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Recovery of endophytic fungi from *Chamaecyparis thyoides*

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A preliminary report describing the species diversity and the frequency of colonization of endophytic fungi in the aerial portions of *Chamaecyparis thyoides* is presented for the purpose of evaluating the efficacy of this substratum as a source of fungi for an industrial screening program. Twenty-four leaf and twig segments were removed from 10 trees at 5 different sites in New Jersey and applied to 3 different isolation media. From 600 twig segments and 600 leaf segments from 50 trees, 961 fungal isolates were cultured representing 88 species of filamentous fungi, yielding 10 ± 2 species/tree. A medium of malt-yeast extract gave the most isolates and highest species recovery rate. Attempts to increase species richness by using media with cycloheximide or benomyl were unsuccessful. These fungitoxic compounds were deleterious to growth of fungi from small foliage segments when compared to a malt-extract medium without inhibitory agents. Nine of 11 dominant species were preferentially isolated on one of the three media. The endophytic mycoflora is similar in taxonomic composition and colonization frequency to that of other conifers. Most isolates represent new reports of fungi inhabiting *C. thyoides*. The total number of isolates was dominated by a few species, but a large percentage of the species were recovered at either a single site or from a single tree. The five sites were relatively homogeneous with respect to number of isolates and number of species recovered. Sampling endophytes over a wide geographic area increased the total species recovered. Although endophytes represent a relatively unexplored source of microbial germplasm for biotechnological applications, screening programs utilizing endophytes as source of isolates should recognize that indiscriminate selection of isolates from the same or similar host plants can lead to unnecessary replication of experiments.

Keywords: endophytes, *Chamaecyparis thyoides*, Pine Barrens, isolation methods, microbial diversity.

Endophytic fungi are being increasingly recognized as an ecological assemblage of microorganisms that may provide sources for new secondary metabolites with useful biological activities. Part of the rationale for exploring these organisms is based on evidence that aerial organs of plants infected by endophytes are conferred with various selective advantages against herbivores or microbial pathogens (Carroll, 1991; Clay, 1988). These adaptive advantages are either the direct result of chemicals produced by the fungal symbiont, changes in host metabolism induced by the presence of the endo-

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phytes, or mediated by yet unknown interactions between the symbionts. A second line of arguments in favor of searching for new chemical structures among these fungal symbionts is based on previous success in discovery of new compounds or biological activities within a limited survey of some major taxonomic groups of endophytes such as the Xylariales (Dreyfuss, 1986; Whalley & Edwards, 1987), the Clavicipitales (Clay, 1988), and other Ascomycetes or their anamorphic stages (Fisher & al., 1984; Tscherter & Dreyfuss, 1982; Dreyfuss, 1986; Clark & al., 1989).

Many endophytic fungi possess a set of characteristics that are desirable for manipulation within an industrial screening program. Firstly, they represent new germplasm, in the sense that they have not undergone intensive biochemical surveys in past screening programs. Theoretically, the likelihood of discovery of new groups of secondary metabolites will be higher than with more well-known groups of fungi, e.g. common genera of soil fungi. With the exception of some xylariaceous anamorphs and some species of coprophilous Sordariales and Pleosporales, endophytic fungi are generally not recovered from decaying vegetation or soil. Cost and availability of materials for isolation are inconsequential, living plants are ubiquitous and plant organs are easily collected and transported to yield desirable results (Dreyfuss & Petrini, 1984). Isolation methods are simple, requiring only surface sterilization and standard mycological media (Petrini, 1986). Many isolates grow readily in culture, can be manipulated with the same ease as other filamentous fungi, and exhibit a heterogeneous array of physiological characteristics when grown in complex media (Carroll & Petrini, 1983).

