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FINAL REPORT

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*Debaryomyces hansenii*: a model system for marine molecular biology.

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**OBJECTIVE:** The major goals for this award were : (1) Construction of a plasmid vector for the transformation of *Debaryomyces hansenii* and its transformation, (2) Mutagenesis of *D. hansenii* (3) Isolation and sequence analysis of a gene ( in this case the SSU rRNA gene ) and its comparison to this gene from other organisms, (4) initial experiments on the isolation of regulatory sequences from inducible genes from *Debaryomyces*.

**ACCOMPLISHMENTS:** In an attempt to construct a vector for the transformation of *Debaryomyces*, two autonomously replicating sequences (ARS) have been isolated. This was accomplished utilizing an auxotrophic strain of *Saccharomyces cerevisiae* (*Ura 3<sup>-</sup>*) and the *URA 3* gene in the plasmid pUC19 as a marker. Two plasmids containing fragments of *Debaryomyces* genomic DNA were autonomous in *Saccharomyces*. These contained inserts of 0.4 kbp and 1.4 kbp respectively. The smaller insert was used for further analysis. The ARS activity resides in 0.15 kbp of the insert and a new plasmid was constructed with the smaller insert. Sequence analysis of the clone revealed it to be A+T rich ( % A+T = 80.76%). It also contains a 12 bp consensus sequence shown to be present in all ARS functional in *Saccharomyces*.

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For the use of the constructed plasmids *Ura 3<sup>-</sup>* mutants had to be generated. Attempts were thus made to generate these mutants by chemical mutagenesis. The *Ura 3<sup>-</sup>* phenotype was selected by the negative selection utilizing 5- fluoro orotic acid. Even though a large number of mutants were generated by this technique they were very unstable. All the mutants that were isolated reverted to their original phenotype. Dr. L. Adler of the University of Goteborg has recently sent me *D. hansenii* strain J-26 which he has successfully used in mutant generation. He has also agreed to generate auxotrophic as well as salt intolerant mutants of his strain.

Transformations of *Debaryomyces* have been attempted with plasmid pLG90 ( kindly supplied by Dr. L. G. Gritz, Applied Biotechnology Inc. ). This plasmid contains a *Saccharomyces* ARS and bacterial hygromycin resistance under the control of the yeast *CYC-1* promoter. The plasmid is functional in providing hygromycin resistance in both *E. coli* and *Saccharomyces*. Several transformation techniques were attempted and low frequencies of transformation of *Debaryomyces* to hygromycin resistance were obtained with the protoplasting technique. The transformants are now being analyzed for the presence of the plasmid. This result, in addition to fact that the ARS sequence from *Debaryomyces* is very similar to that of other organisms such as *Candida albicans*, *Phycomyces blakesleeanus* and *Saccharomyces* indicates that the plasmid that we have constructed will be successful in transforming *Debaryomyces*. The experiments will be performed as soon as stable mutants are generated.

The SSUrRNA from *Debaryomyces* was amplified using polymerase chain reaction and cloned into the polylinker cloning site of the plasmid pUC19. This was then sequenced and the sequences were aligned to organisms across kingdoms based on 23 taxa. *Debaryomyces* clusters among the yeasts but appears more closely related to *Candida albicans* than the group represented by *S. cerevisiae*, *K. lactis* and *T. delbrukii*. *Candida glabarata* clusters more closely to *S. cerevisiae* than either do to *C. albicans*. The fission

yeast *S. pombe* seems to be distantly related to the other yeasts. A second analysis expanding the yeast/fungi group was then performed. This analysis tended to separate *C. albicans* and *D. hansenii* from the other cluster of yeasts. The results indicate that even though *Debaryomyces* is indistinguishable from *Saccharomyces* morphologically and ultrastructurally, it may actually be closer to *Candida*. Greater success could possibly be achieved by adapting molecular techniques that have been developed for *Candida* rather than *Saccharomyces*. It also appears from these analyses that the capacity of these organisms to tolerate high salt is independent of phylogenetic affiliations based on SSU rDNA analysis.

The rationale behind the isolation of regulatory sequences of inducible genes from *Debaryomyces* is that it would help in the construction of expression vectors. This in turn would help in the expression of foreign genes in this organism. As a first step towards this attempts have been made to identify protein changes in *Debaryomyces* in response to high salinities. The experiments involved the radioactive labeling of proteins with <sup>35</sup>S L-Methionine *in vivo* after the addition of sodium chloride. The incorporation of radioactivity in the newly synthesized proteins after the addition of salt decreased ten fold. By eight hours of incubation, the cells completely recovered incorporating comparable amount of radioactivity. Analysis of soluble proteins from the salt treated cultures indicates the presence of a 150 kda. protein in salt treated cultures which is not present in cultures grown in the absence of salt. We are now in the process of purifying this protein with the purpose of raising antibodies.

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List of Publications ( published, in press and submitted ) during the period of support:

1. N. S. Govind, K. L. MacNally and R. K. Trench (1992) Isolation and sequence analysis of the small subunit RNA gene from the euryhaline yeast *Debaryomyces hansenii*. Submitted to *Current Genetics* ( one copy of the manuscript enclosed ).
2. N. S. Govind and A. T. Banaszak (1992) Isolation and characterization of an autonomously replicating sequence (ARSD) from the marine yeast *Debaryomyces hansenii*. Submitted to *Molecular Marine Biology and Biotechnology* ( manuscript enclosed ).
3. R. Iglesias-Prieto, N. S. Govind and R. K. Trench (1992) Apoprotein composition and spectroscopic characterization of the water soluble peridinin-Chlorophyll *a*-proteins from three symbiotic dinoflagellates. *Proceedings of the Royal Society of London B* in press ( copy of galley enclosed, five copies of the reprint will be submitted upon receipt ).
4. L. A. Sadler, K. L. MacNally, N. S. Govind, C. F. Brunck and R. K. Trench (1992) The nucleotide sequence of the small subunit ribosomal RNA gene from *Symbiodinium pilosum*, a symbiotic dinoflagellate. *Current Genetics*, in press ( manuscript enclosed, five copies of the reprint will be submitted upon receipt ).
5. J. L. Matta, N. S. Govind and R. K. Trench (1992) Polyclonal antibodies against iron-superoxide dismutases from *E. coli* cross-react with superoxide dismutases from *Symbiodinium microadriaticum* (Dinophyceae). Communicated to *Journal of Phycology* ( Copy of manuscript enclosed ).
6. R. E. Kochevar, J. J. Childress and N. S. Govind (1991) Carbonic anhydrase in the hydrothermal vent tube-worm *Riftia pachyptila* Jones. *American Zoologist* 31: 71A ( five copies enclosed).

**ISOLATION AND SEQUENCE ANALYSIS OF THE SMALL  
SUBUNIT RIBOSOMAL RNA GENE FROM THE EURYHALINE YEAST  
*DEBARYOMYCES HANSENI***

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**Summary.** The small subunit ribosomal RNA gene (SSU rDNA) from the euryhaline yeast *Debaryomyces hansenii* has been isolated and sequenced. After appropriate alignment of this sequence with SSU rDNA sequences from 30 other taxa, phylogenetic reconstruction using distance matrix and maximum parsimony methods indicates that *D. hansenii* is most closely affiliated with *Candida albicans*, and occurs in the cluster of the yeasts *Saccharomyces cerevisiae*, *Torulaspota delbruekii*, *Candida glabrata*, and *Kluyveromyces fragilis*. It appears that the capacity to tolerate high salt is independent of phylogenetic affiliations based on SSU rDNA analyses.

**Key words:** Small subunit ribosomal RNA; SSU rDNA sequence; phylogenetic reconstruction; yeasts; *Debaryomyces hansenii*.

## Introduction

The sequences of small subunit ribosomal RNA (SSU rRNA) genes has been used extensively in recent times for the reconstruction of phylogeny (Medlin et al. 1988; Dams et al 1988; Hendricks et al. 1991; for review see Schlegel, 1991). New technologies have been developed to facilitate the isolation and characterization of such genes both from isolated microorganisms and from a mixture of organisms in a clinical sample (Medlin et al, 1988; Gobel et al. 1987). Polymerase chain reactions (PCR) utilizing a thermostable DNA polymerase (Saiki et al. 1988) have been successfully employed in the isolation of SSU rDNA from genomic DNA, using specific primers (Medlin et al. 1988 ). Although there are reports on the analysis of yeasts and fungi based on 16S-like rDNA sequences, there are no reported analyses of marine or euryhaline yeasts and fungi.

The euryhaline yeast *Debaryomyces hansenii* has the capacity to tolerate NaCl concentrations of 0 - 24% (Norkans, 1966). While a majority of the studies on this organism have concentrated on the mechanism of halotolerance (Adler, 1986), there is little information available on the relationship of *D. hansenii* to other yeasts and fungi. Recently we have been attempting to standardize various recombinant DNA techniques for this organism. As a first step towards this, and to determine if a correlation exists between phylogeny and halotolerance, we describe here the isolation and sequence analysis of the SSU rDNA from *D. hansenii*, and present a reconstructed phylogeny inferred by comparison with homologous sequences from other yeasts and protists. The results of our analyses indicate that *D. hansenii* is most closely affiliated with *Candida albicans* and occurs in the cluster of the yeasts *Saccharomyces cerevisiae*, *Torulospira delbruekii*, *Candida glabrata* and *Kluyveromyces lactis*, but it appears that the capacity to tolerate high salt is independent of phylogenetic affiliations based on SSU rDNA analyses.

## Materials and Methods

*Strains and Maintenance.* *Debaryomyces hansenii* NRRL Y-7426 was obtained from the United States Department of Agriculture, Peoria, Illinois. Conditions for the cultivation and maintenance of this organism were identical to the ones used for *Saccharomyces cerevisiae* (Campbell, 1988). Plasmids were constructed, propagated and amplified in *Escherichia coli* strain DH5  $\alpha$  [ $F^-$ , *endA1*, *hsdR17*,  $r_k^-$ ,  $m_k^-$ , *supE44*, *thi-1*,  $\lambda^-$ , *recA1*, *gyrA96*, *relA1*,  $\Delta(\text{argF}^-$ , *lacZYA*)U169,  $\Delta 80\text{dlacZ}$   $\Delta$ M15].

*DNA isolation, purification and amplification of SSU rDNA.* DNA from *D. hansenii* was prepared by the method of Cryer et al. (1975). Plasmid DNA was prepared by the method of Ohtsubo et al. (1978). Transformation of *E. coli* was performed by the method of Mandel and Higa (1970). Electrophoretic separation and purification of DNA was conducted as described by Maniatis et al. (1982). Restriction enzymes were purchased from Promega Biotech. and used according to the manufacturer's specifications. The primers, buffer and amplification conditions used for the SSU rDNA from *D. hansenii* have been described by Medlin et al. (1988). The primers carry the restriction sites EcoRI and Sal I at the 5' end and Sma I, BamH I and HindIII at the 3' end to facilitate cloning of the amplified fragments. Amplifications were performed utilizing a Perkin Elmer Cetus DNA Thermal Cycler.

*Cloning and sequencing.* The PCR products were purified by electrophoresis in (low melting) agarose gel, digested with Sal I and Hind III and cloned into the polylinker site of the plasmid pUC19. Synthetic oligonucleotide primers of well conserved regions of eukaryotic 16S-like rRNA genes (Elwood et al. 1985) were used to sequence the clone by the dideoxynucleotide chain termination sequencing method of Sanger et al. (1977).

*Computer Analysis of the SSU rDNA sequences.* The complete SSU rDNA sequences of *Debaryomyces hansenii* (NRRL Y-7426) and 30 other taxa (listed below), were aligned on the



basis of similarity and secondary structure, with the initial alignment provided by the multiple alignment program CLUSTAL (Higgins and Sharp, 1988, 1989), and the final alignment adjusted "by eye". The organisms used for comparison were as follows: *Candida albicans* (Hendriks et al. 1989), *C. glabrata* (Wong and Clark-Walker, 1990), *Saccharomyces cerevisiae* (Rubtsov et al. 1980), *Torulospira delbrueckii* (Hendriks et al. 1990), *Aspergillus fumigatus* (Sogin, pers. commun.), *Neurospora crassa* (Sogin et al. 1986), *Schizosaccharomyces pombe* (Sogin, pers. commun.), *Pneumocystis carinii* (Edman et al. 1988), *Blastocladiella emersonii* (Förster et al. 1990), *Achlya bisexualis* and *Ochromonas danica* (Gunderson et al. 1987), *Prorocentrum micans* (Herzog and Maroteaux, 1986), *Cryptocodium cohnii* and *Saccocystis muris* (Gajadhar et al. 1991), *Plasmodium berghei* (Gunderson et al. 1986), *Oxytricha nova* (Elwood et al. 1985), *Paramecium tetraurelia* (Sogin and Elwood, 1986), *Dictyostelium discoideum* (McCarroll et al. 1983), *Physarum polycephalum* (Johansen et al. 1988), *Giardia lamblia* (Sogin et al. 1989), *Sulfolobus solfataricus* (Olsen et al. 1985), *Kluyveromyces lactis* (Maleszka and Clark-Walker, 1990), *Penicillium noratum*, *Coccidioides immitis* and *Mucor racemosus* (Sogin, pers. commun.), *Aureobasidium pullulans*, *Colletotrichum gloeosporioides*, and *Athelia bombacina* (Illingworth et al. 1991), *Leucosporidium scottii* (Hendriks et al. 1991).

Non-ambiguously aligned positions were analyzed by computation of 50 bootstrap resampled DNA distance matrices, using the maximum likelihood metric (DNADIST). Resolution of the matrices was accomplished by Fitch and Margoliash's algorithm (FITCH), where input order of species added to the topology under construction was randomized ten times. A consensus tree across 500 independent trees was generated using CONSENSE. Using the same alignment, 50 rounds of bootstrap resampled maximum parsimony (DNABOOT) were also conducted. All programs mentioned are from the PHYLIP 3.3 package (Felsenstein, 1990).

