Saccharomyces cerevisiae and Saccharomyces paradoxus coexist in a natural woodland site in North America and display different levels of reproductive isolation from European conspecifics

Paul D. Sniegowski *, Peter G. Dombrowski, Ethan Fingerman

Department of Biology, University of Pennsylvania, 415 S. University Avenue, Philadelphia, PA 19104, USA

Received 2 May 2001; received in revised form 5 September 2001; accepted 27 September 2001

First published online 20 November 2001

Abstract

We report the isolation of multiple strains of Saccharomyces cerevisiae and Saccharomyces paradoxus from a natural woodland site in southeastern Pennsylvania, USA, using enrichment culturing in a medium containing 7.6% (v/v) ethanol. The method was applied to bark and flux material collected from broad-leaved trees (mostly Quercus spp.) and to associated soils. Many candidate wild strains of Saccharomyces were isolated using this method, most of them from soils associated with oaks. Matings to genetically marked tester strains of S. cerevisiae and S. paradoxus identified roughly equal numbers of these two species within this collection. The S. paradoxus isolates showed significant partial reproductive isolation from a conspecific European strain, whereas the S. cerevisiae isolates did not. Variability in both chromosome size and Ty1 element hybridization profiles was observed within both populations at this site. We discuss the relevance of our data to current debates concerning whether S. cerevisiae is a wild species or a domesticated species. ß 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Yeast; Reproductive isolation; Wild Saccharomyces; Saccharomyces paradoxus; Saccharomyces cerevisiae

1. Introduction

Studies of hybrid ascospore viability suggest that the Saccharomyces sensu stricto complex contains at least six species delimited by postzygotic isolation: Saccharomyces cerevisiae, Saccharomyces paradoxus, Saccharomyces bayanus, Saccharomyces carioacanus, Saccharomyces kudriavzevii and Saccharomyces mikatae [1–3]. Relatively little is known about the distribution and population structure of these species. To date, the most extensively studied species in the wild is S. paradoxus, which appears to be present on most continents and exhibits genetic and reproductive differentiation over long distances [2,4–7]. S. paradoxus strains collected from within broad geographical regions show limited allozyme variability [5], but they are clearly variable at the DNA sequence level as judged by DNA fingerprinting methods [8] and hybridization profiles of Ty1 elements to chromosomes [6,8,9]. S. cerevisiae strains isolated from vineyard grapes show considerable variability in metabolic properties, karyotype, and DNA sequence as judged by the results of RAPD analysis [10–13], but no such data are available for S. cerevisiae isolated from uncultivated areas. Indeed, S. cerevisiae has seldom been isolated away from the context of human applications [2,6,14].

The most common uncultivated habitat from which Saccharomyces sensu stricto have been isolated is fluxes of oaks and other broad-leaved trees, although Saccharomyces are not always present at such sites [15]. Previously published methods of isolation from fluxes have involved enrichment in malt extract-based medium, phenotypic screening based on colony, cell, and ascus morphologies, and positive species identification by the analysis of matings to genetically marked tester strains (e.g. [6]). The relative rarity of flux sites and the variable success in obtaining positive enrichments led us to develop a more efficient sampling and enrichment procedure, which we describe here. Although our initial goal was to establish a collection of S. paradoxus for studies of population structure, in the course of this work we obtained comparable numbers of S. cerevisiae isolates from the same habitat. Interest-
ingly, our \( S. \) \textit{cerevisiae} isolates showed no reproductive isolation when crossed with a conspecific tester from Europe, whereas our \( S. \) \textit{paradoxus} isolates showed the expected partial reproductive isolation from a European conspecific. We discuss the implications of our findings for population studies in \textit{Saccharomyces} and for the controversy concerning whether \( S. \) \textit{cerevisiae} is a wild species or a domesticated species.

