummies.

Soil or Sand

Some fungi can be preserved easily and successfully for many years in dry, sterile soil or sand. This low-maintenance and cost-effective method is appropriate for fungi such as *Rhizoctonia* (Sneh et al. 1991), *Septoria* (Shearer et al. 1974), and *Pseudocercosporella* (Reinecke and Fokkema 1979). Dormancy caused by dryness can take time to develop, however, and morphological changes in some fungi have been recorded.

Glass bottles (60 ml) are filled to two-thirds capacity with sand or loam soil (water content 20%) and then sterilized by autoclaving for 20 minutes at 120°C. The bottles are allowed to cool and then sterilized again.

Sterile, distilled water is added to a culture, and the colony surface is scraped gently to produce 5 ml of spore or mycelial suspension. One milliliter of the suspension is added to each bottle of soil or sand. After 2–14 days of growth at room temperature, the bottles are capped loosely and stored in the refrigerator at 4°C. To retrieve the fungus, a few grains of soil are sprinkled onto fresh agar medium. Test tubes or vials can be used in place of glass bottles to save space.

Silica Gel

The silica gel method can be used to preserve sporulating fungi if facilities for freeze-drying or for storage in liquid nitrogen are not available. It originally was developed by Perkins (1962) for *Neurospora* species. He found that sporulating fungi protected by skim milk and stored on silica gel remain viable for 4–5 years. Spores and microcysts of dictyostelids can be preserved for up to 11 years on silica gel (Raper 1984). In general, viability after storage on silica gel depends on the strain of fungus and the medium on which it was grown before storage. When cultures are stored in soil, the initial growth period before storage may permit variant vegetative strains to develop and overgrow the wild type, or saprotrophic segregants to overgrow pathogenic ones. The advantage of silica gel is that it prevents all fungal growth and metabolism. Some researchers use glass beads instead of silica gel.

Revival of cultures from silica gel is easy–a few silica gel crystals are scattered on an agar plate. The same storage container can be used for successive sampling. The Fungal Genetic Stock Center (Appendix III) has used this technique successfully since 1962 for preserving genetic stocks of *Aspergillus nidulans* and *Neurospora crassa*. Fungi such as *Pythium* and *Phytophthora* species, however, do not survive this process.

Protocol A. Screw-cap tubes are filled partially with 6- to 22-mesh silica without indicator dye, which has been sterilized with dry heat for 90 minutes at 180°C and stored in tightly sealed containers (Perkins 1962). Spores are suspended in a 10% (v:v) solution of dry powdered skimmed milk in distilled water, previously cooled to 4°C. The silica gel also is chilled to about 4°C and placed in an ice-water bath. The spore suspension is added to the silica gel to wet about three-fourths of the gel (0.5 ml/4 g) and left in the bath for 30 minutes. Tubes are stored with the caps loose at room temperature for 1–2 weeks. Viability is checked by shaking a few crystals onto a suitable medium. If the cultures are viable, caps are tightened, and the tubes are stored in a tightly sealed container at 4°C.

Protocol B. A slight modification of Protocol A also accommodates nonsporulating strains of fungi (Perkins 1977). Cotton-plugged test tubes (13-mm diameter × 100 mm high) partially filled (to 65 mm) with 12- to 20-mesh silica gel without indicator dye are heat sterilized at 180°C for 2 hours and stored at room temperature in tightly sealed containers until needed. Fungal cultures are grown for a week on suitable agar slants. For sporulating strains, about 0.5-ml sterile water is pipetted gently into the tube of a sporulating culture. Spores are suspended using a vortex-type mixer. For nonsporulating strains, aerial hyphae and mycelia are scraped from the agar surface with a sterile blade and transferred to a test tube (10-mm diameter × 75 mm tall) containing 0.5 ml water. The mixture is ground with a pipette or glass rod against the tube wall to obtain a creamy homogenate. About 0.5 ml sterile skim milk is added and stirred gently. The cotton plug covering the silica is removed, and the suspension is pipetted over it, drop by drop. The tube is agitated briefly with a mechanical mixer to distribute inoculum over as many particles of silica gel as possible and then is placed in an ice-water bath for 15 minutes. After 1 day at room temperature, the particles appear dry, and the tube is sealed against moisture with Parafilm. Tubes are stored at 5°C or −20°C in a moisture-proof box. Storage at low temperatures can increase the survival period twofold to threefold over storage at room temperature.