Intelligent and efficient industrial screening of microorganisms requires that a high α-diversity of organisms be maintained while simultaneously minimizing redundancy among the taxa screened. This can only be achieved through an understanding of the floristic composition and pattern of colonization of the microorganisms within the particular ecological niche being sampled. What species are likely to inhabit the particular endophytic host and their relative abundance? How many species are likely to be found by sampling a single tree or several trees? Does species richness and floristic composition vary among samples throughout a landscape? Does the isolation media or the particular plant organ sampled influence the species recovered? To answer these questions, we chose to examine the endophytic mycoflora of a local plant species that would be likely to have abundant endophytic infections. *Chamaecyparis thyoides* (L.) B. S. P. (white Atlantic cedar, swamp white cedar) was selected because it was common in New Jersey and because the foliage of other species of Cupressaceae was known to harbor a rich endophytic mycoflora (Petrini & Müller, 1980; Petrini & Carroll, 1981). We report

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here on the species diversity and colonization frequency of endophytes in aerial portions of *C. thyoides*, as perceived by our isolation methods, for the purpose of evaluating the efficacy of this substratum as a source of fungi for an industrial screening program.

Materials and methods

Study sites and sample selection

All study sites were located in the coastal plains physiographic region of New Jersey. Within this region, *C. thyoides* is a characteristic and dominant species along streams and in bogs of the Pine Barrens often forming dense, pure stands (Robichaud & Buell, 1983). Four of the 5 sites (except Helmetta Pond) were in the Pine Barrens region.

Ten trees were arbitrarily selected during the the month of July, 1990 at: Helmetta Pond, Middlesex Co. 11, July; Success Lake, near Collier's Mill, Ocean Co., 13 July; Webb's Mill Branch, intersection with State Highway 539, Ocean Co., 18 July; West Branch of Bass River, near Lake Absegami, Ocean Co., 18 July; and Wading River, near State Highway 563, Burlington Co., 18 July. From each tree, a series of small branches with intact foliage were cut from the lower crown with garden clippers from a height of 1.5-2 m above the ground. Branches from each tree were placed in separate wax-paper bags or Manila paper envelopes for transport to the laboratory. Samples were stored overnight at 5 C and used the next day.

Branches from each tree were cut into twelve leaf segments (approximately 2 x 2 cm) and twelve twig segments (2 cm long x 0.2-0.5 cm diameter). No attempt was made to sort leaves into age classes. All age classes were assumed to be sampled with equal likelihood. Segments were surface sterilized by dipping them first in 95% ethanol for 1 min, then into 66% household bleach with a final sodium hypochlorite concentration of 3.3% (leaf segments 3 min, twigs segments 5 min), followed by 30 sec in 95% ethanol (Petrini, 1986). For each tree, four leaf and four twigs segments were applied to three different agar isolation media in quartered 100 mm plastic Petri dishes. Segments were placed in individual compartments of the quartered dishes to minimize merging and overgrowth with fungi from an adjacent segment. Petri dishes were incubated at 20 C, 98% r. h., under 12 hr fluorescent light (Sylvania cool white)/12 hr dark, enclosed in translucent, white, covered plastic boxes. Petri dishes were inspected more or less daily for up to five weeks for the development of fungal colonies on the segments or on the agar. All filamentous fungal colonies were isolated by transfer of mycelial fragments or spores to agar slants. Slants were numbered with the source tree,

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organ type, and isolation media. Strains were not incubated under any special environmental or nutritional conditions to induce sporulation. After three to five weeks growth at 25 C, under 12 hr fluorescent light (Philips Econ-o-Watt)/12 hr dark, slants were sorted and enumerated into morphological "species" based on gross colony morphology and the development of any sporulating structures on the mycelia. Isolates from representative slants of a species or isolates from slants where differences in colony morphology were ambiguous were transferred to 45 mm Petri dishes of agar media for identification, at least as far as possible. Nomenclature of fungi follows that listed in Farr & al. (1989), De Hoog & Hermanides-Nijhof (1977), Ellis & Ellis (1985), Pitt (1979), and Sutton (1980). Representative cultures of all species have been preserved in the Merck Microbial Resources Culture Collection.

Media

The media used for isolation and identification were prepared in 1 liter of distilled water as follows: (1) MYE (10 g malt extract, 2 g yeast extract, streptomycin sulfate 0.05 g, chlorotetracycline 0.05 g, agar 20 g; (2) ACD (Mycosel agar, BBL Laboratories, Cockeysville, MD), phytone 10 g, glucose 10 g, cycloheximide 0.4 g, chloramphenicol 0.05 g); (3) BOP (10 g malt extract, 2 g yeast extract, 1 g sodium propionate, 5 g dehydrated bovine bile, 0.001 g benomyl, 0.05 g streptomycin sulfate, 0.05 g chlorotetracycline, 20 g agar). The malt-yeast extract agar used for enumeration and identification was the same as MYE without antibiotic agents.