2. Materials and methods

2.1. Sample collection and enrichment culturing

All collections were made in July 1999 at the John J. Tyler Arboretum in Lima, PA, USA. The arboretum grounds include 182 ha of mature second-growth forest dominated by stands of native tulip poplar (\textit{Liriodendron tulipifera}), American beech (\textit{Fagus grandifolia}), maples (\textit{Acer} spp.), and oaks (\textit{Quercus} spp.); this forest is contiguous with 1052 ha of similar natural habitat in Ridley Creek State Park. Excudate material, bark, and soil samples were obtained from around the bases of trees using a sterile scalpel or spatula and deposited in sterile 8-ml glass vials in the field. These collection vials were stored for up to 3 days at 4°C before enrichment culturing. Upon return to the laboratory, the vials were filled with a sterile liquid medium consisting of 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g sucrose, 76 ml EtOH, 1 mg chloramphenicol, and 1 ml of 1-M HCl per liter. Vials were then capped tightly, incubated for approximately 10 days at 30°C without shaking, and inspected for signs of fermentation. In many cases, vials effervesced vigorously upon being opened after a few days; in some cases, a visible white sediment on the bottom of the vial indicated proliferation of yeasts or other cells.

A single aliquot of 10 µl from each of these liquid enrichment cultures was then streaked on solid medium containing 15 g of agar, 20 g of methyl-\( \alpha \)-d-glucopyranoside, 6.7 g of yeast nitrogen base with amino acids and ammonium sulfate (Difco, USA) and 4 ml of 1-M sorbitol. Plates were incubated for several days at 30°C and examined daily for the presence of red colonies indicative of adenine auxotrophy. Putative adenine auxotrophs were confirmed by picking to synthetic dextrose (SD) minimal agar [16] and SD agar plus 2 g l\(^{-1}\) of adenine hemisulfate. Confirmed auxotrophs were grown, resporulated and the asci digested to completion to obtain spore suspensions for test matings.

2.2. Sporulation and mutagenesis

Putative \textit{Saccharomyces} strains obtained from the enrichment procedure described above were regrown and sporulated in standard liquid complete presporulation medium and complete sporulation medium [16]. Most isolates sporulated readily, but a few failed to sporulate and were not subsequently analyzed by test matings. Asci for dissection were obtained by digesting sporulated cultures for 10 min at 30°C in 0.5 mg of zymolyase 100T (US Biological, USA) per ml of 1-M sorbitol. Spores for mutagenesis and mating were obtained by digesting asci for 30 min at 30°C, vortexing on high speed for 2 min, centrifuging, and resuspending in 0.015% Igepal (Sigma, USA) to inhibit clumping.

To facilitate mass mating with tester strains, adenine auxotrophs were obtained from the wild isolates. Appropriate concentrations of spores from each wild isolate to be tested were spread on YPD plates and exposed for 5 s to ultraviolet light (254 nm wavelength) on a minitraniluminator (Foto/Phoresis Model 1-1430, FotoDyne, USA). Plates were incubated for several days at 30°C and examined daily for the presence of red colonies indicative of adenine auxotrophy. Putative adenine auxotrophs were confirmed by picking to synthetic dextrose (SD) minimal agar [16] and SD agar plus 2 g l\(^{-1}\) of adenine hemisulfate. Confirmed auxotrophs were grown, resporulated and the asci digested to completion to obtain spore suspensions for test matings.

2.3. Genetic identification of \textit{Saccharomyces} sensu stricto

Adenine auxotrophs derived from the wild isolates were mass-mated to genetically marked tester strains of \( S. \) \textit{cerevisiae} and \( S. \) \textit{paradoxus} on SD agar. The \( S. \) \textit{cerevisiae} tester, DH46, was a \( \Delta \)leu2 ho MATa strain derived from the laboratory strain Y55 and provided to us by Dr. D. Grieg. Strain Y55 was originally isolated by Ø. Winge from wine grapes in France during the 1930s (J.H. McCusker, personal communication). The \( S. \) \textit{paradoxus} tester, N17-13, was an ho \( MATa \) lys2 derivative of wild strain N17, which was originally collected from Tartastan (former USSR) by G.I. Naumov [1]. Strain N17-13 was provided to us by Dr. E.J. Louis. Prototrophic maters between adenine auxotrophic derivatives of the wild isolates and these tester strains were sporulated and approximately 16 asci of each were dissected on YPD agar to determine ascospore viabilities. Viabilities were scored as the proportion of ascospores yielding colonies visible to the unaided eye after 3 days of incubation at 30°C. Where viable ascospores were obtained, segregation and assortment of both adenine auxotrophy and the tester strain auxotrophy confirmed that the strain analyzed was a mater and not a selfed revertant of the wild isolate.
2.4. Ascospore viabilities and phenotypic tests for heterozygosity in the identified strains