Freezing

Most fungal cultures frozen at −20° to −80°C in mechanical freezers remain viable. Freezing with liquid nitrogen is discussed in the following section. Cultures grown on agar slants in bottles or test tubes with screw caps can be placed directly in the freezer (Carmichael 1956). Overall failure rate for mitosporic ascomycetes, Zygomycetes, and yeasts after 5 years in storage at −20°C was 5.1% (Carmichael 1962). The failure rate of medically important fungi, aerobic actinomycetes, and algae stored from 6 months to 13 years at −70°C was 2.3% (Pasarell and McGinnis 1992). Ito (1991) and Ito and Yokoyama (1983) aseptically removed six 6-mm disks from vigorously growing cultures of nonsporulating basidiomycetes and ascomycetes and placed them in sterile cryotubes containing 10% glycerol in water. The cultures were preserved successfully for up to 5 years by mechanical freezing at −80°C. Fungi grown on various organic substrata, such as cereal grains, agar strips, plant parts, and filter paper, and then dried can be frozen (see "Organic Substrata," earlier). In general, vigorously growing and sporulating cultures survive the freezing process better than less vigorous strains. We do not recommend repeated freezing and thawing, which will significantly reduce viability of the cultures.

Liquid Nitrogen

Storage in liquid nitrogen is an effective way to preserve many, if not most, organisms, including those that cannot be lyophilized. It costs somewhat more than lyophilization, however, because liquid nitrogen must be replenished every few days. Liquid-nitrogen storage is recommended for the preservation of dictyostelids (Raper 1984), amoebae (Davis 1956; Evans 1982), Zygomycetes including Entomophthorales (Humber 1994), oomycetes (Nishii and Nakagiri 1991), phytopathogenic fungi (Dahmen et al. 1983), and yeasts (Kirsop 1991). Ascomycetes that sporulate poorly in culture, and higher basidiomycetes that generally grow only as mycelia in culture also can be stored in liquid nitrogen.

Because the rates of mutation in cultured fungi are likely to correspond to those of cell division and metabolic activity, storage methods that stop cell division completely and totally arrest metabolism, while still retaining viability, are best. Freezing at or below −139°C, the temperature at which ice crystals do not grow and rates of other biophysical processes are too slow to affect cell survival, accomplishes this. All fungi can be cryopreserved, but this method generally is reserved for fungi that do not sporulate in culture, fungi that have large or delicate spores that will not survive freeze-drying, genetic stocks, and dangerous human pathogens. In addition, many culture collections, such as the American Type Culture Collection (ATCC; Appendix III), store their seed stock in liquid nitrogen so that when distribution stock is depleted, the material used for replenishment will be as genetically close to the original deposit as possible.

Because living cells can be damaged severely by freezing and thawing, chemical cryoprotectants are used in most protocols, Cryoprotectants are of two types: penetrating agents such as glycerol and dimethyl sulfoxide (DMSO), which readily pass through the cell membrane and protect intracellularly and extracellularly, and nonpenetrating agents such as sucrose, lactose, glucose, mannitol, sorbitol, dextran, polyvinyl-pyrrolidone, and hydroxyethyl starch, which exert their protective effect external to the cell membrane. Glycerol and DMSO have proved to be most effective for fungi, although polyethylene glycol, another penetrating agent, can be used also (Ohmasa et al. 1992).

The major advantages of liquid nitrogen storage include prevention of increased genetic variability of distributed culture stocks; timesaving, reduced labor

requirements compared to handling of living stocks; elimination of the need for repeated pathogenicity tests, prevention of culture loss from contamination; and increased assurance of long-term availability of cultures. The major disadvantages of this technique are the relatively high cost of the apparatus and the liquid nitrogen that must be replaced every 2 days, the space requirement for refrigeration units, and the need for constant surveillance. Culture collections, such as the ATCC, usually maintain a backup off-site liquid-nitrogen storage facility. One disadvantage affecting distribution of strains preserved in liquid nitrogen is that they first must be grown on agar or in liquid medium to avoid the expense of shipping frozen materials.