Statistical methods

Statistical computations were made using software provided by Statistical Analysis Systems (release 6.06, SAS Institute Inc., Cary, NC, USA). The SAS summary procedure was used to compile totals and means for the different species, trees, sites and media treatments. The SAS anova procedure was use to perform analysis of variance on species means among sites and among media treatments and to compare the values of different means by Duncan's multiple range test. The chi-square goodness-of-fit tests were calculated with the SAS tabulate procedure.

Results and discussion

From 600 twig segments and 600 leaf segments from 50 trees, 961 fungal isolates were cultured representing 88 species of filamentous fungi (Tab. 1). Overall, this sampling and isolation technique yielded 10 ± 2 species/tree (N=50, Tab. 2).

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Tab. 1. – Fungi isolated from living twigs and leaves of 50 trees of *Chamaecyparis thyoides* in New Jersey. Species ordered by decreasing number of isolates recovered.

Species	Number of isolates	Number of trees	Number of sites
Nodulisporium sp.	162	49	5
Mycoleptodiscus atromaculans Bills & Polishook	119	41	5
Sterile mycelium A	81	35	5
Coniochaeta sp. A	72	36	5
Xylaria longipes Nitschke	72	38	5
Xylaria cubensis (Mont.) Fr.	53	28	5
Geniculosporium sp. A	41	22	5
Cryptosporiopsis sp.	37	18	5
Diplodia sp.	31	20	5
Geniculosporium sp. R	25	18	5
Sporidesmium sp.	20	15	5
Geniculosporium sp. H	18	16	5
Pestalotiopsis funerea (Desmaz.) Steyaert	17	10	2
Hormonema dematioides Lagerberg & Melin	16	13	4
Sterile mycelium B	16	8	4
Microdochium sp.	14	11	4
Alternaria alternata (Fr.) Keissl.	13	10	3
Phyllosticta sp.	13	6	4
Geniculosporium serpens Chesters & Greenhalgh	12	9	3
Coniochaeta sp. B	10	9	4
Coniochaeta tetraspora Cain	8	6	3
Cladosporium cladosporioides (Fres.) De Vries	7	4	2
Pleurophoma sp.	7	4	3
Epicoccum nigrum Link	6	4	3
Penicillium spinulosum Thom	6	5	2
Gelasinospora tetrasperma Dowding	4	4	2
Sterile mycelium C	4	4	4
Tubercularia vulgaris Tode	4	1	1
Geniculodendron sp. ?	3	2	2
Nigrospora sphaerica (Sacc.) E. Mason	3	3	1
Sterile mycelium F	3	2	1
Cladosporium herbarum (Pers.) Link	2	2	2
Glomerella cingulata (Stoneman) Spauld. & H. Schrenk	2	2	2
Harknessia thujina Ellis & Everh.	2	2	1
Phomopsis sp.	2	1	1
Neocosmospora endophytica Polishook, Bills & Rossman	2	2	2
Sterile mycelium V	2	2	1
Sterile mycelium Y	2	2	2
Chaetophoma sp.	1	1	1
Discomycete sp. A	1	1	1
Exophiala sp.	1	1	1
Fusarium sp.	1	1	1
Hypoxylon deustum (Hoffm.) Lind.	1	1	1
Penicillium pseudostromaticum Hodges, Warner & Rogerson	1	1	1
Pseudoeurotium ovalis Stolk	1	1	1
Penicillium thomii Maire	1	1	1
Sordaria sp.	1	1	1
Verticicladium trifidum G. Preuss	1	1	1
Virgaria nigra (Link) Nees	1	1	1
Wallemia sebi (Fr.) von Arx	1	1	1
Xylaria curta Fr.	1	1	1
Unidentified Hyphomycete A	1	1	1
Unidentified Coelomycete spp. (isolated only once from a single tree)	4	–	–
Sterile mycelial spp. (isolated only once from a single tree)	29	–	–
Total	961	–	–