## Results and Discussion

DNA samples after PCR were separated on a 1% agarose gel and the approximate size of the amplified band was determined to be 1.8 kbp (data not presented). This was the only promi-

ment product of the PCR. Utilizing this strategy for amplification, fewer than one error per 15,000 positions have been observed in the rRNA sequences (Gelfand and White, 1990). The size of the amplified product is very similar to the sizes obtained for the marine diatom *Skeletonema costatum* and the ascomycete *Kluyveromyces lactis* (Medlin et al. 1988). On the other hand *Plasmodium falciparum* gives a 16S-like rRNA of approximately 2.0 kbp (McCutchan et al. 1988). The amplification procedure yielded approximately 5 µg of product and this quantity was sufficient to clone into pUC19. The SSU rDNA was cloned into the *Sal*I and *Hind*III sites of the polylinker sequence of the plasmid pUC19 and the resulting plasmid was called pGN5. Double stranded DNA sequence analysis was performed on the cloned region, and the results are presented in Figure 1. The nucleotide sequence of the SSU rRNA gene from *Debaryomyces hansenii* shows a size of 1798 nucleotide pairs including both the 5' and the 3' primers used for the amplification.

Fig. 1 also shows the alignment of the SSU rDNA from *D. hansenii* with homologous sequences from six other yeasts. Using this alignment, phylogenetic reconstruction was conducted using maximum parsimony (DNABOOT) and distance matrix (Maximum Likelihood) methods. The resulting topologies of the trees generated by both methods were in good agreement.

Fig. 2 shows a reconstructed phylogeny across Kingdoms based on 23 taxa (with *Physarium polycephalum*, *Giardia lamblia*, and *Sulfolobus solfataricus* used as outgroups). The relationships of the ciliates, the apicomplexans, and the dinoflagellates are as has been established by others (Lenaers et al. 1989; Hendriks et al. 1989, 1991; Gajadhar et al. 1991; Sadler et al. 1991). The relationships of the fungi to other groups is also consistent with those indicated by Hendriks et al. (1989, 1991), and Schlegel (1991). *D. hansenii*, a budding yeast, clearly clusters among the yeasts, but appears more closely related to *Candida albicans*, a dimorphic budding yeast, than to the group of yeasts represented by *S. cerevisiae*, *K. lactis*, *T. delbruekii*, other budding yeasts. By this criterion, *C. glabrata* may appear to be out of place. In our analyses, and in those of Barns et al. (1991), *Candida* (= *Torulopsis*) *glabrata* clusters more closely to *S. cerevisiae* than either do to *C. albicans*. However, the distances among these taxa are quite short. The fission yeast *S. pombe* is distantly related to the other yeasts.

In order to expand the analysis of the yeasts/fungi groups, the same set in Fig. 1a (with the exception of *A. pullulans*) were combined with six other fungal and yeast taxa (for a total of 19 species). One thousand six hundred and ten unambiguously aligned positions were subjected to the same series of programs used above, this time using *Ochromonas danica* and *Achlya bisexualis* as outgroups. Again, DNA distance and bootstrap resampled maximum parsimony resulted in consistent topologies. This second analysis, Fig. 3 tended to separate *D. hansenii* and *C. albicans* from the other cluster of yeasts including *T. delbrueckii*, *C. glabarata*, *S. cerevisise* and *K. lactis*, but even so, the yeasts cluster quite separately from the remainder of the fungi. Our analysis places *Coccidiodes immitis* among the ascomycetes. The systematics of this fungus is ambiguous; in the saprophytic form it is regarded as a zygomycete, while in the parasitic form it is regarded as an ascomycete (Rippon, 1988).

According to Molitoris and Schaumann (1986), based on the definition provided by Kohlmeyer and Kohlmeyer (1979), there are about 500 known marine fungi. Of these, about 180 are yeasts, in the genera *Candida*, *Kluyveromyces*, *Rhodotorula*, *Trichosporum*, *Pichia* and *Debaryomyces*. Although we have not analyzed examples from all these genera, it appears that the capacity to tolerate high salt is independent of phylogenetic affiliations based on SSU rDNA analyses.

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## Figure legends

Figure 1. The complete sequence of the SSU rDNA from *D. hansenii* aligned to homologous sequences from six other fungal taxa. Abbreviations: D.h., *Debaryomyces hansenii*; C.a., *Candida albicans*; K.l., *Kluyveromyces lactis*; S.c., *Saccharomyces cerevisiae*; C.g., *Candida glabrata*; T.d., *Torulospora delbrueckii*; S.p., *Schizosaccharomyces pombe*.

Figure 2. An across-Kingdom tree based on distance matrix analysis with *Physarum polycephalum*, *Giardia lamblia*, and *Sulfolobus solfataricus* used as outgroups. Distances were calculated using a maximum-likelihood estimate for transition/transversions based on actual base frequencies for 1487 unambiguously aligned positions with 50 bootstrap resamplings of the data set. Each bootstrap-resampled distance matrix was resolved into a topology ten times by randomizing input order of species added by Fitch and Margoliash's method. A consensus tree was generated across 500 trees with one having the same topology as the consensus tree being shown.

Figure 3. A "fungal" tree using *Ochromonas danica* and *Achlya bisexualis* as outgroups. The methodology employed was the same as that used to generate the tree shown in Fig. 2 except 1610 unambiguously aligned positions were used for the distance calculations.

D.h. GTCGACAACCTGGTGGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCCATGCTAAGTATAAGCAA-TTTATACAGTGAAACTGCCAA  
C.a. -----T.T.....  
K.l. -----T.T.....  
S.c. -----T.T.....  
C.g. -----T.T.....  
T.d. -----T.T.....  
S.p. -----T.....T.G.T.....

D.h. TGGCTCATTAAATCAGTTATCGTTTATTGATAGTACCTTTACTACTTGG-ATAACCGTGGTAATCTAGAGCTAATACATGCTAAAAATCCCGACTGT-  
C.a. ....T.....  
K.l. ....T.....T.T.....T.....T.....C.....  
S.c. ....T.....A.....T.....T.....T.....C.....  
C.g. ....T.....A.....T.....T.....T.....T.....CTC.....  
T.d. ....T.....A.....T.....T.....T.....T.....CT.....  
S.p. ....CA.....-GACT.T

D.h. -TTGGAAGGGATGTATTTATTAGATAAAAAATCAATGCTTTTCGGAGCTCT-TTGATGATTCATAATAAGCTTTTCGAAATGCCATGCGCTTGTGCTGGCAG  
C.a. ....C..CG..--.....GA.....  
K.l. ....A.....TC..CG.A--..C.....GA.....  
S.c. ....A.....TC..CGCA--.....GA.....  
C.g. ....A.....TC..CG.A--..T.....GA.....  
T.d. ....A.....TC..CG.A--.....GA.....  
S.p. T.....C.....C.-...GCT.T.T..G..G.....C..C..GA

D.h. TGGTTCATTCAAATTTCTGCCCTATCAACTTCGATGGTAGGATAGTGGCTACCATGGTTTCAACGGGTAACGGGGATAAGGGTTGCGATTCCGGAGAG  
C.a. ....  
K.l. ....  
S.c. ....  
C.g. ....  
T.d. ....  
S.p. ....A.....T.....T.....

D.h. GGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGGCCCAATTACCCAATCCCGACAC-GGGGAGGTAGTGACAATAAATAACGATACAGG  
C.a. ....TT.A.....  
K.l. ....TA.TT.A.....  
S.c. ....TA.TT.A.....  
C.g. ....T.....A.....  
T.d. ....TA.T.A.....  
S.p. ....G.....A..G....

D.h. GCC-TTTCGGGTCTTGTAAATGGAATGAGTACAATGTAATACCTTAACGAGGAACAATGCGAGGGCAAGCTCTGGTCCAGCAGCCGGGTAATCCAGC  
C.a. ...C..T.....  
K.l. ...CA.....C.....  
S.c. ...CA.....  
C.g. ...CA.....A.....  
T.d. ...CA.....  
S.p. ...C.....

D.h. TCCAATAGCGTATATTAAGTTGTTGCCAGTTAAAAAGCTCGTAGTTGAACCTTGGGCTTTTGGCCGGTCC---GCCTTTTGGC-GAGTACTGGAC-  
C.a. ....A.....T.....C.....AT.....C..AT.C.....  
K.l. ....G.....T.....TC.....T.....A.....A.-..TC.C.C.....TTT  
S.c. ....T.....CC.....AT.....--TC.T.....TT  
C.g. ....T.....C..G.A.....AT.....--TC.T.....AT  
T.d. ....T.....C.....AT.....--TC.T.....TTT  
S.p. ....T.....A..C.....C.A.T.....GGC-----G.TCTT.T.GAGC---

D.h. -CCAACCGAGCCTTTCCTTCTGGCTAACCTTTGCCCTTGTGGTGTGTTG---CGGAACCGAGACTTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGC  
C.a. ...G.....TAGC.A..A.....  
K.l. T-...GAT.....G.ACT.....G---CAG.....  
S.c. T...G.G.....GA.T.....CTC..GG--.....  
C.g. .G..C..G.....CCA.T.....C---GG.....  
T.d. ...G.....GG.T.....CC..G.....  
S.p. ....GTA.G.....C.....

D.h. --CT-TTCTCGAATATATTAGCATGGAATAATAGAATAGGACGTTATGCTTCTATTGTTGGTTTCTAGGACCATGTAATGATTAAATAGGACCTGT  
C.a. ....G.....  
K.l. .GAAA-.....G.....  
S.c. .G.A.....  
C.g. .G.A.....G.....  
T.d. .G.A.....  
S.p. AAG.T.....C.....A.....G.....GC.....T-A..

D.h. CCGGGGCATCAGTATTCAGTTGTC-AGAGCTGAAATTCCTGGATTACCTGAAGACTAAGTACTGCGAAAG-ATTTCGCAAGGACGTTTTCATTAAATCAAG  
C.a. ....T.....A.....G.A.....TA.....A.....  
K.l. ....A.....TAT.....C.....  
S.c. ....G.....A.....TAT.....C.....  
C.g. ....A.....TAT.....C.....  
T.d. ....A.....TAT.....C.....  
S.p. ....TC.....A.....TAT.....G.....T.....



D.h. AAGGAAAGTTAGGGGATCGAAGATGATCAGATACCGTCTAGTCTTAACCATAACTATGCCGACTAGGGATCGGGTGTGTTCTTTTTTTGACGGACT  
C.a. ....T.....A.....A.  
K.l. ....G.....C..A...C...  
S.c. ....T.G.....G.....AA...C...  
C.g. ....G.....AG...C...  
T.d. ....G.....AC...C...  
S.p. ....C.....CAA...TCA...A.C...TTG..

D.h. CGGCACCTTACGAGAAATCAAAGTCTTTGGGTTCTGGGGGAGTATGGTCCCAAGGCTGAAACTTAAGGAATTGACCGAAGGGCACCACCA-GGAGTGG  
C.a. ....  
K.l. ....  
S.c. ...T.....T.....  
C.g. ....  
T.d. ....  
S.p. ....C.....A.T.....

J.h. AGCCTGGG-CCTAATTTGACTCAACACGGGAACTCACCAGTCCAGACACAATAGGATTGACAGATTGAGAGCTCTTCTGATTTGTGGTGGTG  
C.a. ....G.....  
K.l. ....  
S.c. ....GC.....  
C.g. ....  
T.d. ....  
S.p. ....G.....T.G.....C.A.....

D.h. GTGCATGGCCGT-CCTAGTTGGTGGAGTGATTGCTCTGCTTAATTCCGATAACGAACGAGACCTTAACCTACTAAATAGTCTGCTAGCTTTGCTGGT  
C.a. ....A.....  
K.l. ....G.T...G..AC...C...  
S.c. ....T..C.....G.....A.....  
C.g. ....G.....A.....  
T.d. ....G.....A.....  
S.p. ....G.....