Storage in the vapor phase of the liquid-nitrogen freezer is an attractive alternative to immersion in the liquid nitrogen. Tubes that are immersed must be sealed carefully to prevent entry of liquid nitrogen. A tube filled with liquid nitrogen that is quick-thawed at 37°C is likely to explode from the pressure created by the expanding nitrogen. Another concern is that cultures in leaky vials may be contaminated with bacteria or spores that may be present in the liquid-nitrogen freezer. Various alternative techniques for liquid-nitrogen storage, such as using plastic straws instead of vials or tubes, have been reported (Stalpers et al. 1987; Kirsop 1991). Other protocols such as the one used by Gulya et al. (1993) to preserve zoosporangia of downy mildew for up to 4 years in liquid nitrogen require neither cryoprotectants nor controlled freezing regimens.

Procedures used to harvest materials for preservation in liquid nitrogen differ depending on whether the fungus sporulates, has mycelia that penetrate below the surface of the agar, or grows only in liquid culture. Samples of human pathogens are scraped from the agar surface, or agar plugs are cut from the cultures. Such samples are never macerated in a mechanical blender because of the hazard of aerosol dispersion of the pathogen.

Protocol A. For fungal cultures that do not sporulate or that produce mycelia that grow deep into the agar, sterilized 2-ml screw-cap polypropylene vials are filled with 0.5–1.0 ml sterile 10% glycerol. Plugs 4mm in diameter are cut from vigorously growing cultures using a sterilized plastic straw. Several plugs are placed in the vial, the cap is tightened, and the tube is placed directly into the vapor phase (temperature is about −170°C) of a liquid-nitrogen tank. The accession number should be written on each cryovial with a cryoresistant-ink lab marker, printed onto paper, and then taped to the vial, or it should be printed onto a special cryoresistant adhesive label, which is readily available from biotechnology supply companies. The location of storage in the freezer must be indexed for rapid retrieval. Frozen preparations are retrieved by removing the vials from the freezer and rapidly thawing them in a 37°C water bath. The thawed agar plugs are placed on appropriate agar plates. Viability of the cultures should be checked from 2–7 days after storage.

Protocol B. To make suspensions of spores or mycelial fragments from cultures growing on the surface of agar slants or plates, the colony surface is flooded with 10% glycerol or 5% DMSO and gently scraped with a pipette. Preparation continues according to the "General Protocol,)) described later in this chapter.

Protocol C. The mycelium of a fungus that grows only in liquid culture must be macerated before it can be pipetted into vials. The broth culture is fragmented for a few seconds in a sterile mini-blender and mixed with equal parts of 20% glycerol or 10% DMSO to give a final concentration of 10% glycerol or 5% DMSO, respectively. The mixture is then treated as described in the following section.

General Protocol. The fungal mycelial and spore suspension is pipetted in aliquots of 0.5 ml into sterile 2-ml screw-cap polypropylene vials. Filled vials are placed into prelabeled cans in racks that then are put into the freezing chamber of a programmable freezer and allowed to equilibrate at 4°C for about 10 minutes. They are then cooled from 4° to 40°C at a rate of 1C° per minute and from −40° to −90°C at 10C° per minute. After reaching −90°C, vials are transferred immediately to liquid-nitrogen vapor at −150° to −180°C. Polypropylene vials are not immersed in liquid nitrogen.

Cultures are thawed rapidly by placing vials in a warm-water (37°–55°C) bath until the last trace of ice dissipates. Cultures in glass ampoules and straws thaw in less than a minute; those in polypropylene containers take longer. Culture samples are then transferred aseptically to appropriate growth media.