Isolates that were identified by genetic analysis as either *S. cerevisiae* or *S. paradoxus* were sporulated and scored for ascospore viability as described above. In addition, colonies derived from dissected complete asci of these identified strains were examined phenotypically for heterozygosity in utilization/fermentation of four sugars, prototrophy, and homothally/sporulation. Colonies were replica-plated to indicator agars containing the pH-sensitive dye bromothymol blue and the sugars galactose, sucrose, maltose and melibiose [16], to SD agar, and to minimal sporulation agar. Colonies replicated to the indicator medium and SD medium were inspected visually after 2 days at 30°C; colonies replicated to sporulation medium were inspected microscopically after several days for the presence of asci indicative of homothallic selfing and subsequent sporulation.

2.5. Karyotype and Ty1 element hybridization analyses

Karyotypes of monosporic clones derived from the identified strains were analyzed using contour-clamped homogeneous field (CHEF) gel electrophoresis. Two standard laboratory strains of *S. cerevisiae*, S288C [17] and YPH80 (obtained commercially from New England Biolabs, USA), were included in the analysis for comparison, as well as the type strain of *S. paradoxus*, CBS 432 [1]. Preparation of chromosomal DNAs followed a published protocol [18]. Chromosomes were separated on a Bio-Rad CHEF Mapper using the manufacturer’s supplied algorithm for separating DNA fragments within the 220–2200-kb size range. Depurinated and denatured chromosomal DNAs were transferred to positively charged nylon membranes using a vacuum blotter (Bio-Rad, USA, Model 785) and cross-linked with a UV transilluminator. Membranes were probe with a 974-bp internal fragment from the open reading frame of the Ty1 transposable element. The probe was amplified from the laboratory strain S288C with the primer pair 5'-AATAGCTGTGAGTCCAACCGATT-3' and 5'-TTGATTGACCTTTCTTGTGATGATCC-3' and was PCR-labeled with digoxigenin-11-dUTP. Hybridization and detection were carried out using the DIG Nucleic Acid Labeling and Detection Kit (Roche Molecular Biochemicals, USA) according to the manufacturer’s protocol; blots were allowed to develop in color substrate solution for 16 h.

3. Results

3.1. Natural distribution of *S. cerevisiae* and *S. paradoxus*

We collected a total of 84 samples for enrichment from 40 different individual trees and their associated soils: 17 white oaks (*Quercus alba*), 11 red oaks (*Quercus rubra*), five black oaks (*Quercus velutina*), two chestnut oaks (*Quercus prinus*), two American beeches (*F. grandifolia*), one tulip poplar (*L. tulipifera*), one red maple (*Acer rubrum*), and one undetermined oak species. Only the samples associated with oaks yielded putative *Saccharomyces* yeasts as judged by colony and vegetative cell morphology. Of 79 such oak-associated samples processed through enrichment culture, 18 yielded isolates that were highly fertile with either the *S. cerevisiae* or the *S. paradoxus* tester strain. Where fertility in hybrids with both tester species was observed it was always high in one hybrid and very low in the other; in no case was high fertility observed in hybrids with both species.