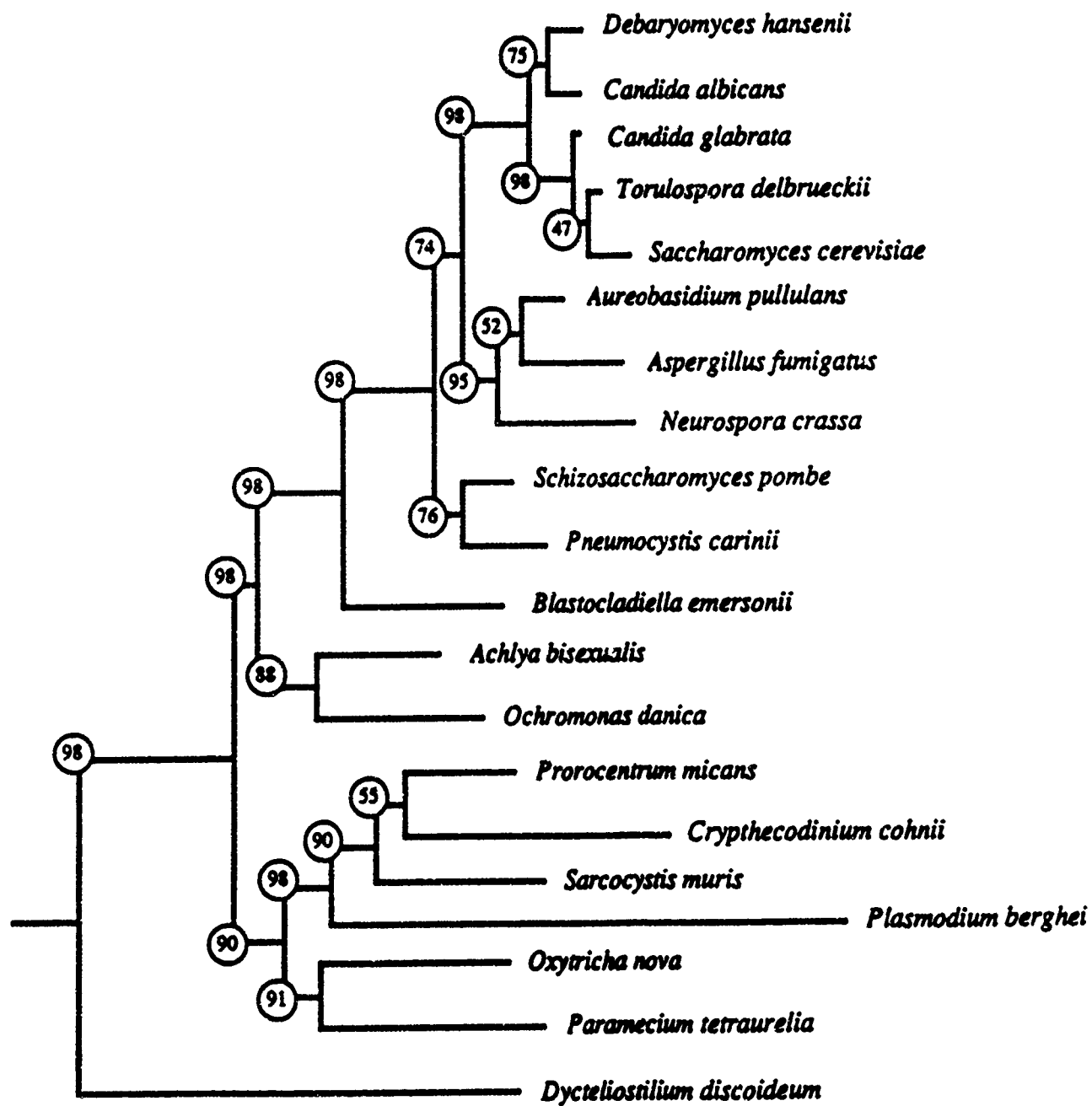
D.h. ATAGTC-----ACTTCTTAGAGGGACTATCGATTTCAGTGGATGGAAGTTTGAGCCAATAACAGGCTCTGTGATGCCC  
C.a. ....C.C.....  
K.l. -.GACT.....G.....C.....  
S.c. -.TC.....G.....C.....  
C.g. -.GTC.....G.....C.....  
T.d. -.TC.....G.....C.....  
S.p. ---CTGGATCAGCCATTTGGCTGATCATTAG-.....T.GCA.A..C.A.....

D.h. TTAGA-CGTTCTGGGCGGACGGCGCTACACTGACGGAGCCAACGAGTATTAA-----CCTTGGCCGAGAGGCTCTGGGAAATCT  
C.a. ....G.....G.....  
K.l. ....G.....CA-.....C.T.....  
S.c. ....A.....G.....C.A-.....T..T.....  
C.g. ....G.....C.A-.....T..T.....  
T.d. ....G.....C.A-.....T.....  
S.p. ....T-.....TGA..AAAATCTTTGATTTTAT.....GA.....T.....

D.h. TGTGAAACTCCGTCGCTGGGATAGACCATGTAATATTGCTCTTCAAGGAGGAATTCCTAGTAGCCCAAGTCAATCACTTGGCTTGATTACGTC  
C.a. ....G.....  
K.l. ....C.....  
S.c. ....  
C.g. ....  
T.d. ....  
S.p. ...T.....C.....A.....

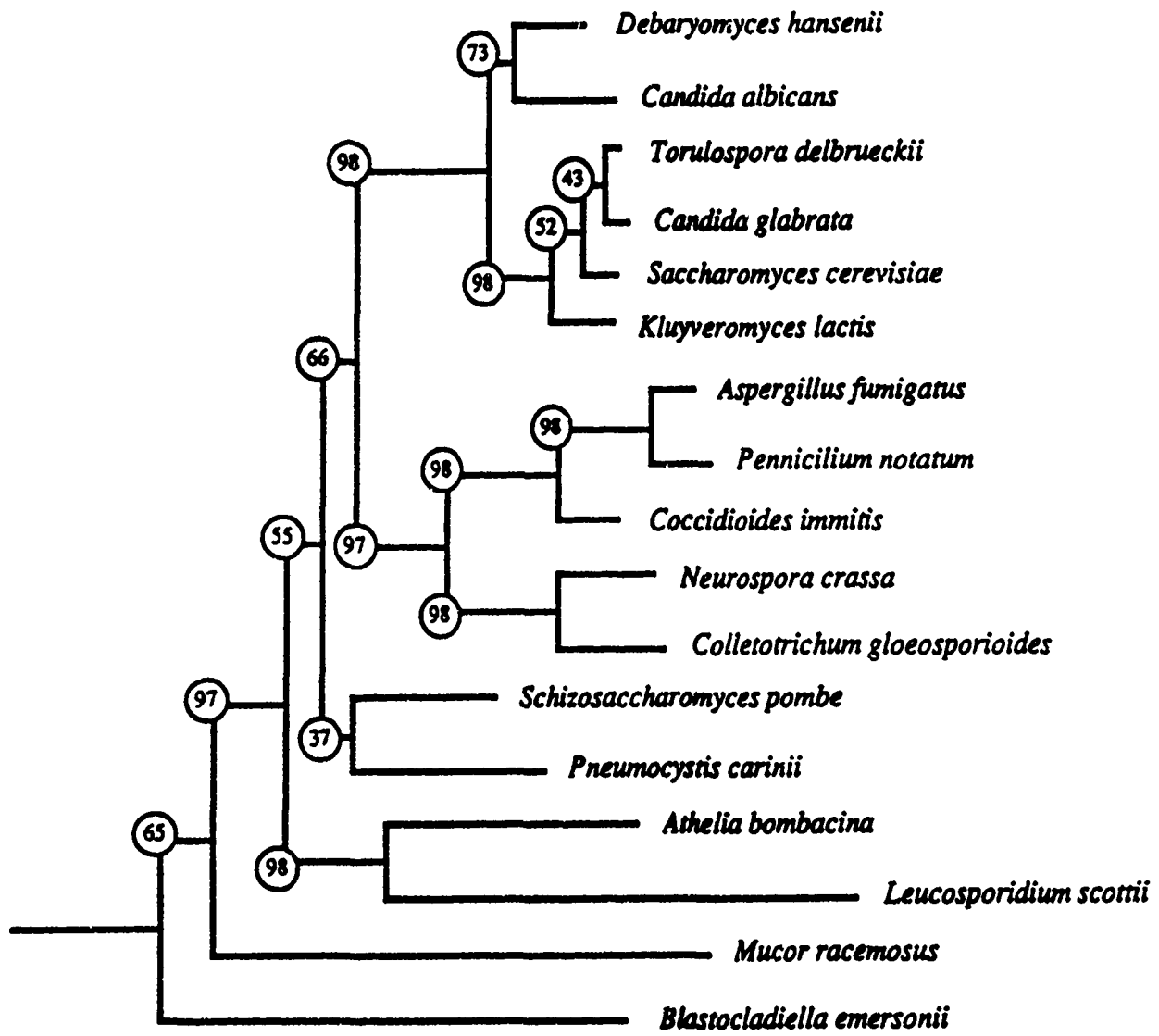
D.h. CTGCCCTTTGTACACACCCCGCTCCCTACTACCGATTGAAATGGCTTAGTGAAGCCCTCCGGATTGGTTTAAAGAAAGGGGCAACTCCATCTT----GGA  
C.a. ....GGA.....TC.....  
K.l. ....G.....A...T.C..G.....C...A..  
S.c. ....G.....A...CT.C..G.....C...A..  
C.g. ....G.....A...CT.C..G.AG.....G.....CT.C...A..  
T.d. ....G.....A...CT.C..G.....C...A..  
S.p. ....T.....C..GTTCT.CT.....GG.G---GAAACATT

D.h. ACCGAAAAGCTGCTCAAAGTTCCTCATTAGAGGAAGTAAAGTCCATAACAAGGTTTCCGTAAGTGAACCTGCAGAAGGATCAAGCTT  
C.a. ....G.....G.....T--.A  
K.l. G.GA.G..T.....C.....G.....T--.A  
S.c. G.G..G..TT..A.....C.....G.....T--.A  
C.g. G.G..G..T.....C.....G.....T--.A  
T.d. G.G..G..T.....C.....G.....T--.A  
S.p. G...G..T..A.....TTA--



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Isolation and characterization of an autonomously  
replicating sequence (ARSD) from the marine  
yeast *Debaryomyces hansenii*

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Running Title: ARS from *D. hansenii*

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### Abstract

The marine yeast *Debaryomyces hansenii* is known to tolerate salinities ranging from 0% - 24%. As a first step toward the molecular analysis of halotolerance in this organism we report here the isolation of an autonomously replicating sequence (ARS) and its use in the construction of a shuttle vector. The ARS from *D. hansenii* (ARSD) is 0.4 kbp long and the function rests in 0.13 kbp of the sequence. Sequence analysis of ARSD shows strong homology to ARS from other organisms including a 12 bp consensus sequence common to all ARS functional in *Saccharomyces cerevisiae*.

**Key Words:** Autonomously replicating sequences, *Debaryomyces hansenii*, halotolerance, marine yeast.

## Introduction

The marine occurring yeast *Debaryomyces hansenii* may provide an ideal model system for studying the mechanisms of salt tolerance in marine organisms. *D. hansenii* is strongly halotolerant with the ability for growth in 0% - 24% sodium chloride (Adler, 1986). When grown in high salt conditions, this organism extrudes sodium and selects for potassium uptake (Norkans and Kylin, 1969; Hobot and Jennings, 1981). Polyhydroxy alcohols (polyols) are also produced and accumulated within the organism to counterbalance the decreased osmotic potential of the environment (Adler and Gustafsson, 1980; Gustafsson and Norkans, 1976). The two major polyols synthesized are glycerol during the exponential growth phase (Gustafsson and Norkans, 1976) and arabinotol during the stationary phase (Adler and Gustafsson, 1980).

When the salinity of the growth medium is increased, there is a corresponding proportional increase in the intracellular glycerol content in growing cells (Gustafsson and Norkans, 1976). In the presence of high concentrations of sodium chloride, *D. hansenii* retains glycerol intracellularly more readily than *Saccharomyces cerevisiae* (Larsson and Gustafsson, 1987). These authors have suggested that the capacity to regulate glycerol metabolism allows *D. hansenii* to optimize growth under conditions of high salinity. Using this mechanism, *D. hansenii* can tolerate more readily far higher concentrations of sodium chloride than can *S. cerevisiae* (Norkans, 1966; Hobot and Jennings, 1981).

Recently, we have initiated studies aimed at understanding the genetic regulation of halotolerance in this organism. As a first step towards this we describe here the isolation of an autonomously replicating sequence from *D. hansenii* (ARSD) and its use in the construction of a shuttle vector. To our knowledge this study represents the first of its kind with marine eukaryotes.

## Results

The plasmid pJL2 (Revuelta and Jayaram, 1986) contains the *S. cerevisiae* URA 3 as an auxotrophic marker. This 1.1 kbp URA 3 fragment was excised from pJL2 with the restriction endonuclease Hind III and cloned into the Hind III site of the plasmid pUC19. The resultant plasmid was called pGN3.

*Debaryomyces* genomic DNA fragments (average size 0.4 - 1.6 kbp) were generated by partial digestion with Sau 3AI and purified on a linear (10% - 40%) sucrose gradient. These fragments were then ligated into the Sal I site of pGN3 by the technique of partial filling of cohesive ends as described by Zabarovsky and Allikmets (1986). This method is efficient because it avoids self ligation of the vector and prevents multiple insertions of genomic DNA. Two to three micrograms of the ligation mixture were used to transform *S. cerevisiae* strain SEY.2108 to URA prototrophy. Twenty to thirty transformants were obtained on selective medium. Total DNA was prepared from 12 randomly selected yeast transformants and used to transform *Escherichia coli* strain DH5 $\alpha$ . Two of the 12 clones contained autonomous plasmids by virtue of the fact that they could be recovered in *E. coli* and could subsequently transform *S. cerevisiae* to URA prototrophy. Analysis of these two recombinant plasmids by restriction enzyme digestion and subsequent agarose gel electrophoresis showed the presence of ARS inserts of the sizes 0.4 kbp and 1.4 kbp respectively. The plasmid with the 0.4 kbp ARS insert (pAB81) was selected for further analysis (Fig. 1A). Southern blots of total DNA isolated from *S. cerevisiae* transformed with pAB81 and probed with the parent plasmid pUC19 indicated the presence of an autonomous plasmid. Using restriction mapping of pAB81 an Eco RI site was found within the insert. To map the functional limits of the ARS, two subclones were constructed. pAB81 was digested with Eco RI to excise a 0.25 kbp portion of the ARS and the plasmid recircularized (pAB83, Fig. 1A). The excised portion of the ARS (0.25 kbp) was cloned

into the Eco RI site of pGN3 (pAB82, Fig. 1B). The three plasmids (pAB81, pAB82, and pAB83) were then used to transform *Saccharomyces* (Fig. 1B). pAB81 and pAB83 transformed *Saccharomyces* with high frequencies (equivalent to pJL2, data not shown) while pAB82 showed very inefficient transformation (< 10 transformants/ $\mu$ g plasmid DNA) indicating that the ARS from *Debaryomyces* resides within 150 bp of the 400 bp insert.