Lyophilization

Lyophilization, or freeze-drying, a low-cost form of permanent preservation, is not appropriate for all fungi. In fact, the technique is used primarily with species that form numerous, relatively small propagules. However, Croan (2000) demonstrated that mycelial isolates of basidiomycetes can be lyophilized effectively in the presence of trehalose. Lyophilized spores of dictyostelids, with associated bacteria, were maintained successfully for up to 30 years (Raper 1984). This procedure is the preservation method of choice for many

spore- forming fungi that produce large numbers of spores 10-µm or less in diameter. Larger spores tend to collapse during the lyophilization process, and the structural damage caused is not reversible by hydration. A significant number of the spores of appropriate size also are physically damaged and killed during the freezing process by the formation of ice crystals. Thus, each ampoule initially must contain many viable spores. Rapid freezing and the addition of a menstruum that dissolves ice crystals minimize growth of ice crystals. The two most common menstrua are nonfat dry milk powder (sterile 5% or 10% solution) and filter-sterilized bovine serum, although other proteinaceous materials also can be used.

Equipment and Supplies. Material required for lyophilization include the following: high-quality mechanical vacuum pump (e.g., Edwards two-stage pump); vacuum gauge; vacuum manifold; cold trap; hoses to connect pump, trap, and manifold; insulated bath; support stand for manifold; oxygen-gas torch; oxygen supply; 10-cm lengths of 6-mm soft glass tubing with one end heat sealed, or lyophilization ampoules; cotton for plugging tubes; Pasteur pipettes; mechanical or electrical pipetting aid; sterile menstruum; and permanent ink suitable for writing on glass.

General Protocol. An agar slant with medium that supports good growth and sporulation is inoculated with the organism, which is allowed to grow until it reaches the resting phase. Lyophilized preparations from cultures much younger or older than resting phase often exhibit very low postlyophilization viability. Five or more lyophilization tubes are sterilized and labeled for immediate use. About 1.5–2.0-ml sterile menstruum is added to an agar slant; spores are suspended in the menstruum by gently scraping the agar surface with a Pasteur pipette. If sporulation has been light, the menstruum-spore mixture is transferred to a second agar slant, whose spores are added to the mixture. Approximately 200 µl 4 the spore suspension is added to each of several iyophilization tubes. Tubes are: plugged loosely with cotton, the open end of the glass is lubricated with castor oil, and the tubes are placed on the vacuum manifold. The manifold is lowered until the lyophilization tubes are immersed in a dry ice and ethylene glycol bath that is maintained between 40°C and −50°C while the contents of each tube freezes (about 5 minutes). A vacuum is applied to the system for about 30 minutes while the bath warms to about 0°C. The manifold then is raised to remove the tubes from the solvent bath. Drying of the lyophilization preparations continues at room temperature until the pressure in the system

reaches about 30 milliTorrs. Evaporative cooling keeps the samples frozen during the drying process. The tubes then are sealed under vacuum using a gas-oxygen torch. Finished lyophilization ampoules are stored in numbered plastic boxes or sealed plastic bags in a 4°C refrigerator. The purity and viability of the preparation in one lyophilization vial should be checked 1–2 weeks after preservation. Viability can be roughly categorized as poor (10–50 colonies/ampoule), moderate (50–100 colonies/ampoule), or good (1 00–1 000 colonies/ampoule).

Bunse and Steigleder (1991) described an alternative method for the lyophilization of cultures grown on agar slants in glass ampoules that preserves the fungus as well as the macromorphology of the cultures. Good results were obtained with *Penicillium, Aspergillus, Cladosporium, Alternaria, Mucor, Candida,* and *Rhodotorula* species but not with any dermatophyte species.

RECORDS AND RECORD KEEPING

For a small research collection (i.e., fewer than 500 cultures) the least costly, yet effective, method of keeping records is through the use of index card files. Records for the Agricultural Research Service Culture Collection (NRRL; Peoria, Illinois) filamentous fungus collection records originally were kept on three sets of 3" x 5" index cards. One set of cards was arranged alphabetically by species name; the other two sets were arranged by accession number. In this way one could readily look up species using the alphabetical file and quickly identify cultures labeled with their accession numbers using the numerical file. The cards were the only database kept for many years and included information on more than 10,000 fungal strains. The third set of cards was used as an inventory of vials of lyophilized specimens and specimens stored in liquid nitrogen available for distribution.