Table 1 lists all of the strains identified as *S. cerevisiae* or *S. paradoxus* and gives the substrate from which each was isolated. As shown in the table, these two species were identified in roughly equal numbers and from similar substrates. Table 2 gives the number of dissected asci and the proportion of viable spores in hybrids with both testers for each identified strain. Auxotrophic mutations in the tester strains (leu2 and his2) and in the wild isolates (ade1 or ade2) segregated and assorted normally in the viable spores (data not shown). The rare viable spores in interspecific hybrids invariably gave tiny and slowly growing colonies.
3.2. Geographic reproductive isolation

The wild isolates of *S. cerevisiae* and *S. paradoxus* differed in their degree of reproductive isolation from their conspecific European tester strains: for *S. cerevisiae* UY55 fertile hybrids, the average spore viability + S.E.M. was 89 + 1.8%, whereas for *S. paradoxus* UN17-13 fertile hybrids, it was 52 + 8.1%. This difference is highly significant (two-tailed *t*-test of arcsin square root transformed viability proportions: *t* = 10.58; *P* < 0.0001; df = 16). The reduction of hybrid spore viability observed in *S. paradoxus* is not a property of the tester strain, as the *S. paradoxus* tester parent strain N17 itself has a previously observed spore viability of 100% [1]. To address whether hybrid spore viabilities in both species were a property of the wild isolates or a property of the hybrids, we obtained data on spore viabilities in the original diploid wild isolates as shown in Table 3. Average spore viability in the *S. cerevisiae* isolates was 82 + 5.5%, which is not significantly different from that observed in hybrids between these isolates and the *S. cerevisiae* tester, Y55 (paired *t*-test of arcsin square root transformed viability proportions: *t* = 0.78; two-tailed *P* = 0.456; df = 9). Average spore viability in the *S. paradoxus* isolates was 81 + 4.1%, which is significantly higher than that observed in hybrids with the *S. paradoxus* tester parent strain N17-13 (paired *t*-test of arcsin square root transformed viability proportions: *t* = 4.98; two-tailed *P* = 0.0016; df = 7). There was no significant difference in spore viability between the *S. paradoxus* and *S. cerevisiae* isolates themselves.

### Table 3
Spore viabilities in wild strains of *S. cerevisiae* and *S. paradoxus* identified in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Wild isolate</th>
<th>No. of tetrads dissected</th>
<th>Ascospore viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>YPS 128</td>
<td>15</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>YPS 129</td>
<td>16</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>YPS 133</td>
<td>15</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>YPS 134</td>
<td>16</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>YPS 139</td>
<td>16</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>YPS 141</td>
<td>16</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>YPS 142</td>
<td>16</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>YPS 143</td>
<td>16</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>YPS 154</td>
<td>16</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>YPS 163</td>
<td>16</td>
<td>98</td>
</tr>
<tr>
<td><em>S. paradoxus</em></td>
<td>YPS 125</td>
<td>16</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>YPS 138</td>
<td>16</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>YPS 145</td>
<td>16</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>YPS 150</td>
<td>16</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>YPS 151</td>
<td>16</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>YPS 152</td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>YPS 155</td>
<td>16</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>YPS 158</td>
<td>16</td>
<td>83</td>
</tr>
</tbody>
</table>

Spore viabilities were recorded after 3 days of incubation at 30°C.
rose and melibiose was confirmed on SD plates containing these sugars as the sole carbon source.

3.4. Molecular karyotyping and Ty1 element hybridization

Fig. 1 illustrates electrophoretic karyotypes of monosporic isolates from a subset of the 18 wild strains along with those of standard \textit{S. cerevisiae} strains YPH80 and S288C and the type strain of \textit{S. paradoxus}, CBS 432. All isolates displayed chromosome numbers and sizes typical of those previously observed in the \textit{Saccharomyces sensu stricto} group. As expected [9], no consistent differences in karyotype between \textit{S. cerevisiae} and \textit{S. paradoxus} were observed. In contrast to results previously reported for wild \textit{Saccharomyces}, however [9,19], we observed substantial size variation in chromosome XII in both species. Fig. 2 shows profiles of hybridization of the Ty1 probe to chromosomes from monosporic clones of a subset of the wild isolates. Hybridization signal was strong in both the wild and laboratory standard \textit{S. cerevisiae} strains, and visible signal was obtained from most chromosomes. The \textit{S. paradoxus} type strain CBS 432 also exhibited strong hybridization signal on many chromosomes, but the wild \textit{S. paradoxus} strains isolated in this study gave a much weaker signal that was detectable on only a small number of chromosomes. Within the wild isolates of each species, multiple strains appeared to share chromosomal Ty1 hybridization profiles; however, at least two distinct hybridization profiles were distinguishable for each species.