The 0.4 kbp ARS was excised from pAB81 by digestion with Bam HI and Hind III (from the polylinker sequence of pUC19), purified by agarose gel electrophoresis and radioactively labelled with  $^{32}$ P utilizing the random priming technique (Sambrook *et al.*, 1989). This was then used as a probe against *Debaryomyces* genomic DNA digested with Bam HI (Fig. 1C, Lane A) and Hind III (Fig. 1C, Lane B). The Southern hybridizations were performed at high stringencies (Wahl *et al.*, 1987). Our results indicate that strong homology exists between the ARS from pAB81 and the genomic DNA from *Debaryomyces* suggesting that the ARS in pAB81 originates from *Debaryomyces*.

The ARS from pAB83 was sequenced by the dideoxy chain termination method of Sanger *et al.* (1977). The sequence of 130 bp, as shown in Figure 2 has a high A + T content (% A + T = 80.76). It also has a 12 bp consensus sequence (Fig. 2, the underlined region) which is common to all DNA sequences that function as ARS in *Saccharomyces* (Broach and Hicks, 1980).

### Discussion

We have described here the isolation of an ARS from the marine yeast *Debaryomyces hansenii*. We have demonstrated that the ARS gives the plasmids pAB81 and pAB83 the capability of autonomous replication in *Saccharomyces cerevisiae*. Restriction mapping and subsequent transformation of *S. cerevisiae* indicate that the ARS activity



resides in 130 bp of the clone. In *Phycomyces blakesleeanus* the ARS activity was found to reside in less than 160 bp along a 900 bp fragment (Revuelta and Jayaram, 1986). Araki and Oshima (1989) identified a 30 bp sequence from *Zygosaccharomyces rouxii* that is functional both as an ARS in *S. cerevisiae* and its native host.

Sequence analysis of the functional region of ARSD showed that it has a high %A + T content and includes a 12 bp consensus sequence. Both of these observations are consistent with sequences of ARS from other species (Revuelta and Jayaram, 1986; Cannon *et al.*, 1990; Broach and Hicks, 1980).

Our results do not demonstrate whether the isolated ARSD is functional in *D. hansenii* although plasmids that contain the 2 $\mu$  circle ARS transform *D. hansenii* with low efficiencies (Govind, unpublished data). This observation, coupled with the fact that the sequence of ARSD is very similar to the ARS from *Saccharomyces* (Broach and Hicks, 1980) as well as ARS from both *P. blakesleeanus* (Revuelta and Jayaram, 1986) and the core regions of the ARS from *Candida albicans* (Cannon *et al.*, 1990) indicates that the sequence would be functional in *D. hansenii*. Experiments are now underway to isolate *Ura*<sup>3</sup> strains of *D. hansenii*. When this is accomplished, transformation of the organism will be attempted with pAB81 and pAB83.

One useful attribute of pAB81 and pAB83 is that these plasmids could be conceivably used to transform both *D. hansenii* and *S. cerevisiae*. It is also possible to use these plasmids to complement auxotrophic mutations in *Saccharomyces* and facilitate the isolation of a marker. This is particularly significant since there are no auxotrophic markers available for *Debaryomyces* at the present time. A shuttle vector (i.e., pAB81 or pAB83) that is capable of autonomous existence in *Debaryomyces*, *Saccharomyces*, and *E. coli* would be especially important for the study of gene regulation. It would also be possible to express *Saccharomyces* genes in *Debaryomyces* and vice versa. With the isolation of auxotrophs and their respective genes, it should then be feasible to isolate salt responsive genes and to study their regulation.

## Materials and Methods

### *Strains and Maintenance*

The *Saccharomyces cerevisiae* strain SEY 2108 (*MAT $\alpha$  ura 3-52 leu 2-3,-112 suc2 $\Delta$ 9  $\Delta$ prc1::LEU2<sup>+</sup>*) was obtained from Dr. Scott Emr, California Institute of Technology, Pasadena, California, and described by Bankaitis *et al.* (1986). Complete as well as dropout media and conditions for cultivation of *Saccharomyces* have been described by Campbell (1988). The *Debaryomyces hansenii* strain NRRL Y-7426 was obtained from the United States Department of Agriculture, Peoria, Illinois. The conditions and media for the cultivation of *Debaryomyces* were identical to that of *Saccharomyces*. Plasmids were constructed, propagated and amplified in *Escherichia coli* strain DH5 $\alpha$  (*F<sup>-</sup>, endA1, hsdR17, r<sub>k</sub><sup>-</sup>, m<sub>k</sub><sup>+</sup>, supE44, thi-1,  $\lambda$ -, recA1, gyrA96, relA1,  $\Delta$ (argF-lacZYA)U169,  $\phi$  80dlacZ M15*).

### *Miscellaneous Methods*

Plasmid DNA from *E. coli* was isolated by the method of Ohtsubo *et al.* (1978). Transformation of *E. coli* was performed by the method of Mandel and Higa (1970). DNA from *S. cerevisiae* and *D. hansenii* were prepared by the method of Cryer *et al.* (1975). Transformation of *Saccharomyces* was performed as described by Ito *et al.* (1983). Electrophoretic fractionation of DNA and Southern hybridization were accomplished as described by Wahl *et al.* (1987). Double stranded DNA sequencing was performed by the method of Sanger *et al.* (1977). Restriction and modification enzymes were purchased from Promega Biotechnology and used according to the manufacturer's recommendations.

### Acknowledgments

We are grateful to Dr. R. K. Trench for the use of his laboratory. We thank Dr. R. K. Trench and Dr. J. L. Matta for their critical evaluation of this manuscript. We thank Dr. S. Emr for *Saccharomyces cerevisiae* strain SEY 2108 and Dr. M. Jayaram for plasmid pJL2. This study was supported by a grant from the Office of Naval Research ( N00014-89-J-3027 to NSG).

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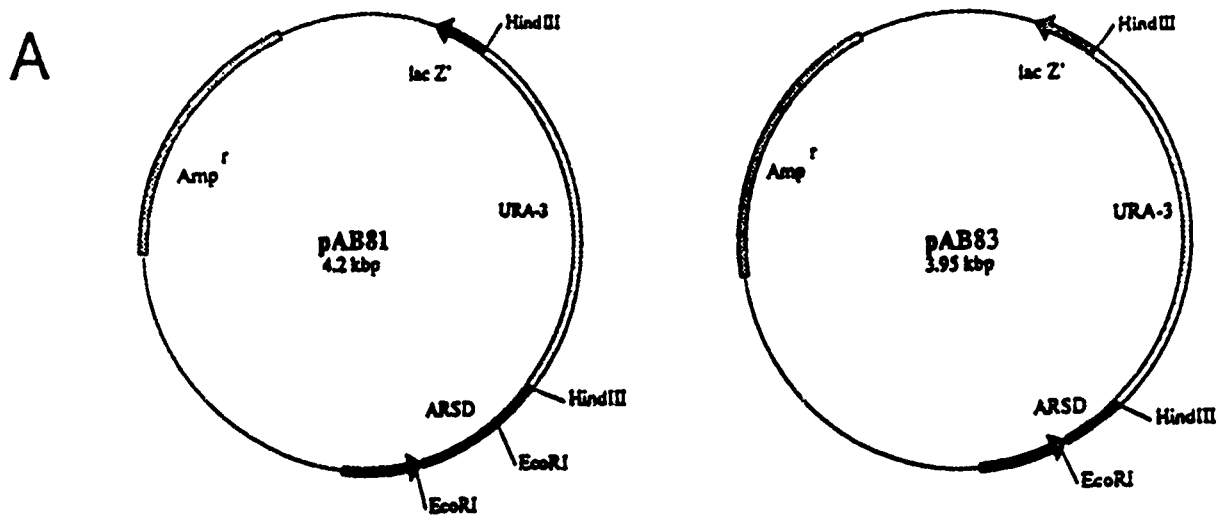
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### Figure Legends

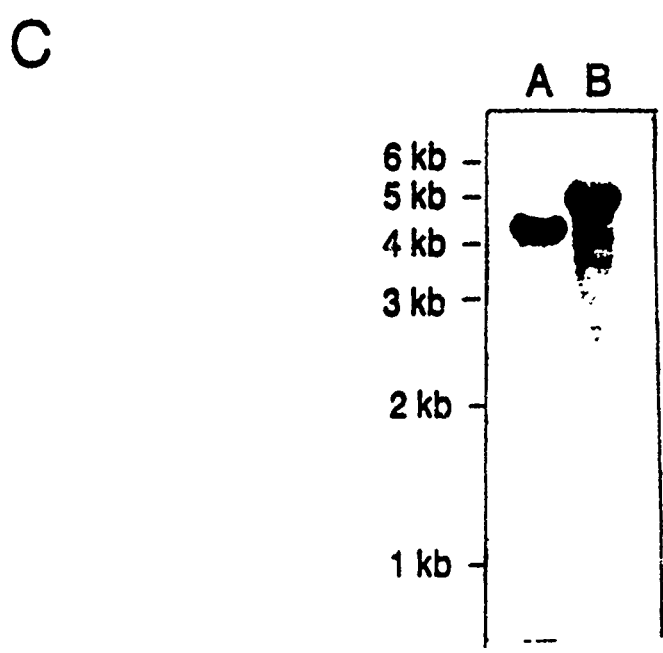
Figure 1: Isolation of ARSD from *Debaryomyces hansenii*. (A) Plasmid pAB81 contains the yeast *URA 3* gene (1.1 kbp) cloned at the Hind III site of of the plasmid pUC19. It also has a 0.4 kbp fragment from *Debaryomyces* (cloned at the Sal I site) that functions as an autonomously replicating sequence in *Saccharomyces*. Plasmid pAB83 was constructed as follows: pAB81 was digested with Eco RI and the plasmid was recircularized after excision of 0.25 kbp of ARSD. The plasmid contains approximately 0.15 kbp of the ARS. (B) Transformation of *S. cerevisiae* with pAB81, pAB82 (construction of pAB82 has been detailed in the results section), and pAB83. Both pAB81 and pAB83 show high frequency transformation of *S. cerevisiae* SEY 2108 to *URA* prototrophy while pAB82 shows very inefficient transformation. (C) Southern blot analysis of genomic DNA from *Debaryomyces* digested with Bam HI (Lane A) and Hind III (Lane B) and probed with ARSD from pAB81.

Figure 2: Sequence analysis of ARSD from pAB83. The consensus sequence TTTTATRTTTTT, common to all DNA fragments that are functional as ARS in yeasts is underlined.



**B**

Plasmid	ARS Insert	Transformants/ $\mu$ g DNA
pAB81		>1000
pAB82		<10
pAB83		>1000



5' TTCCATATTA ATACCCAATT TATATTATT CGTTAAAAGA GATTTATTT  
AAATCTCAA CCTTTATTC GGTATAGACT TTCATAACAT AATATTATT  
TAATTAATAT TACTAATTCG ATGAGATTAA 3'



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# Apoprotein composition and spectroscopic characterization of the water-soluble peridinin-chlorophyll *a*-proteins from three symbiotic dinoflagellates

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## SUMMARY

The water-soluble peridinin-chlorophyll *a*-proteins (sPCP) from three symbiotic dinoflagellates, *Symbiodinium microadriaticum*, *S. kawagutii* and *S. pilosum*, have been analysed for their quaternary structure (by using immunoblotting techniques) and spectroscopic characteristics (by using absorption and fluorescence spectra). The sPCP from *S. kawagutii* is comprised exclusively of a monomeric apoprotein of 35 kDa, whereas sPCP from *S. pilosum* possesses only a dimeric apoprotein with subunits of 15 kDa each. The sPCP from *S. microadriaticum* simultaneously contains both. Spectroscopically, sPCP from *S. kawagutii* is very similar to the 35 kDa species in *S. microadriaticum*; sPCP from *S. pilosum* is similar to the 15 kDa species from *S. microadriaticum*. Gaussian deconvolution analyses of absorption and fluorescence emission spectra show that each holoprotein is comprised of two spectrally distinct forms of chlorophyll *a*. We propose molecular topologies for sPCP consistent with our findings.

## 1. INTRODUCTION

The water-soluble peridinin-chlorophyll *a*-proteins (sPCP) are the major light-harvesting complexes in dinoflagellates (Siegelman *et al.* 1977; Prézélin 1987; Mimuro *et al.* 1990*a*). The function of these chromoproteins is to capture light energy and transfer the excitation to the reaction centres, where primary photochemistry takes place. The presence of xanthophylls in the antenna complexes of chlorophyll *a*-containing algae extends their light-harvesting capacity into the blue-green region of the photosynthetically active radiant spectrum, which is the dominant spectral component of light in the ocean (Larkum & Barrett 1983; Kirk 1983; Owens 1988). Among the various chlorophyll-protein complexes isolated from dinoflagellates, sPCP is the best characterized (Prézélin & Alberte 1978; Boczar *et al.* 1980; Boczar & Prézélin 1986, 1987; Govind *et al.* 1990).

In different dinoflagellates, the apoproteins of sPCP occur as a monomer of about 31–35 kDa or as a homodimer, with subunits of about 15 kDa (Prézélin & Haxo 1976; Siegelman *et al.* 1977; Chang & Trench 1984; Govind *et al.* 1990). The quaternary structure of sPCP is species specific; some species possess the monomeric or dimeric form exclusively, but others possess both (Govind *et al.* 1990). The sPCP isolated from different species can be resolved into several isoelectric forms, the patterns being species specific (Chang & Trench 1982; Trench 1987).