Given the common availability of personal computers and the low cost of database programs, it is now easy to enter the records for even small culture collections into electronic databases. The primary considerations when choosing a computer and a database program are universality, or intercompatibility. Most of the common commercial (and some shareware) database programs can export data in a standard format (e.g., comma-delimited fields) or a system data format. If a database can export data in one of those formats, the data can be transported to practically any database platform at will. A second consideration is ease of searching. Some database systems have a user-friendly visual interface that

leads the user through all the steps necessary to search fields in the database. Probably the largest single expense in creating the database (after procuring the computer) is data entry. Making a card index or an electronic file, however, would require similar investments of time. Once the data are in electronic form, they can be transferred to other programs and media. It is essential that electronic data files be backed up and stored in different locations to minimize the possibility of their catastrophic loss (e.g., to weather-related factors, fires, mechanical failures of the computer).

Each strain in a culture collection is recorded in the database, which should include separate fields for (1) strain number; (2) genus; (3) species; (4) variety; (5) taxonomic authority; (6) required growth conditions; (7) date of isolation; (8) locale of isolation; (9) isolation substrate or host; (10) person who isolated the culture; (1 1) provenance of the strain (e.g., isolated by Thom 1917, sent to CBS in 1934 as CBS 225.34, CBS 225.34 sent to IMI as IMI 101557 in 1956, IMI 101557 sent to NRRL as NRRL 28994 in 1995); (12) isolate numbers in other collections that reference the same strain (i.e., CBS 225.34 = IMI 101557 = NRRL 28994 = ATCC 1076); (13) person who identified the strain; (14) person, if anyone, who has reidentified the strain; (1 5) subjective synonyms; and (16) comments that do not fit well into other fields. Other useful information that might be maintained includes published references to the isolate, metabolites produced, transformations, and so forth. The manner of the preservation of each strain also is recorded, usually in a separate database, along with particulars of the long-term preservation of the strain, number of replicate preservation vials and dates they were prepared, and test viability of the organism following preservation. In addition, if germ plasm is distributed on a regular basis, it is handy to keep a listing of the individuals to whom particular preservation vials have been sent. Such individuals can be contacted to determine (via user feedback) how well a preservation technique is working, whether a strain is pure, and whether a strain conforms to the original description and produces the expected products.

Data-entry audit should be an integral part of any database. In an optimal situation, data are entered into the system by a laboratory technician or a data-entry technician, the entered data are checked for correct spelling using an electronic spell checker, and the checked data are printed out for review by someone who did not participate in the original data entry. In many database systems, data may be entered and then held in a temporary file until audited. Once checked and approved, the data can be added to the database by a batch append command.

DISTRIBUTION AND EXCHANGE

GENERAL PRACTICES

Service culture collections will supply any culture if one is a bona fide scientific investigator associated with a properly equipped laboratory. Some cultures in a service collection, such as plant or animal pathogens, require permits from the U.S. Department of Agriculture (USDA) or the U.S. Public Health Service (USPHS), an export license for shipment abroad, or the acceptance of responsibility statements on certain pathogens. The use of most of the cultures in service collections, including biological materials cited in expired or invalid patents, or in abandoned patent applications is not restricted. Use of cultures cited in valid patents, however, is restricted. Such materials are distributed to the scientific community for research purposes only. In contrast, some patent applicants do not restrict access to their cultures while a patent is pending, but limit use of such cultures to research.

Also available from service collections is biological material that is patented per se (i.e., material that is itself the subject of a patent). Use of such patented material during the life of the patent is limited to research purposes. Other strains to which special conditions apply include safe-deposit strains held on a confidential basis for the depositor and strains isolated by a staff member or currently forming part of a staff member's research. Those strains are not catalogued or generally made available to the public, although they may be supplied under special terms on a case-by-case basis.