4. Discussion

We have shown that the \textit{Saccharomyces sensu stricto} species \textit{S. paradoxus} and \textit{S. cerevisiae} can be obtained from uncultivated habitats by enrichment culturing in a medium containing 7.6\% (v/v) ethanol. Ethanol enrichment has been used to isolate \textit{S. cerevisiae} from vineyard grapes [20,21], but to our knowledge this is the first report of its successful application in uncultivated habitat. The presence of \textit{S. cerevisiae} in our collection from an uncultivated site is not a local anomaly, as previous studies have reported occasionally finding \textit{S. cerevisiae} in uncultivated habitats in Central Siberia, Japan, Europe [14], North America [6,22], and South America [23,24]. Nonetheless, this is the first time that substantial numbers of isolates of \textit{S. cerevisiae} and \textit{S. paradoxus} have been isolated contemporaneously from the same site.

Somewhat surprisingly, most of our isolates were obtained from oak-associated soils rather than from oak fluxes as usually reported. The numbers of oak soil and oak flux/bark samples that we collected were approximately equal, yet 12 of our 18 identified isolates originated from soil samples, four from oak bark, and only two from fluxes. It is obviously not necessary to sample oak fluxes in order to have a good chance of isolating \textit{Saccharomyces} from uncultivated habitat. Indeed, both \textit{S. cerevisiae} and \textit{S. paradoxus} have previously been isolated from soils, though never in substantial numbers as reported here [19,25–30].
There is an ongoing debate as to whether *S. cerevisiae* is exclusively a domesticated organism [12,20,21,31–33]. Many isolates identified as *S. cerevisiae* have been obtained from damaged grape berries and fermented grape must at wineries practicing natural (no added yeast) fermentation [10,11,13,20,21], and human pathogenic strains have been isolated and studied [34,35], but the species has seldom been obtained from habitats less closely associated with humans. At present it remains impossible to rule out any of three explanations for the presence of *S. cerevisiae* in natural habitats: (1) *S. cerevisiae* is a domesticated organism that occasionally establishes synanthropic natural populations; (2) a wild population of *S. cerevisiae* has recently expanded its range from a single region of origin [36], perhaps in association with humans; (3) there are diverse, globally distributed wild populations of *S. cerevisiae* (like those apparent in *S. paradoxus* that predate domestication and have existed independently all along. Some combination of these scenarios is also possible; for example, there could be ongoing gene flow between domesticated and wild populations of *S. cerevisiae*.

Previous studies have shown that *S. paradoxus* populations from different geographic regions exhibit partial reproductive isolation [2,4–7]. As expected, hybrids between our isolates of *S. paradoxus* and the *S. paradoxus* tester strain N17-13, which is originally from Tartastan, showed significantly reduced fertility compared with the parent strains. However, hybrids between our *S. cerevisiae* isolates and the Y55-derived *S. cerevisiae* tester, which is originally from France, were as fertile as their parent strains. Naumov et al. [22] previously reported similar results for ten *S. paradoxus* and three *S. cerevisiae* strains isolated from multiple North American sites: hybrids between their *S. paradoxus* strains and an *S. paradoxus* tester from Denmark showed an average spore viability of 31.6 ± 3.9%, whereas hybrids between their *S. cerevisiae* strains and *S. cerevisiae* testers from Russia showed an average spore viability of 87.7 ± 1.5%. Perhaps *S. cerevisiae* populations from diverse regions share a far more recent common ancestor than comparable *S. paradoxus* populations and hence have had much less time in which to evolve reproductive divergence.