The chromophores of sPCP from several dinoflagellates consist of peridinin and chlorophyll *a* in a

molar ratio of 4:1 (Prézélin & Haxo 1976), but a 9:2 ratio has been reported for the sPCP from *Amphidinium carterae* Plymouth 450 (Haxo *et al.* 1976; Siegelman *et al.* 1977). Based on spectroscopic evidence, Song *et al.* (1976) and Koka & Song (1977) suggested a molecular topology for sPCP consisting of two dimers of peridinin closely associated with one chlorophyll *a* molecule. They suggest that this configuration extends the fluorescence lifetime of the peridinin molecules, and accounts for 100% efficiency of excitation energy transfer from peridinin to chlorophyll *a*, which occurs in less than 10 ps (Song *et al.* 1976; Mimuro *et al.* 1990*a*). The sPCP is unique among the chlorophyll *a*-containing proteins because, to the best of our knowledge, it is the only water-soluble example, although Matthews *et al.* (1979) have reported a water-soluble bacteriochlorophyll *a*-protein complex from *Prosthecochloris aestuarii*. This characteristic of sPCP allows the study of the spectroscopic behaviour of chlorophyll *a* inside the apoprotein, without the interference associated with the use of detergents necessary to solubilize other chlorophyll-protein complexes (Brown & Schoch 1981).

In this paper, we characterize the spectroscopic properties and the quaternary structure of the different isoelectric forms of sPCP from the symbiotic dinoflagellates *S. microadriaticum* (which has both the monomeric and dimeric apoproteins), *S. kawagutii* (which has only the monomeric apoprotein), and *S. pilosum* (which has only the dimeric apoprotein). We find that the spectroscopic characteristics of sPCP appear to be dependent on the quaternary structure of

the apoprotein, and are not species specific. This evidence is supportive of the presence of two spectral forms of chlorophyll *a* associated with the 15 kDa apoprotein, and two different spectral forms of chlorophyll *a* associated with the 35 kDa apoprotein.

## 2. MATERIALS AND METHODS

### (a) Algal cultures

Axenic cultures of *Symbiodinium microadriaticum*, *S. konoaguai* and *S. pilanum* (Trench & Blank 1987) were grown in 2.8 l Fernbach flasks, containing 1 l sterile ASP-8A (Blank 1987) at 26 °C, and illuminated with cool white fluorescent lamps delivering 25  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation (PAR) on a 14 h:10 h (light:dark) photoperiod.

### (b) Harvesting cells and isolation of PCP

Algal cells were harvested by centrifugation at 8000 *g*. PCP was partly purified following the methods described by Prézelin & Haxo (1976) and Chang & Trench (1982) with minor modifications. Algal cells were resuspended in ice-cold TM buffer [10 mM Tris-HCl (pH 8.0), 2 mM  $\text{MgCl}_2$ ]. The cells were broken by three passes through a French pressure cell (Aminco) at  $8.3 \times 10^7$  Pa, and the lysates centrifuged at 100000 *g* at 4 °C for 2 h. The pellets were repeatedly extracted until no more pigment was observed in the supernatant. As, depending on the algal species, varying quantities of peridinin remained in the pellet, we distinguish between the water-soluble (sPCP) and the water-insoluble (iPCP) PCP fractions. The crude sPCP was treated with  $(\text{NH}_4)_2\text{SO}_4$  (50% and 100% saturation). The precipitated PCP-containing fractions were dialysed overnight against TMS buffer (TM, with 500 mM NaCl), concentrated against polyethylene glycol (molecular mass, 10000), applied onto a Sephadex G-75 (Pharmacia) sieving column, and eluted with TMS. The eluate was monitored at 478 nm (Chang & Trench 1982), and fractions containing PCP were pooled, dialysed overnight against 60  $\text{g l}^{-1}$  glycine solution at 4 °C, and analysed by IEF-PAGE (isoelectric focusing in polyacrylamide). About 6% of the sPCP did not precipitate at 100%  $(\text{NH}_4)_2\text{SO}_4$  saturation. This fraction was dialysed and processed as just described. All procedures were conducted under dim light to minimize photodestruction of the chromophores.

### (c) Isoelectric focusing electrophoresis

Partly purified samples of PCP were separated into their composite isoelectric forms by preparative isoelectric focusing (IEF) in a granulated bed (pH range, 3.5–9.5) as described in Chang & Trench (1984), and by analytical IEF-PAGE, using pre-poured Ampholine-PAG plates (pH range 3.5–9.5), and a flat bed electrophoresis apparatus, according to the directions of the manufacturer (LKB). The pH gradient in the gels was measured with a surface pH electrode. After preparative or analytical IEF, regions of the gel containing PCP were excised and eluted with TM buffer.

### (d) PCP purification and production of anti-PCP polyclonal antibodies

*Symbiodinium californium*, the dinoflagellate symbiont harboured by the sea anemone *Anthopleura degumiersis*, was isolated by described methods (Trench 1971). The algal cells were ruptured, and sPCP purified to electrophoretic hom-

modifications. The fractions containing PCP eluted from the Sephadex G-75 column were pooled and concentrated. The solution was mixed with DEAE-cellulose (Pharmacia) for 2 h and centrifuged at 1500 r.p.m. in a clinical centrifuge. The resulting supernatant was dialysed overnight against 10 mM Na-acetate (pH 6.0), applied onto a Sephadex CM-50 column, and eluted with a linear (0.01–0.3 mM) NaCl gradient in Na-acetate buffer. The two resulting fractions were combined, and applied to a preparative gradient (10–20% polyacrylamide) SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). The gel was lightly stained with Coomassie Brilliant Blue. A single polypeptide of 15 kDa was visualised and excised. Anti-sPCP (antibodies against sPCP from *Symbiodinium californium*) were generated commercially (GABCos, Berkeley, California).

### (e) Immunoblotting analyses

Portions of sPCP containing 26  $\mu\text{g}$  chlorophyll *a* were separated by SDS-PAGE and electroblotted onto nitrocellulose as described previously (Roman *et al.* 1988; Govind *et al.* 1990). The blots were treated with diluted (1:500) anti-sPCP serum. Pre-immune serum was used in control assays.

### (f) Spectroscopic analyses

Absorption spectra were obtained at room temperature by using a Varian Techtron series 634 UV/VIS spectrophotometer. The spectra were digitized using an A/D board (SABLE Systems), attached to a computer (Systek). By using this configuration, it was possible to capture 640 data points per spectrum. Typically, a slit width of 0.5 nm was used. The absorption measurements were captured at 0.64 nm intervals while scanning the full visible spectrum (338–750 nm), and at 0.23 nm intervals when scanning the red portion of the spectrum (600–750 nm). The absorption spectrum of each sample was obtained by averaging 15 scans. A linear regression was fitted to the residual absorption in the 730–750 nm range. The spectra were corrected for molecular scattering by subtracting the extrapolated linear regression of the residual absorption from the original data. Chlorophyll *a* was quantified using a molar extinction coefficient of  $60 \text{ mm}^{-1} \text{ cm}^{-1}$  (Shionawa *et al.* 1974).

Room temperature fluorescence emission spectra were recorded with a Perkin-Elmer LR-50 spectrofluorometer, equipped with a red-sensitive photomultiplier (Model R-928). The instrument automatically digitized the spectra with resolution of 0.5 nm. The apparatus was operated with both excitation and emission slit widths of 2.5 nm. Samples were excited at 433, 463 and 520 nm. Concentration of 20  $\mu\text{M}$  chlorophyll *a* were used to minimize reabsorption. Each final spectrum is the result of averaging 25 uncorrected spectra.

Fourth derivatives of the absorption spectra were calculated as described by Butler & Hopkins (1970). Gaussian deconvolutions of both absorption and fluorescence emission spectra (600–750 nm) were done by using a nonlinear fit procedure with the Marquardt (1963) algorithm (PeakFit, Jandel). The goodness of fit was evaluated by the distribution of the residuals as well as by the values of  $\chi^2$ . We routinely tried fitting the spectra to different combinations of Gaussian and Lorentzian components, as well as with the Gaussian-Lorentzian cross-product function (French *et al.* 1972). The best fits were consistently obtained when pure Gaussian, or the Gaussian-Lorentzian cross-product function were used. For simplicity, we report here only the results obtained by using the Gaussian analysis. In all cases, the difference between the areas below the observed and predicted spectra was less than 0.3%.

3. RESULTS

The anti-ScPCP antibodies cross-reacted with PCP apoproteins from *S. microadriaticum*, *S. kaseguti* and *S. pilosum* (figure 1), and an indication that the 15 kDa and the 35 kDa apoproteins possess common antigenic sites. Pre-immune sera showed no cross-reactivity. These results are identical to those reported by Govind *et al.* (1990) using antibodies against PCP from the dinoflagellate *Heterocapsa pygmaea*, and confirm the observation that the sPCP from *S. microadriaticum* contains a monomeric apoprotein of about 35 kDa, and an apparent homodimer of 15 kDa, whereas sPCP

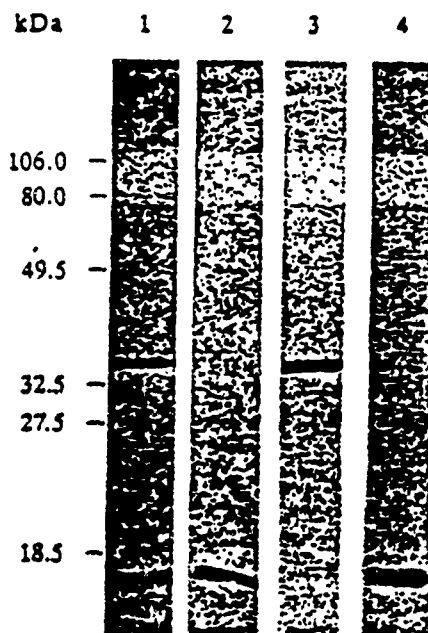


Figure 1. Immunoblot analysis of water-soluble extract, separated by SDS-PAGE, transferred onto nitrocellulose and probed with anti-ScPCP serum. Lane 1, *S. microadriaticum*, partly purified sPCP. Lane 2, *S. microadriaticum*, sPCP fraction that did not precipitate after 100% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation. Lanes 3 and 4, partly purified sPCP from *S. kaseguti* and *S. pilosum*, respectively.

from *S. kaseguti* and *S. pilosum* are composed exclusively of the 35 and 15 kDa apoproteins, respectively. The sPCP from *S. microadriaticum*, which failed to precipitate after 100% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation, is composed exclusively of the 15 kDa apoprotein (figure 1, lane 2).

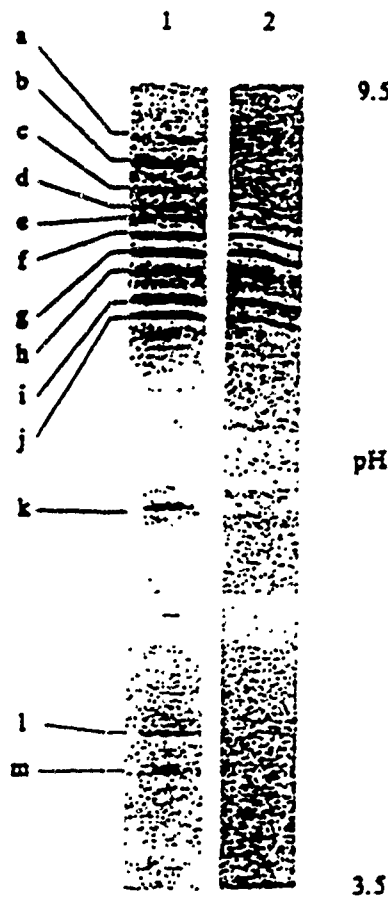


Figure 2. Unstained SDS-PAGE gel of separated sPCP isolated from *S. microadriaticum*. Lane 1, sPCP precipitated by 50-100% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation. Lane 2, *S. microadriaticum*, sPCP fraction that did not precipitate after 100% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation.

Table 1. *Symbiodinium microadriaticum*: molecular mass of PCP/apoproteins and spectro characteristics at room temperature of the different isoforms

isoform	pI	molecular mass/kDa	absorption maximum (nm)	fluorescence emission maximum (nm)
a	8.4	35	669.8	673.0
b	8.2	35	669.8	673.0
c	8.0	35	669.8	673.0
d	7.9	35 15	671.0	674.0
e	7.8	35 15	671.4	674.3
f	7.7	35 15	672.4	675.0
g	7.6	35 15	673.4	675.5
h	7.5	35 15	673.4	676.0
i	7.3	35 15	673.4	676.5
j	7.2	35 15	673.4	676.5
k	6.0	15	673.4	676.5
l	4.5	15	673.4	676.5
m	4.3	15	673.4	676.5

sPCP spectral

eq #5

cont table

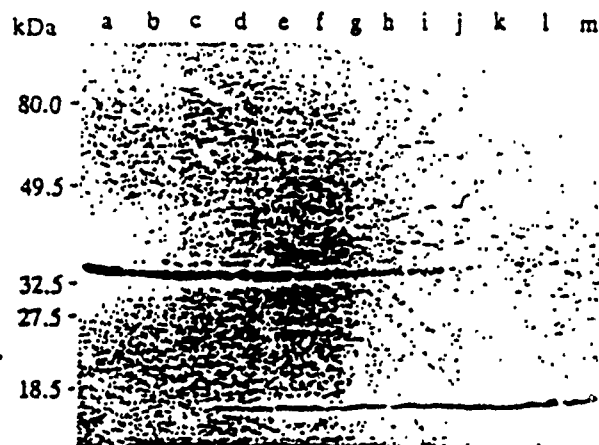


Figure 3. Immunoblot of 13 isoelectric forms of sPCP isolated from *S. microadriaticum* after SDS-PAGE. The blot was probed with anti-sPCP serum. For identification of the 13 lanes, see table 1.