PERMITS AND REGULATIONS

Because of the pathogenic or hazardous nature of certain microorganisms, containment and security for domestic and international shipments receive particular attention. Such shipments may require permits or licenses depending on the type of pathogen (human, animal, or plant) and its destination. Permits for shipment serve the following purposes: (1) to ensure that persons receiving the cultures are qualified to use them and that their laboratories are properly equipped to do so; (2) to inform government agencies about the volume of material being shipped and its destination; and (3) to provide information about the shipment in the event of an accident during shipment. For more information on international import and export restrictions, see Rohde and colleagues (1995) and Rohde and Claus (1999).

Regulations are well defined by several U.S. government agencies, including the USPHS, the USDA, and

the U.S. Department of Transportation (DOT). International shipments are governed by the rules and regulations of the U.S. Department of Homeland Security (Bureau of Customs and Border Protection) and the U.S. Department of Commerce (DOC). The USPHS, in the Code of Federal Regulations (42 CFR Part 72), defines a hazardous organism or etiological agent as a viable organism or its toxin that causes or may cause human disease. The USPHS requires a permit (CDC0.753) for importation of such organisms into the United States. The regulation is administered by the Centers for Disease Control and Prevention (CDC) through its Foreign Quarantine Program. The CDC also regulates the packaging and shipping of human pathogens for interstate transport (42 CFR 72.3).

The USPHS designated organisms as Class I, II,III, or IV in *Classification of Etiologic Agents* on the Basis of Hazard (USPHS 1974) and described biosafety levels for infectious agents in *Biosafety in Microbiological and Biomedical Laboratories* (Richmond and McKinney 1999).

1. Class I organisms — no recognized hazards, special packaging not required.
2. Class II organisms — ordinary potential hazard, distribution restricted to bona fide laboratories, special packaging required.
3. Class III organisms — special hazards, special packaging and a letter from the receiving institution acknowledging the hazard required.
4. Class IV organisms — potentially dangerous to human health, USPHS and/or USDA permit required for shipping.

Plant pathogens are described in APHIS (Animal and Plant Health Inspection Service of the USDA) 7 CFR 330.100 as organisms that can directly or indirectly injure, cause disease in, or damage any plant or plant part, or any processed, manufactured, or otherwise produced products of plants. The USDA regulates the movement of all plant pathogens and mycotoxin-producing strains across interstate or international boundaries and requires a permit from the Plant Protection and Quarantine (PPQ) Program for doing so. The investigator who receives the culture must complete Form PPQ-526, "Application and Permit to Move Live Plant Pests." The form then must be submitted to the plant regulatory official of the state in which the culture will be used. The state in turn mails the form to PPQ for approval, These safeguards are set in place by USDA and similar authorities in foreign countries to protect the environment from introduced pests and diseases.

The APHIS requires Form VS 16–3 for the importation of extremely virulent pathogens of livestock or poultry or of those for which a national eradication control program exists. The regulations are stated in 9 CFR Part 122.2. An organism that has been genetically engineered via recombinant-DNA techniques from a donor organism, vector, or vector agent that is a plant pest or contains plant pest components requires APHIS Form 2000 (7 CFR Part 340).

The DOT is responsible for establishing and enforcing regulations for safety aspects of transportation (Appendix III), which include, but are not limited to, infectious substances in domestic transport (49 CFR Parts 171–178). The regulations cover (1) classification of materials, (2) packaging, (3) hazard communication, (4) transportation and handling, and (5) incident reporting.

The U.S. Bureau of Customs regulates the importation of materials into the United States. It determines if materials are admissible and if they should be referred to other government agencies for examination, permits, and release. The Customs Bureau judges if import duties should be paid and if the packages being imported do contain the goods that are manifested or declared.

The DOC regulates the export of biological materials through the Bureau of Export Administration. Any organism or toxin that appears under Export Control Classification Number 1C61B requires a validated export license for all foreign destinations except Canada. Materials that do not require a validated license may be shipped under a general license (G-DEST). Institutions apply for this general license once and label each appropriate shipment as such. There is a mandate for the DOC to identify organisms that might be involved in biological warfare and to place export controls on them. Several other countries, such as Australia, New Zealand, and Germany, also require import permits and licenses.