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When both twigs and leaves are combined, MYE medium yielded the highest species richness (54 species, 5 ± 2 species/tree, N=50, Tab. 2). However, the mean species/tree recovered on MYE was not significantly greater than from BOP. Numbers of isolates and species richness recovered from twigs alone were comparable among the three media (Tab. 2). If only MYE is considered, then nearly equal proportions of species were recovered from leaves and twigs. When all media were considered, more isolates (642) and more species (63) were recovered from twigs than from leaves (319 isolates, 53 species, Tab. 2). The inhibitory media, ACD and BOP, drastically reduced the number of isolates and species recovered from leaves. The few leaf isolates that were recovered on ACD and BOP emerged from the leaf tissue only after 3-5 weeks incubation, rather than in 1-4 weeks on MYE. In a previous experiment with discs of tree bark (Bills & Polishook, 1991), greater species richness was recovered using media with benomyl or cycloheximide to reduce the growth of rapidly spreading species. The small size of the leaves (0.5-1.5 mm diam) probably permitted greater diffusion of the cycloheximide or benomyl into the leaf tissue than into the twigs, thus nearly all species failed to grow. Only 28 (31%) of the species were recovered from both twigs and leaves indicating that a majority of fungal species exhibit organ specificity as in other host plants (Petrini, 1986).

Tab. 2. – Number of isolates and species of fungi recovered on 3 different media from 50 trees of *Chamaecyparis thyoides* . Two-hundred twig segments and 200 leaf segments were placed onto each medium. See methods for media formulations.

	Media			
	MYE	ACD	BOP	All media
Combined twig and leaf segments sampled	400	400	400	1200
Isolates from twig segments	199	185	258	642
Isolates from leaf segments	208	46	65	319
Total isolates	407	231	323	961
Species from twig segments	36	31	32	63
Species from leaf segments	37	14	20	53
Total species	54	38	41	88
Species/tree* (mean ± std)	$5^{a}\pm2$	$3^{b}\pm2$	$4^{a}\pm2$	10± 2
Species/tree (range	2–12	0–7	0–8	6–15

*Data are means of 50 trees at 5 sites. Different letters above the means indicate significant differences at P=0.05 according to Duncan's mutiple range test.

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Species with an isolation frequency greater than 2% (the first 11 species of Tab. 1) were analyzed with respect to media preference using a chi-square goodness-of-fit test ($P=0.05$, 2 d.f.). When compared to expected isolation frequencies assuming no media preference for isolation media, only *Cryptosporiopsis* sp. ($P=0.73$) and *Geniculosporium* sp. R ($P=0.58$) did not differ significantly from the expected values. All other dominant fungi exhibited a significant preference and/or aversion for isolation media (all $P<0.05$). *Nodulisporium* sp. A, *Mycoleptodiscus atromaculans* , and *Diplodia* sp. were preferentially isolated on MYE. On BOP, *Coniochaeta* sp. A, *Xylaria cubensis*, and *Xylaria* sp. A were isolated more frequently than expected. On ACD, Sterile Mycelium A and *Sporodesmium* sp. were preferentially isolated. *Xylaria hypoxylon* was isolated more frequently on BOP and ACD than expected.

The endophytic mycoflora of *C. thyoides* (Tab. 1) is similar in taxonomic composition and colonization frequency to that of other conifers. The dominant flora is characterized by the first 12 species of Tab. 1. These species were recovered at every site and from 30% or more of all trees. The majority of the species recovered can be grouped according to the categories provided by Petrini (1986) and include anamorphs of Xylariaceous fungi (*Xylaria* anamorphs, *Nodulisporium*, *Geniculosporium*), coprophilous genera (*Coniochaeta*, *Gelasinospora*, *Sordaria*), non-specific, epiphytic genera (*Epicoccum*, *Cladosporium*, *Alternaria*, *Nigrospora*, *Penicillium*, *Virgaria*, *Wallemia*), and a group of genera that are almost exclusively endophytes (*Phyllosticta*, *Diplodia*, *Cryptosporiopsis*, *Harknessia*, *Hormonema*, *Pestalotiopsis*, *Phomopsis*, *Glomerella*, *Pleurophoma*). Many of the unidentifiable Coelomycetes probably could be included in the last category. Sterile isolates accounted for 35 (40%) of the species. These cultures were examined for 6-8 weeks after isolation. They exhibited a bewildering array of colony morphologies and growth rates but failed to produce any recognizable sporulating structures under our incubation conditions. As in previous studies of endophytes, these isolates are problematic because their taxonomic affinities are uncertain, but it is likely that many represent host-specific species.