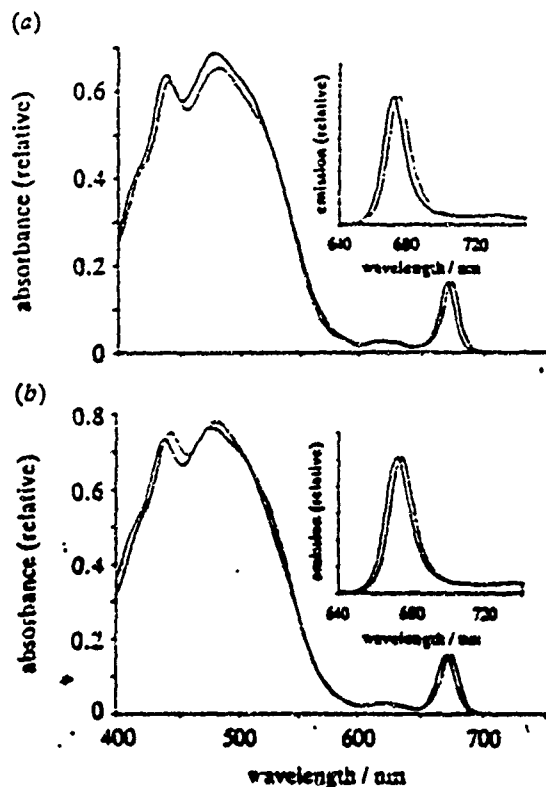


Figure 4. Room temperature absorption, and fluorescence emission spectra (inserts) of sPCP. (a) *S. kawagutii*, 35 kDa apoprotein (continuous lines), and *S. pilanum*, 15 kDa apoprotein (broken lines). (b) *S. microadriaticum*, 35 kDa apoprotein (continuous lines) and 15 kDa apoprotein (broken lines). Excitation was at 435 nm.

After separation of sPCP from *S. microadriaticum* by analytical IEF-PAGE, 13 isoelectric forms of sPCP were resolved, with pI values ranging from pH 4.3 to 8.4 (figure 2, lane 1). Elution of the individual isoelectric forms, and subsequent analysis by SDS-PAGE and immunoblotting methods, revealed that they are composed of apoproteins of either 35 or 15 kDa (figure 3). The PCP fraction that did not precipitate in 100%  $(\text{NH}_4)_2\text{SO}_4$  is composed of four isoelectric forms with

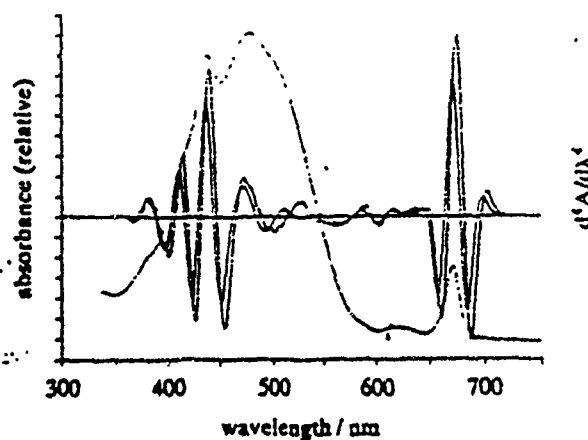


Figure 5. Fourth derivative analysis of absorption spectra of sPCP from *S. kawagutii* (—) and *S. pilanum* (---). The absorption spectrum of sPCP from *S. kawagutii* (···) is provided as reference. Identical results were obtained with the fourth derivative analyses of the monomeric and dimeric forms of sPCP from *S. microadriaticum*.

pI values ranging from pH 7.3 to 7.9 (figure 2, lane 2), and are composed exclusively of the 15 kDa apoprotein. The existence of isoelectric forms that appear to be composed of apoproteins of 15 and 35 kDa (isoelectric forms d-j in table 1; figure 3) is very probably the result of contamination during excision from the IEF-PAGE gels, or may represent different holoproteins with the same isoelectric points. **INSERT**

The results of absorption and fluorescence emission spectral analyses (table 1) suggest that there are two distinct spectral forms of sPCP in *S. microadriaticum*. Isoelectric forms containing the 35 kDa apoprotein show absorption maxima at 669.8 nm and fluorescence emission maxima at 673 nm, whereas those with the 15 kDa apoprotein show absorption maxima at 673.2 nm, and fluorescence emission maxima at 676.5 nm.

To examine whether the spectral characteristics of sPCP are species specific, as suggested by Koka & Song (1977), or dependent on their apoprotein composition, we compared the absorption and fluorescence emission spectra of sPCP from *S. microadriaticum*, *S. kawagutii* and *S. pilanum*. The results (figure 4) show that the sPCP from *S. kawagutii* is similar to the sPCP with 35 kDa apoproteins from *S. microadriaticum*, and the sPCP from *S. pilanum* is similar to the sPCP with 15 kDa apoproteins from *S. microadriaticum*. The absorption spectra of both the 15 kDa and the 35 kDa sPCP were normalized to the absorption peak in the red, and the difference spectrum obtained suggested that the spectral differences of the monomeric and dimeric forms are not restricted to the red absorption band of chlorophyll *a*, but can be found in the Soret band as well. Similar results were obtained by fourth derivative analyses of the absorption spectra (figure 5), which also suggest that the peridinin absorption bands in the monomeric sPCP are red-shifted in relation to the dimeric forms.

The red (600–750 nm) absorption spectra of sPCP at room temperature are the result of absorption of only chlorophyll *a* (see table 2 and figure 6). Gaussian deconvolution analyses of the spectra suggest that the

Table 2. *Symbiodinium* spp.: Gaussian deconvolution of the absorption (600–750 nm) and fluorescence emission (600–750) spectra of PCP:

absorption			fluorescence emission		
maximum	bandwidth	amplitude	maximum	bandwidth	amplitude
<i>S. microadriaticum</i> 15 kDa					
Qx transition			primary maximum		
605.5	42.7	0.031	675.7	4.6	0.511
623.1	15.9	0.0093	678.1	8.4	0.361
Qy transition			secondary maximum		
670.8	9.2	0.354	693.8	20.7	0.070
673.6	4.4	0.522	739.2	12.6	0.058
<i>S. pilosum</i> 15 kDa :					
Qx transition			primary maximum		
613.4	42.7	0.068	675.8	4.9	0.528
623.1	11.7	0.068	678.3	9.2	0.351
Qy transition			secondary maximum		
670.8	8.8	0.414	698.5	23.2	0.068
673.6	4.5	0.450	740.8	12.4	0.053
<i>S. microadriaticum</i> 35 kDa					
Qx transition			primary maximum		
607.4	12.2	0.069	672.5	5.5	0.655
627.7	12.8	0.104	676.2	10.5	0.268
Qy transition			secondary maximum		
666.5	9.7	0.324	717.1	36.5	0.061
670.2	4.9	0.503	734.4	6.1	0.016
<i>S. kaseguttii</i> 35 kDa					
Qx transition			primary maximum		
602.8	7.3	0.025	672.6	5.3	0.657
621.5	15.2	0.129	676.3	10.3	0.268
Qy transition			secondary maximum		
666.8	9.6	0.330	719.7	35.6	0.061
670.1	4.8	0.516	733.4	5.5	0.014

Qx envelopes are dominated by two components with peaks between 602–623 nm. The Qy envelopes are composed of two peaks with maxima at 670.9 nm and 673.5 nm (15 kDa apoproteins), and at 666.6 nm and 670.2 nm (35 kDa apoproteins). The proportions and shape of these two transitions as judged by their bandwidths and amplitudes are conserved in all the samples.

Gaussian deconvolution of the fluorescence emission spectra require four Gaussian components, two in the primary maximum around 674 nm, and two more in the secondary maximum in the vicinity of 733 nm, to be accurately described (figure 6). The Gaussian components of the primary maximum are conserved in terms of both their bandwidths and amplitudes. The first Gaussian element of the primary maximum has peaks at 675.7 nm and 678.1 nm (for the 15 kDa apoprotein), and 672.5 nm and 676.2 nm (for the 35 kDa apoprotein) (table 2).

#### 4. DISCUSSION

The anti-ScPCP antibodies cross-reacted with all the isoelectric forms of sPCP from *S. microadriaticum*, *S. kaseguttii* and *S. pilosum*. They also reacted with the different apoproteins of all three algal species. Roman *et al.* (1988) also found that antibodies generated against the sPCP from *Heterosigma pygmaea* reacted with all isoelectric forms of sPCP from that alga, and

Govind *et al.* (1990) found that the same antibodies cross-reacted specifically with the apoproteins from a wide range of dinoflagellates, and did not cross-react with chlorophyll-protein complexes from other algae. These observations are identical to those reported by Friedman & Alberte (1987), and Hiller *et al.* (1988).

The spectroscopic similarities of the 35 kDa isoelectric forms from *S. microadriaticum* and the 35 kDa isoelectric forms from *S. kaseguttii*, are reflected in the positions of their absorption and fluorescence emission maxima and the presence of virtually identical Gaussian components. The same applies to the 15 kDa isoelectric forms from *S. microadriaticum* and the 15 kDa isoelectric forms from *S. pilosum*. Based on these observations, we conclude that local effects characteristic of the different apoproteins are responsible for the spectroscopic characteristics of the holoprotein.

Spectroscopic differences between sPCP isolated from different dinoflagellate species have been reported previously. At room temperature, sPCP from *H. pygmaea* has an absorption maximum at 672 nm (Prézelin & Haxo 1976) and a fluorescence emission maximum at 675 nm (Song *et al.* 1976) whereas sPCP from *Corycaux polyedra* and *A. carterae* Plymouth 450 have an absorption maximum at 670 nm and a fluorescence emission maximum at 672 nm (Song *et al.* 1976; Koka & Song 1977). Our data suggest that the spectroscopic characteristics of sPCP are correlated with the molecular mass of the apoproteins, and are

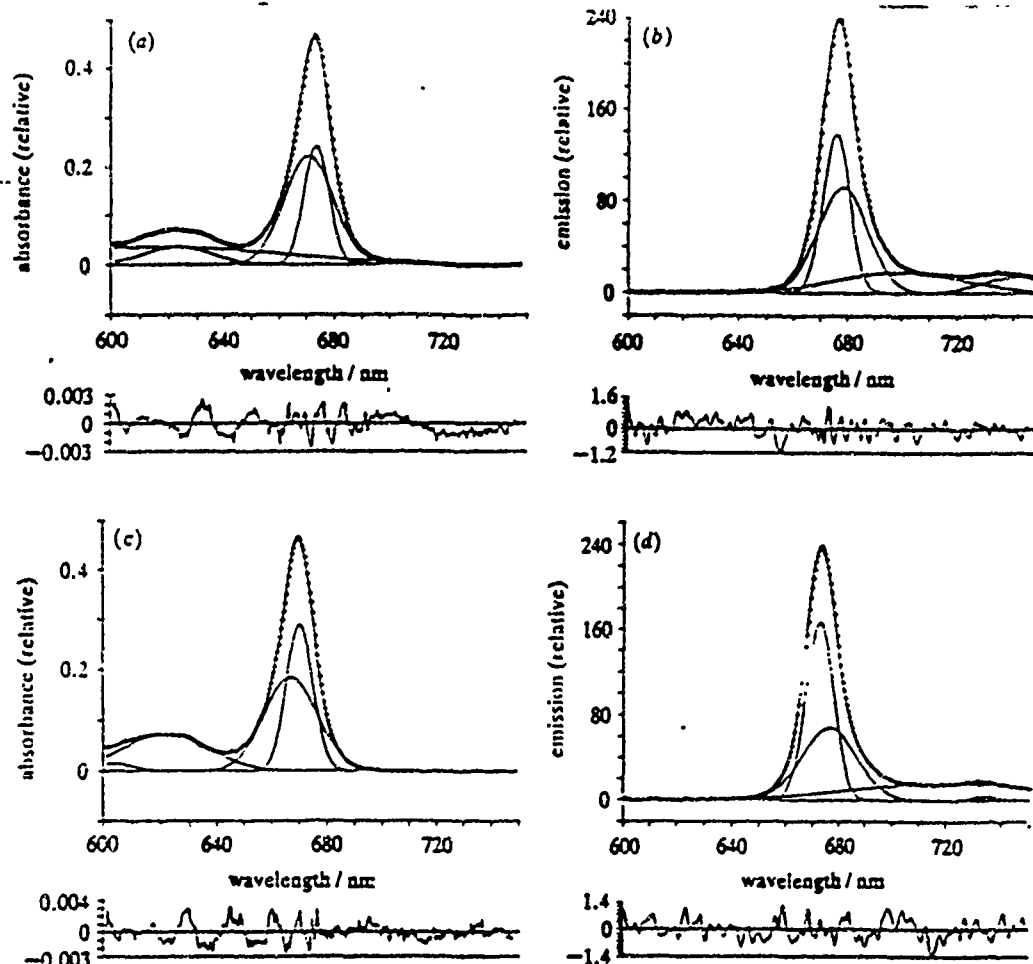


Figure 6. Gaussian deconvolution analyses of (a) room temperature absorption, and (b) fluorescence emission spectra of sPCP from *S. pilanum*, and (c) and (d) from *S. kanevskii*. Excitation wavelength as in figure 4. The experimental data are represented by crosses, and the full lines are the Gaussian components and their sum. Below each spectrum is a plot of the residuals.

consistent with the differences just cited, as *H. pygmaea* sPCP has the 15 kDa apoprotein, whereas the sPCP of both *G. polyedra* and *A. carterae* Plymouth 450 possess the 35 kDa apoprotein (Prézelin & Haxo 1976; Govind *et al.* 1990).