The United Nations (UN) Economic and Social Council staff (2001) provides the international shipping regulations in "Recommendations of the Transport of Dangerous Goods." The current DOT regulations are based on the UN recommendations applicable to hazardous material transport in the United States. Those who export biological material to foreign countries are required to follow the UN regulations. Mandatory requirements for shippers of dangerous goods have been in effect since 1990. A 24-hour emergency number allows for rapid responses to numbers of hazardous materials. These international regulations are explained in detail by Rohde and Claus (1999). In addition, the World Federation for Culture Collections Committee on Postal, Quarantine and Safety Regulations (Appendix III) should be consulted to keep abreast of the ever-changing regulations.

In Canada microorganisms are assigned to one of four risk groups ranging from a low individual and

community risk, level 1, to a high individual and community risk, level 4 (Anonymous 1996b). Each risk group has a corresponding containment level. Containment level 1 is found in a basic microbiology laboratory, whereas containment level 4 represents a geographically isolated unit functionally independent of other areas. Agriculture Canada requires an annual import permit for all plant and animal pathogens shipped to Canada. Some pathogens require a single-entry permit; others can be shipped under a multipurpose permit. As of September 1994, all human pathogens (Risk Groups II–IV) require a Health Canada permit. To import a pathogen that is both a human and animal pathogen, permits from Health Canada and Agriculture Canada are required. Shipments of extremely hazardous agents require a Canadian Department of Transport Emergency Response Assistance Plan.

The Working Party on Safety in Biotechnology of the European Federation of Biotechnology (EFB) has classified organisms as harmless (EFB Risk Class 1); low risk (EFB Risk Class 2), which means that they may cause disease and might be a hazard to laboratory workers but are unlikely to spread in the environment; medium risk (EFB Risk Class 3), organisms that severely threaten the health of laboratory workers but represent a comparatively small risk to the population at large; and high risk (EFB Risk Class 4), organisms that cause severe illnesses. Classes 3–4 fall into special containment categories.

SHIPPING REGULATIONS

Shipping of noninfectious, perishable biological organisms within the United State is generally not a problem once required permits are obtained. Freeze-dried cultures, agar slants, and broth cultures may be shipped via the U. S. Postal Service (USPS) or private freight carrier. Shippers are responsible for the safety of those handling and receiving the culture. However, the USPS, in its Domestic Mail Manuals and International Mail Manuals, and the DOT, in 49 CFR Part 173, all require that etiological agents be packaged in accordance with USPHS guidelines (42 CFR Part 72). Requirements for packages containing infectious substances are described in 49 CFR Part 178.609, Federal regulations and permit requirements are summarized in Richmond and McKinney (1999). Some state governments also may require special permits. For more information on international regulations, see Rohde et al. (1995) and Rohde and Claus (1999).

Etiological agents shipped domestically via the USPS must be sent as first-class mail, priority mail, or express mail. Exceptions are agents listed in 42 CFR 72.3 (f), which must be sent by registered mail or an equivalent

system that notifies the sender when the package has been received. International shipments must be sent via registered mail. For shipping to Canada, any package labeled "infectious substances" must be sent by private carrier following International Air Transport Association (IATA, a trade organization of airlines) regulations. At the present time, only Federal Express will carry such materials.

The USPS will accept only properly packaged cultures. A nonpathogenic culture is placed in a foam insert, which is then put into a fiberboard cylinder. For freight carriers, the cylinder is then placed inside a corrugated box. Both domestic and international shipments of etiological agents or etiological agent preparations are limited to a volume of 50 ml. The primary container (e.g., test tube, vial) must be securely sealed and watertight and must be enclosed in a second, durable, watertight container (secondary container). Several primary containers may be enclosed in one secondary container. Enough absorbent material must be placed between primary and secondary containers to absorb the contents of the primary container(s) if it breaks. For cultures shipped by freight carriers, the set of containers must be enclosed in an outer shipping container constructed of corrugated fiberboard, cardboard, wood, or other material of similar strength.