Published genetic analyses of population structure in *Saccharomyces* are limited to a single allozyme study documenting significant genetic differentiation between European and Far East Asian populations of *S. paradoxus* [5]. That study provided little evidence of intrapopulation variation, but a recent study analyzing multilocus sequence data from a British population of *S. paradoxus* has documented genetic variation and a low but detectable rate of outcrossing (L. Johnson, personal communication). Our karyotype and Ty1 element hybridization data indicate that both *S. paradoxus* and *S. cerevisiae* at the Tyler site are also genetically variable. First, in contrast with some
previous studies of wild strains collected from numerous regions [6,9], we observed substantial variation in the size of chromosome XII among our wild isolates (Fig. 1). Size variation in chromosome XII is commonly observed between *S. cerevisiae* wine strains and has been attributed to length variation in the rDNA gene cluster present on this chromosome [37,38]. Second, although several of our wild isolates within both species shared Ty1 chromosomal hybridization profiles, at least two different hybridization profiles were present in each species. (The possibility that strains sharing hybridization profiles have different Ty1 insertion sites at the nucleotide level also cannot be ruled out.) Taken together, our data on chromosome XII size variation and Ty1 hybridization indicate at least five unique genotypes within the isolates of each species from the Tyler site.

*S. cerevisiae* and *S. paradoxus* at the Tyler site differed markedly in Ty1 hybridization profile and signal intensity (Fig. 2). The signal intensity difference may reflect Ty1 sequence divergence or a copy number difference between the two species; at present we cannot say which. Whatever the cause of the differences in Ty1 hybridization, their presence supports the conclusion that these two populations are reproductively isolated from one another.

All *S. cerevisiae* and *S. paradoxus* strains isolated from the Tyler site yielded identical spore phenotypes: all spores from multiple complete asci in each isolate were prototrophic, homothallic and sporulation proficient, able to ferment or utilize sucrose and galactose, and unable to ferment or utilize maltose and melibiose. Analyses of a wider range of such phenotypic characters in vineyard strains indicated high levels of heterozygosity [10–13]. Because of differences in the way data were reported, only one statistical comparison between those previous studies and ours is possible: Mortimer et al. ([11], Table 1) found that eight of 28 *S. cerevisiae* strains isolated from the Emilia Romagna region of Italy were heterozygous for one or more of the characters homothally, sucrose fermentation, maltose fermentation, and galactose fermentation. Although this suggests a higher overall proportion of heterozygous strains in these vineyard isolates, it is not significantly different from our finding of zero out of 10 heterozygous *S. cerevisiae* strains at the Tyler site for these same four phenotypic characters (two-tailed Fisher exact test, \( P = 0.082 \)). (Mortimer et al. assayed their strains for fermentation capacity using bromthymol blue indicator agars rather than the standard Durham tube method [39]. Nonetheless, because our strains were assayed using the same indicator agar method, the comparison is a legitimate one.) It is noteworthy that most vineyard *S. cerevisiae* strains are able to ferment or utilize maltose, whereas all of our strains from uncultivated sites were unable to ferment or utilize maltose. Variation in maltose fermentation capacity has been observed previously in wild and cultivated isolates of *S. cerevisiae* and *S. paradoxus* [40].

*S. cerevisiae* and *S. paradoxus* show almost indistinguishable profiles for fermentation and assimilation reactions and other characteristics [3,32] and their life cycles are identical as far as is known. Our finding that these species coexist in similar habitat thus raises an ecological question: do *S. cerevisiae* and *S. paradoxus* coexist stably in the wild, and, if so, how?

**Acknowledgements**

P.D.S. thanks G.I. Naumov and E.S. Naumova for introducing him to the genetic identification of species in *Saccharomyces*. We are grateful to E.J. Louis and D. Greig for the tester strains, M.-A. Lachance for advice on the enrichment protocol, J. Sweeney for technical assistance, M. Bucan for use of the CHEF Mapper, T. Pugh for advice on CHEF interpretation, and L. Johnson for sharing data before publication. We also thank Richard Colbert, Director of the Tyler Arboretum, for permission to collect on the arboretum grounds. We thank H. Kuehne, M.-A. Lachance, and G.I. Naumov for comments on the manuscript. This research was supported by startup funds to P.D.S. from the University of Pennsylvania and by a grant from the University of Pennsylvania Research Foundation to P.D.S.

**References**


[10] Valviali, D., Barberio, C., Casalone, E., Pinzauti, F., Sebastiani, E.,