The Gaussian deconvolution analyses of the absorption spectra of sPCP comprised of the 15 kDa and the 35 kDa apoprotein suggest the existence of two spectral forms of chlorophyll *a* associated with each apoprotein, as rendered evident by the presence of two components in the Q<sub>y</sub> envelope. Justification for the use of Gaussian analyses of room temperature absorption spectra is provided by the studies of Zucchelli *et al.* (1990) and Mimuro *et al.* (1990b). The proposed models for the structure of sPCP (Prézelin & Haxo 1976; Prézelin 1987), suggest the presence of a single chromophoric unit (four peridinin and one chlorophyll *a*) associated with a 29–35 kDa polypeptide, and our high derivative analyses of the absorption spectra revealed a single peak in the Q<sub>y</sub> region probably caused by the broadening of the transition bands at room temperature (French *et al.* 1972). We therefore tried to fit a single Gaussian to the Q<sub>y</sub> envelope. To account for the apparent asymmetry of some transition bands, we also attempted to fit the spectra to a single asymmetrical Gaussian (French *et al.* 1972; Zucchelli *et*

*al.* 1990). By neither approach were we able to obtain successful fits using a single component. Based on these results, we tried to fit the Q<sub>y</sub> transitions to a set of two Gaussian elements, where we treated the Gaussian peak positions as parameters to be fit by the computer program. This approach produced a very good fit in all cases. Increasing the number of Gaussian elements did not result in any increase in the goodness of fit. Analyses of the relative areas below the Gaussian components suggest that the two spectral forms are present in similar proportions (59:41 ± 4%). The symmetry of the Gaussian elements may be associated with weak interactions between the chlorophyll molecules in the local environment (Zucchelli *et al.* 1990). The absorption maxima of the two spectral forms of chlorophyll *a* that we observed are very similar to those obtained by the room temperature Gaussian deconvolution analyses of Mimuro *et al.* (1990b) for the fucoxanthin–chlorophyll *a/c*–protein aggregates isolated from the brown sea weed *Dictyota dichotoma*.

Consistent with the two spectral forms of chlorophyll *a* suggested by the data on absorption, Gaussian deconvolution analyses of the fluorescence emission spectra suggest the existence of two components in the primary peak resulting from the relaxation of the Q<sub>y</sub> transitions (Schmidt 1988). The results (figure 5; table

2), suggest that, for the 15 kDa apoprotein, the spectral form of chlorophyll *a* absorbing at 670.9 nm emits at 675.7 nm, and the one absorbing at 673.5 nm emits at 678.1 nm. For the 35 kDa apoprotein, the spectral form of chlorophyll *a* absorbing at 666.6 nm emits at 672.5 nm, and the one absorbing at 670.2 nm emits at 676.2 nm. Therefore, in *S. microadriaticum*, where sPCP is composed of both the 15 and the 35 kDa apoproteins, there are four distinct spectral forms of chlorophyll *a* present.

The absorption-fluorescence spectra of chlorophyll *a*, *in vivo* and in organic solvents, show a characteristic Stokes shift of 5–15 nm (Shipman & Housman 1979; Govindjee *et al.* 1979). Prézélin & Haxo (1976), Song *et al.* (1976), and Koka & Song (1977) reported very small Stokes shifts (2–3 nm) for sPCP, and in the case of the membrane-bound peridinin-chlorophyll *a*-protein complex isolated from *H. pygmaea* by Boczar & Prézélin (1986), there is no Stokes shift at all. Our results are very similar to those described, showing a Stokes shift of approximately 3 nm when the peaks of the Q<sub>y</sub> envelopes are compared with the peaks in the primary emission envelopes. But when the two Gaussian components of the Q<sub>y</sub> envelope and the two components of the emission spectra are compared simultaneously, each Gaussian component in the Q<sub>y</sub> envelope shows a Stokes shift of about 6 nm.

Gaussian deconvolution analyses of the absorption spectra of light-harvesting complexes from green plants show that they contain all the chlorophyll *a* spectral forms described by French *et al.* (1972), with the exception of the form with absorption maximum at 680 nm (Brown & Schoch 1981; van Dorssen *et al.* 1987). However, Zucchelli *et al.* (1990), and Jennings *et al.* (1990), using Gaussian deconvolution of spectra taken at room temperature, have reported the existence of long wavelength chlorophyll *a* forms in chlorophyll *a/b* light-harvesting complex II of spinach and pea. The sPCP exhibits less diversity of spectral forms than the antennae of green plants, and in addition, lacks long wavelength components. These observations are consistent with the assignment of sPCP as the most peripheral antenna complex in dinoflagellates (Prézélin & Alberte 1978; Govindjee *et al.* 1979; Mimuro *et al.* 1990 *a*).

Based on the lack of chlorophyll-chlorophyll interactions, as determined by circular dichroism, the apparent molecular mass of the native holoprotein, and the assumed steric difficulty of accommodating more than one chromophoric unit within an apoprotein of 30–35 kDa, a molecular topology consisting of peridinin:chlorophyll *a*:protein ratio of 4:1:1 has been proposed (Prézélin & Haxo 1976; Song *et al.* 1976; Koka & Song 1977; Prézélin 1987). Haxo *et al.* (1976) suggested a peridinin:chlorophyll *a*:protein ratio of 9:2:1 for the sPCP from *A. carteras* Plymouth 450, based on spectroscopic determinations of the chromophore content, and the difference in molecular mass between the holoprotein (39.2 kDa) and the apoprotein (31.8 kDa). The determination of the number of chromophoric units in the native complex is difficult because of the limitations of molecular sieving techniques and the small size of the chromophoric unit

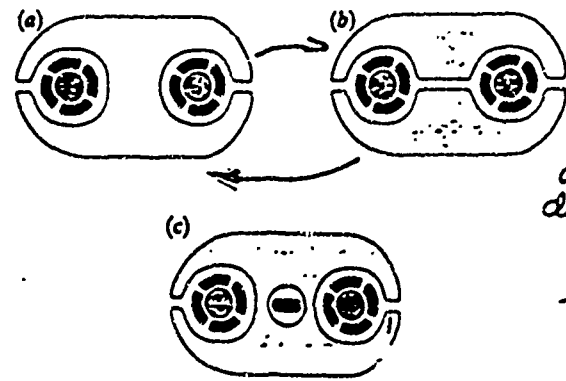


Figure 7. Hypothesized molecular topologies of sPCP from different dinoflagellates. For details of the structure of the chromophoric unit, see Song *et al.* (1976). The diagrams show peridinin monomer (—), chlorophyll *a* (●) and protein (○). (a) Two chlorophyll *a* molecules (spectroscopically different), each associated with two dimers of peridinin, with two homodimeric (15 kDa) apoproteins, as found in *P. pilonum*. This arrangement would reflect a chlorophyll *a*:peridinin:apoprotein ratio of 2:8:2. (b) Two chlorophyll *a* molecules (spectroscopically different), each associated with two dimers of peridinin, with one monomeric (35 kDa) apoprotein, as found in *S. kasegahii*. This arrangement would reflect a chlorophyll *a*:peridinin:apoprotein ratio of 2:8:1. (c) Two chlorophyll *a* molecules (spectroscopically different), each associated with two dimers of peridinin, with one monomeric (35 kDa) apoprotein. The two chromophoric units are separated by a 'silent' peridinin monomer. This arrangement will reflect a peridinin:chlorophyll *a*:apoprotein of 2:9:1, as suggested by Haxo *et al.* (1976) and Seigelman *et al.* (1977) for *A. carteras* Plymouth 450.

(3.4 kDa) (Prézélin & Haxo 1976; Prézélin 1987). Koka & Song (1977) analysed the molecular topology of sPCP from *A. carteras* Plymouth 450, and found that this molecule had spectral characteristics identical to those found in sPCP having peridinin:chlorophyll *a* ratios of 4:1. They concluded that the peridinin:chlorophyll *a* ratio of 9:2 was inconsistent with the proposed molecular topology, and proposed an alternative model in which two chromophoric units are contained by a 30–35 kDa polypeptide. However, they considered this latter model unlikely, principally because the apoprotein was believed to be too small to house two chromophoric units.

Our analyses are consistent with two chromophoric units associated with each sPCP apoprotein. Crystallographic analyses of the water-soluble bacteriochlorophyll *a*-protein from *Prosthecochloris aestuarii* revealed the presence of seven bacteriochlorophyll *a* molecules within a 50 kDa monomer (Matthews *et al.* 1979; Tronrud *et al.* 1986), and Kühlbrandt & Wang (1991), using electron crystallography, corroborated the presence of 15 chlorophyll molecules (eight chlorophyll *a* and seven chlorophyll *b*) per 25 kDa apoprotein in chlorophyll *a/b* light-harvesting complex II of green plants. These two observations suggest that, in the case of sPCP, apoproteins of 30–35 kDa can possess enough space to accommodate two chromophoric units, separated by enough distance to account for the absence of chlorophyll *a* ellipticity (Song *et al.*

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1976; Koka & Song 1977). Based on these considerations, we postulate two models for sPCP (figure 7) in which two chromophoric units are associated with either: (i) a homodimer of 15 kDa apoprotein subunits; or (ii) a monomer of 35 kDa apoprotein. Finally, we also speculate the existence of an inactive peridinin molecule in the case of sPCP from *A. carterae* Plymouth 450 (figure 7c) similar to the inactive fucoxanthin detected by Mimuro *et al.* (1990b), which reconciles the 9:2 peridinin:chlorophyll *a* ratio reported by Haxo *et al.* (1976) and Siegelman *et al.* (1977), and the molecular topology that we propose. Ultimately, the resolution of the correct protein-chromophore stoichiometries will require the use of crystallographic techniques.

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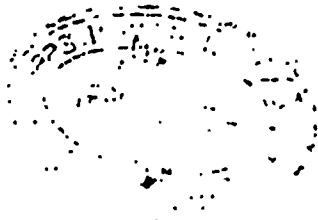
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THE NUCLEOTIDE SEQUENCE OF THE SMALL SUBUNIT  
RIBOSOMAL RNA GENE FROM  
SYMBIODINIUM PILOSUM, A SYMBIOTIC DINOFLAGELLATE

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**Summary.** The complete sequence of the small subunit ribosomal RNA (SSU rRNA) gene was determined for the symbiotic dinoflagellate *Symbiodinium pilosum*. This sequence was compared with sequences of 2 other dinoflagellates (*Prorocentrum micans* and *Cryptocodinium cohnii*), 5 Apicomplexa, 5 Ciliata, 5 other eukaryotes and one archaeobacterium. The corresponding structurally conserved regions of the molecule were used to determine which portions of the sequences could be unambiguously aligned. Phylogenetic relationships were inferred from analysis of distance matrices, where pair-wise distances were determined using a maximum likelihood model for transition and transversion ratios, and from maximum parsimony analysis, with bootstrap resampling. By either analytical approach, the dinoflagellates appear distantly related to prokaryotes, and are most closely related to two of the Apicomplexa, *Sarcocystis muris* and *Theileria annulata*. Among the dinoflagellates, *C. cohnii* was found to be more closely affiliated with the Apicomplexa than either *P. micans* or *S. pilosum*.

**Key words :** Molecular phylogeny, dinoflagellates, polymerase chain reaction, small subunit ribosomal RNA gene, *Symbiodinium pilosum*.

## Introduction

Among the Protista, the phylogeny of the dinoflagellates is somewhat ambiguous. By virtue of the absence of nuclear histones and nucleosomes (Herzog and Soyer, 1981; Rizzo, 1981, 1987), the similarity of the organization of dinoflagellate chromosomes to the bacterial nucleoid (Soyer and Herzog, 1985), and the substantial substitution of thymine by hydroxymethyluracil (Steele and Rae, 1980; Herzog and Soyer, 1982), some investigators have regarded them as being closely affiliated to the prokaryotes. But dinoflagellates also show distinct eukaryotic features; large quantities of DNA per cell (ranging from 2.7 pg/cell in *Amphidinium carterae* to 200 pg/cell in *Gonyaulax polyedra*; with 50-60% repeated sequences); typical eukaryotic range in G+C content (36.8 - 52.7%; Rae, 1976); mRNA splicing mechanisms, and the maturation of a 38S rRNA precursor (Rae, 1970), similar to eukaryotes (Hinnebusch et al. 1980; Rizzo, 1987). The apparent "midway position" of the dinoflagellates which prompted Dodge (1966) to the idea of the mesokaryotes, has not gained support from molecular biology. Molecular phylogeny places the dinoflagellates firmly within the eukaryote lineage (Lenaers et al. 1989; Hendriks et al. 1989, 1991; Gajadhar et al. 1991), and lends credence to Loeblich's (1984) idea that the so-called prokaryotic characters of dinoflagellates are derived, and are not a reflection of their ancestral state.

The phylogenetic relationships within the division Pyrrhophyta (=Dinophyta) are poorly understood. Three current schemes on the phylogeny of dinoflagellates (Loeblich, 1984; Dodge, 1984; Taylor, 1987) show some major disagreements. For example, some investigators regard the procenteroids as primitive (Herzog and Soyer, 1981), while others regard the ancestral dinoflagellate state to be represented by an *Oxyrrhis*-like organism (Loeblich, 1984). The ordinal affiliations of several dinoflagellates remain unresolved. Taylor (1987) regards *Cryptecodinium cohnii* as a member of the Gonyaulacales, while Dodge (1984) places it among the Peridinales.

The systematics of dinoflagellates known to occur as endosymbionts in a variety of marine invertebrates, is also poorly understood (Blank and Trench, 1986). However, based

on biochemical, morphological, and karyotypic analyses of algae isolated from various hosts and maintained in culture (Schoenberg and Trench, 1980; Trench and Blank, 1987), or analysed as fresh isolates (Kowan and Powers, 1991a,b), it is currently recognized that all symbiotic dinoflagellates are not conspecific, and that distinct dinoflagellate taxa, representing six genera (*Prorocentrum*, *Amphidinium*, *Gymnodonium*, *Symbiodinium*, *Gloeodinium* [= *Hemidinium*] and *Scrippsiella*) in four orders, occur as endosymbionts (Trench, 1991).