Nearly all the isolates represent new reports of fungi from *C. thyoides* and the Pine Barrens region. *Pestalotiopsis funerea* and *Harknessia thujina* previously have been reported from this host in the index compiled by Farr & al. (1989). The list of Farr & al. includes 19 species of fungi on *C. thyoides*, excluding rusts and wood-decay Basidiomycetes, and is based primarily on species observed to fruit on various organs of the tree. Quite possibly many of the taxa we have isolated represent anamorphic or vegetative stages of some of the fungi listed by Farr & al. (1989), but without direct comparison of

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our cultures with those derived from identified fruiting structures on the host, definitive identifications are difficult. Conversely, many of the ubiquitous epiphytic fungi we have recovered were not included in the list of Farr & al. (1989). This discrepancy emphasizes the limited usefulness of traditional surveys of plant-inhabiting fungi to investigators of endophytes. The accumulating body of information on distribution of the diversity of internal fungi of vascular plants is certain to impact future compilations and interpretations of host indices.

The second most frequently isolated species, *Mycoleptodiscus atromaculans*, was recently described as a new species (Bills & Polishook, 1992) and is very similar to *Mycoleptodiscus taiwanensis* Matsushima. *Neocosmospora endophytica*, producing a *Penicillifer* anamorph in culture, (Polishook & al., 1991) was another new species discovered during this investigation. This species was also recovered as an endophyte in stems of *Hudsonia ericoides* L. at the Success Lake site. We recognized three *Penicillium* spp. (*P. thomii*, *P. pseudostromaticum*, and *P. spinulosum*) that are common taxa of the Pine Barrens region and are frequently recovered from the local soils or from other plants. Litter of adjacent trees of *Pinus rigida* Mill. may have been the inoculum source for the isolate of *Verticicladium trifidum,* a species common on decaying needles of *Pinus* spp.

Tab. 3. – Number of isolates and species of fungi recovered from twigs and leaves of *Chamaecyparis thyoides* on 3 media from 10 trees at 5 different sites in New Jersey. Two-hundred twig segments and 200 leaf segments were placed onto each medium.

	Sites				
	Helmetta Pond	Success Lake	Webb's Mill Branch	Bass River	Wading River
Total isolates	212	161	201	189	196
Total species	44	38	31	30	30
Species/tree* (mean ± std)	12±2	10±2	10±2	10± 2	9±1
Species/tree (range)	9–15	6–14	9–14	8–15	7–12

*Data are means of 10 trees at each site. Means are not significantly different according to analysis of variance (P= 0.09).

The five sites were relatively homogeneous with respect to the number of isolates and species recovered (Tab. 3). Slightly more isolates and species were recovered at the Helmetta Pond site. The *C. thyoides* bog at the Helmetta Pond was located slightly north of the Pine Barrens region and was surrounded by a more mesic deciduous forest than the other 4 sites that were within the typical xeric *Pinus*

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rigida-Quercus forest of the Pine Barrens. However the number of species/tree were not significantly different among the sites (Tab. 3).