When volumes of greater than 50 ml are shipped, a layer of shock-absorbent material must be placed between the secondary container and the outer container. Nonpathogenic cultures are limited to 1000 ml per primary container and 4000 ml per outer container.

According to 42 CFR Part 72, if cultures are shipped on dry ice, the ice must be placed outside the secondary container in such a way that the container does not become loose as the ice sublimes. Outer containers must be vented to allow carbon dioxide gas to escape. Dry ice cannot be sent by international mail; cultures in dry ice must be shipped by freight carrier. If wet ice is used, it must be placed between the secondary container and the outer container. The outer container should be designed so that it does not collapse after the ice melts, and the entire package should be leak-proof. Liquid-nitrogen containers must be able to withstand ultra-low temperatures, and DOT regulations in 49 CFR Part 173.316 must be observed.

The Universal Postal Union, the International Civil Aviation Organization (ICAO), and the International Maritime Organization (IMO) regulate the transport of cultures between countries. The United Nations Economic and Social Council Committee of Experts on the Transport of Dangerous Goods (2001) provides recommendations that are enforced by ICAO and IMO. The IATA publishes a manual of air-transport procedures in agreement with the ICAO regulations, which are followed by freight carriers worldwide. Because most cul-

tures are sent by air, IATA regulations must be followed whether the material is sent by the USPS or a freight carrier.

The only requirement when transporting less than 50 ml of an etiologic agent by registered airmail is compliance with packaging regulations. If more than 50 ml of an etiologic agent is shipped, the containers must be specially tested and the material can be sent only by cargo aircraft (DOT 49 CFR 173.387). In addition to the required Biological Substance mailing labels and forms set forth by USPS, private shipping companies require that a telephone number at which a person knowledgeable about the shipment and its contents can be reached 24 hours a day be provided. Growing cultures, which are not acceptable for mailing to certain countries, are sent by freight carrier.

Several agencies require labels for etiological agents or infectious substances, depending on the destination, the state of the shipment (dry or frozen), and the carrier (USPS or freight carrier). All shipments of etiologic agents within the United States must carry the CDC etiologic agent label. Shipments of more than 50 ml of a culture also must carry a "Cargo Aircraft Only" label. Packages sent by air-freight carrier must carry an IATA infectious substance label and a Shipper's Declaration for Dangerous Goods from the IATA and must be marked as a UN 2814, Class 6.2 shipment with the name of the agent, volume, and name and telephone number of the responsible party noted.

All foreign shipments of biological materials, regardless of hazard, must have a Shipper's Export Declaration (DOC Form 7525-V), which verifies the existence of a validated export license or a general license. If sent by the USPS (air mail only), volumes of culture exceeding 50 ml also must carry the green customs label (PS 2976) and a "Cargo Aircraft Only" label. Etiologic agents must carry an IATA infectious substance label and a Shipper's Declaration for Dangerous Goods and must be labeled as a UN 2814 Class 6.2 shipment, specifying the volume, the name of agent, and the name and telephone number of the responsible party.

When a culture is shipped in dry ice, an additional UTA Miscellaneous 9 label and a UN 1845 designation with the amount of dry ice noted in kilograms is required. If the net weight of the dry ice is greater than 5 lb (ca. 2.3 kg), a Shipper's Declaration for Dangerous Goods must be included. When a frozen shipment uses liquid nitrogen, an UTA nonflammable gas label and "Do Not Drop" and "Handle With Care" labels also are required. The words "Keep Upright" with arrows for proper orientation must be placed on each side or at 120° intervals around the package. The outer shipping container is marked "Nitrogen, Refrigerated Liquid, Class 2.2 UN 1977."

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Inventory and Monitoring Methods

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Front cover: The slime mold *Physarum roseum* on a decaying leaf. Slime molds are fungal-like organisms traditionally studied by mycologists. Photo by Ray Simons.

Back cover: A species of *Mycena* found in Yunnan, China. Species of the mushroom genus Mycena are commonly encountered throughout the world. Photo by Gregory M. Mueller.