To examine the effect of increasing the number of trees, and hence the number of isolates sampled, the 50 trees were ordered randomly and the increment in numbers of species and isolates was plotted (Fig. 1). Although the first twelve species in Tab. 1 were very widespread and all were encountered after the first few trees were sampled, most species were very restricted in distribution. Fifty-four of the 88 species (61%) occurred at a single site, Forty-nine species (55%) were only recovered from a single tree, and 47 species (53%) were represented by a single isolate. As is the case with other conifers studied (Carroll & Carroll, 1978; Petrini, 1986), most endophytic species of *C. thyoides* were encountered sporadically, possibly because of environmental factors influencing their spatial distribution. The number of isolates/tree was relatively constant (Fig. 1, 19.2 ± 4.5 isolates/tree, N=50). The initial increment in new species was rapid, but continued sampling of trees yielded additional species at decreasing rate (Fig. 1). Clearly, retrieving multiple samples of a host plant over a landscape, coupled with recognition and constant dis-

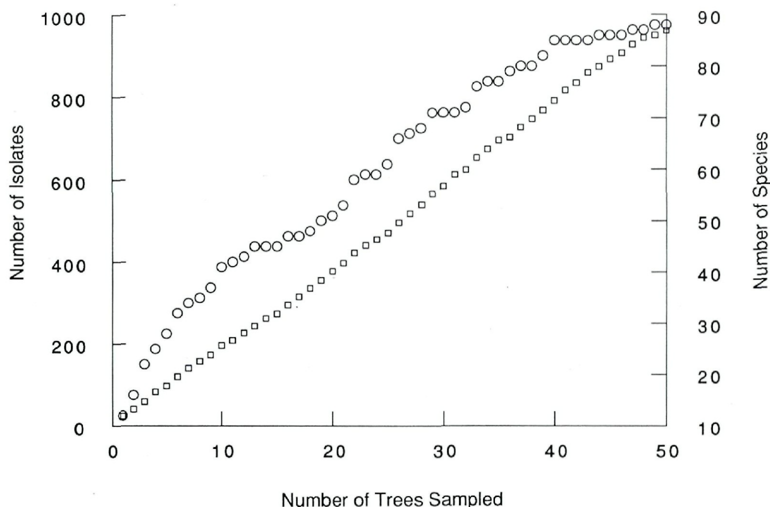

Fig. 1. – Increase in species richness (○) and number of isolates (◇) as a result of sampling more trees. Trees were selected at random from the population of 50 included in this study.

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carding of the common dominant species, is a useful strategy for increasing α-diversity of endophytes selected for a screening program.

The number of species encountered per isolate recovered in our entire study is in the same order of magnitude (88 taxa in 961 isolates. Fig. 1) to that reported for soils (Christensen, 1981). However, our individual small samples of living leaves and twigs (6-15 species/sample) fell short of the large numbers of species that can be expected from a single soil sample of a few grams. Individual soil samples from temperate regions examined by simple dilution-plating can be expected to yield in the range of 15-100 species/sample (Christensen, 1981). As with soil fungi, the number of species isolated from *C. thyoides* was a function the number of isolates examined (Christensen, 1981; Fig. 1). Sampling endophytes, with a scheme similar to that described here, for input into an industrial screening program would be much more labor intensive with regard to sample collection and preparation to obtain the same number of species as from soil samples. However, the value of examining endophytes lies in the probability that a relatively high proportion of the taxa from a host plant may never have been examined for novel metabolites and that a few of the species may be so host-specific they are restricted only to that family, genus, or species of vascular plant (Dreyfuss, 1989).

From our study we would conclude that the foliage of *C. thyoides* holds a large reservoir of fungi that are easily recovered into culture, many of which are of unknown practical importance. In general the floristic composition and its colonization frequency is similar to that of endophytes from internal tissues of other conifers. Relatively little overlap occurs with fungal communities from more conventional substrata used for industrial screening such as soil and litter. Sampling over a large area increases the species richness recovered from a particular host. Precaution needs to be taken when undertaking large-scale screening of endophytes, especially with non-specific endophytes or with closely related species of the same genera from similar host plants. Because many of the genera and species recovered in this study were the same or similar to those of other conifers and woody angiosperms, unnecessary replication of experiments will occur when the inevitable multiple isolates of the dominant taxa, occurring within a single sample or co-occurring among similar samples are not eliminated. Future experiments to increase diversity of endophytes recovered from leaves or small diameter twigs by use of selective media should consider adding smaller concentrations of inhibitory substances, incorporating only non-toxic, colony-restricting compounds into the media, or using selective carbon or nitrogen sources.

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