In initiating a study on the molecular phylogeny of symbiotic dinoflagellates, which must be done in the context of free-living dinoflagellates, we have sequenced the SSU rRNA gene from the symbiotic dinoflagellate *Symbiodinium pilosum* (Trench and Blank, 1987). The sequence has been submitted to Genbank and given the accession number M . This sequence was aligned to the SSU rDNA sequences from the non-symbiotic dinoflagellate species *Prorocentrum micans* (Herzog and Maroteaux, 1986) and *Crypthecodinium cohnii* (Gajadhar et al. 1991) and several protists. We have generated phylogenetic trees which show that the dinoflagellates are more closely related to some of the Apicomplexa (*Sarcocystis muris* and *Theileria annulata*) than to the Ciliata, as suggested by Johnson et al. (1990), Gajadhar et al. (1991) and Barta et al. (1991). The analyses also show that among the three dinoflagellates studied, *C. cohnii* is more closely linked to the Apicomplexa than are *S. pilosum* (Gymnodiniales) and *P. micans* (Prorocentrales).

#### Materials and Methods

The symbiotic dinoflagellate *Symbiodinium pilosum* Trench and Blank, originally isolated from the zoanthid *Zoanthus sociatus* was cultured axenically in ASP-8A as previously described (Trench and Blank, 1987). The cells were harvested by centrifugation at 1500 xg, at 4°C.

*DNA isolation and purification.* The pelleted cells were resuspended in a small volume of ASP-8A. The thick slurry was added dropwise to liquid nitrogen in a mortar, and ground with a pestle to a fine powder which was dissolved to 50 ml of extraction buffer [5M guanidine

thiocyanate, 10 mM Na<sub>2</sub>EDTA, 50mM HEPES, pH 7.6 and 5% (by volume) β-mercaptoethanol]. The viscous solution was transferred to centrifuge tubes and rendered 4% (by weight) with Sarcosyl. The suspension was centrifuged at 8,000 xg for 5 min at 4°C to remove cell debris, and the supernatant was brought to 1.42M with solid CsCl. The solution was layered on to a 5.7M CsCl cushion and centrifuged at 160,000 xg at 20°C for 18hr. DNA was removed from the interface of the step gradient and exhaustively dialyzed against TE buffer (10mM Tris-HCl, pH 8.0 and 1mM NA<sub>2</sub>EDTA). The solution was rendered 100mM with NaCl, and the DNA precipitated with 2.5 volumes of ethanol, dried and redissolved in an appropriate volume of TE.

*Polymerase Chain Reaction:* Amplification of the SSU rDNA was accomplished (Saiki et al. 1988) using the amplification primers described by Medlin et al. (1988), using a Perkin Elmer CETUS DNA Thermal Cycler. The amplified product was purified by electrophoresis in low-melting agarose.

*Cloning and Sequencing the SSU rDNA.* The SSU rDNA fragment was completely digested with restriction enzyme Bam HI and partially digested with Eco RI, and cloned into the polylinker sequence of pUC19 (Messing, 1983). Bacterial transformation, plasmid amplification, and isolation were performed as described by Maniatis et al. (1982). Double stranded DNA sequencing was accomplished by the dideoxy chain-termination method of Sanger et al. (1977). The sequencing primers used have been described by Sogin and Gunderson (1987). Restriction and modification enzymes were obtained from Promega Biotech. (Madison, Wisconsin) and used according to the manufacturer's specifications.

*Computer analysis of sequences.* The complete SSU rDNA sequences of *Cryptosporidium parvum*, *Cryptosporidium parvum* (Gajadhar et al. 1991), *Prorocentrum micans* (Herzog and Maroteaux, 1986), *Plasmodium berghei* (Gunderson et al. 1986), *P. falciparum* (Waters et al. 1989), *P. falciparum* (McCutchan et al. 1988), *Oxytricha nova* (Elwood et al. 1985) and *Symbiodinium pilosum* were aligned on the basis of similarity and secondary structure, with the initial alignment provided by the multiple sequence program

CLUSTAL (Higgins and Sharp, 1988, 1989). One thousand five hundred non-ambiguously aligned positions were analyzed by (1) maximum parsimony, with 500 bootstrap resamplings employing the PAUP program (Swofford, 1989), and (2) computation of bootstrap resampled DNA distance matrices using the maximum likelihood model (DNADIST), with resolution of the matrices by Fitch and Margoliash's algorithm (FITCH). A consensus tree was generated using CONSENSE. All programs are from the PHYLIP 3.3 package (Felsenstein, 1990).

A secondary structure for the SSU rRNA from *S. pilosum* was generated using the eukaryotic model of Gutell et al. (1985) and Huss and Sogin (1990).

### Results and Discussion

The model of the secondary structure of the SSU rRNA from *S. pilosum* is shown in Fig. 1. This structure is based on the model for *Chlorella vulgaris* given by Huss and Sogin (1990), with helix numbering according to Dams et al. (1988). This model for a dinoflagellate differs from that given for *Prorocentrum micans* by Herzog and Maroteaux (1986) primarily for the regions encompassed by stem helices E21-1 to E21-6, 16 - 19, and the pair of helices consisting of 43 and 44. Since the primary sequences of the SSU rRNA of *S. pilosum* and *P. micans* demonstrate about 93% conservation, the observed differences are due to the different models employed. The model based on *C. vulgaris* contains only those structures which are considered to be proven by compensatory base changes among sequences in a large data base of aligned 16S-like rRNAs (Huss and Sogin, 1990). The short region given as "insert\*" is one in which the secondary structure can not be represented with confidence (Gutell et al. 1985). We therefore believe that the model presented here is an accurate representation of the structure of dinoflagellate SSU rRNA.

The complete sequence of the SSU rRNA gene from *S. pilosum* is shown in Fig. 2. aligned to similar sequences from eight other protists. There are seven regions of major divergence among these sequences. Of these seven, the three most variable regions correspond to

stems 10 through 11, E21-1 through E21-6, and 41 and 42. These are recognised as being the hypervariable regions among eukaryotes.

Included with the group of protists indicated in Fig. 2, were sequences from *Paramecium tetraurelia*, *Tetrahymena thermophila*, *Euplotes aediculatus*, *Stylonychia pustula*, with *Dictyostelium discoideum*, *Ochromonas danica*, *Saccharomyces cerevisiae*, as outgroup. The outgroup species were chosen for their close proximity to the dinoflagellate-apicomplexa-ciliate cluster. These sequences (obtained through GenBank v67; Bilofsky et al. 1986) were aligned to the other group with the same weighting applied (Fig. 2). Five hundred bootstrap resamplings were performed using the maximum parsimony program in PAUP. Fig. 3 shows the resulting cladogram. A frequent alternative to this cladogram places the ciliates closer to the group including the dinoflagellates, *S. muris* and *T. annulata*, than to the *Plasmodium* group.

Using the same sequence alignments and weighting, with *Sulfolobus solfataricus*, *Giardia lamblia* and *Physarum polycephalum* (sequences obtained through Genbank) as outgroups, pair-wise distance matrices were computed using DNADISTANCE with Felsenstein's maximum likelihood model for transitions-transversion values on the actual frequencies of nucleotides, with 50 bootstrap replicates. Fitch and Margoliash's method (FITCH) for resolution of the bootstrap resampled distance matrices was used with both global optimization, outgrouping to *S. solfataricus*, and randomization of species added, ten times for each bootstrap replicate. A consensus topology across the 500 resulting phenograms was generated using CONSENSE. One of these phenograms, having the same topology for all groups as the consensus tree, is shown as Fig. 4.

The two trees shown are consistent in their topologies. By either parsimony or distance matrix methods, the dinoflagellates cluster more closely with some of the Apicomplexa (*Sarcocystis* and *Theileria*) than with the Ciliata. This observation is consistent with those of Johnson et al. (1990), Gajadhar et al. (1991) and Barta et al. (1991), and indicates that dinoflagellates are securely grouped among the eukaryotes. Thus, consistent with the suggestion of Hinnebusch et al. (1981) and Loeblich (1984), those characteristics of

dinoflagellates regarded as indicating their "primitive" status with prokaryote-like features, are probably derived. Again, consistent with the conclusions of Ariztia et al. (1991), the dinoflagellates are very distantly related to the chromophytes (as represented by *O. danica*).

In their analysis, using 5.8S rDNA sequence data, Hinnebusch et al. (1981) concluded that *C. cohnii* was distantly related to prokaryotes, and was joined to the tree at the point of divergence between plant and animals, and above the fungi. Although subsequent analyses using SSU rDNA (Hendriks et al. 1991) and partial large subunit (LSU) rDNA (Lenaers et al. 1991) sequences have shown that dinoflagellates are indeed distantly related to prokaryotes, they consistently group the dinoflagellates with the ciliates.

The results of our analyses show that the dinoflagellates are closely linked with the apicomplexans *S. muris* and *T. annulata*. Although the three *Plasmodium* species are always grouped together, they are distant from the other two apicomplexans. A frequent alternative to the cladogram shown in Fig. 3 places the ciliates closer to the group including the dinoflagellates, *S. muris* and *T. annulata*, than to the *Plasmodium* group. Similarly, one alternative to the phenogram in Fig. 4 places the *Plasmodium* group closer to *S. muris*, *T. annulata* and the dinoflagellates. These results raise a possibility that the Apicomplexa are polyphyletic. Johnson et al. (1990) suggest that the Apicomplexa may not be monophyletic, with *Sarcocystis* closely related to the dinoflagellates and the *Plasmodium* group closely related to the ciliates; the latter appear to have a high rate of evolutionary change relative to other species. Alternatively, Barta et al. (1991) suggest that the Apicomplexa are monophyletic, although their analysis has few informative sites. Based on the data available to us, it is difficult to confidently assess apicomplexan phylogeny. More apicomplexans should be analyzed to resolve the details of their phylogeny.

The close affiliation of the ciliates, the apicomplexans and the dinoflagellates based on molecular criteria, prompts the search for independent characters that the three phyla may share. There are various structural analogies found in the ciliates and dinoflagellates (the pellicle, basal bodies associated with the flagella, ejectosomes [trichocysts, mucocysts] and



the possession of multiple copies of genetic information (Orias, 1976). The microtubule-based structure of the apical complex of apicomplexans (Russell and Burns, 1984) shows a great deal of similarity to that found in the peduncle of dinoflagellates (Spero, 1982). In addition, there is immunological evidence for the presence of ferrisuperoxide dismutase in dinoflagellates (Mata et al, submitted), a character they share in common with ciliates (Barra et al. 1990).

Our results also indicate that among the dinoflagellates, *C. cohnii* is more deeply rooted to the Apicomplexa than either *P. micans* or *S. pilosum*. In fact, in some analyses, by either method, *C. cohnii* was rooted to the Apicomplexa. This observation is at variance with Taylor's (1987) phylogenetic scheme for dinoflagellates, which renders the prorocentroids as ancestral. By contrast, Loeblich (1984) represents the prorocentroids as being relatively recent, diverging from the "Zooxanthellales" (the group representing the symbiotic dinoflagellates in the genus *Symbiodinium*, or *Zooxanthella* by Loeblich's taxonomy) in the Triassic. At present, our results are more consistent with this latter scheme, which is also in agreement with the inferred phylogeny based on partial LSU rDNA sequence analysis (Lenaers et al. 1991). It should also be indicated that, although no rDNA sequence is yet available, a congener of *P. micans*, *P. concavum* has been identified as the symbiont of a marine flatworm (Yamasu, 1987). We are currently sequencing the SSU rDNA from *Oxyrrhis marina* (an alternate candidate to the prorocentroids as representing the ancestral dinoflagellate), and it is anticipated that we will soon be able to resolve its phylogenetic position.

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## Figure Legends

Figure 1. A model of the secondary structure of the SSU rRNA from *S. pilosum*. Stem helices are numbered according to Dams et al (1988). Canonical base-pairings are indicated by —, while non-canonical base-pairings are indicated by • or °.

Figure 2. The complete SSU rDNA sequence of *S. pilosum* aligned to similar sequences from eight other eukaryotes. Abbreviations: S.p., *S. pilosum*; P.m., *P. micans*; C.c., *C. cohnii*; S.m., *S. muris*; T.a., *T. annulata*; P.b., *P. berghei*; P.l., *P. lophurae*; P.f., *P. falciparum*; O.n., *O. nova*.

Figure 3. The phylogenetic tree based on 500 bootstrap resamplings of maximum parsimony. SPIL=*S. pilosum*; PMIC=*P. micans*; CCOH=*C. cohnii*; SMUR=*S. muris*; TANN=*T. annulata*; PBER=*P. berghei*; PLOP=*P. lophurae*; PFAL=*P. falciparum*; ONOV=*O. nova*; SPUS=*S. pustulata*; EAED=*E. aediculatus*; PTET=*P. tetraurelia*; TTHER=*T. thermophila*. The length of each branch is indicated. The numbers at the nodes indicate the percentage of bootstrap resamplings that group the clade descending from that node.

Figure 4. A tree topology produced from Fitch and Margoliash's resolution of 50 bootstrap-resampled pair-wise distance matrices (which were calculated using Felsenstein's maximum likelihood model for transition-transversion events), globally optimized and randomized 10 times each for order of species added to the topology under construction. The out-group organisms are not shown. Abbreviations as in Fig. 3, with additionally, SCER=*S. cerevisiae*; ODAN=*O. danica*; DDIS=*D. discoideum*. The scale bar represents 10 changes per 100 nucleotides. The numbers at the nodes indicate the percentage of trees (out of 500 trees) having the group to the right of the node.

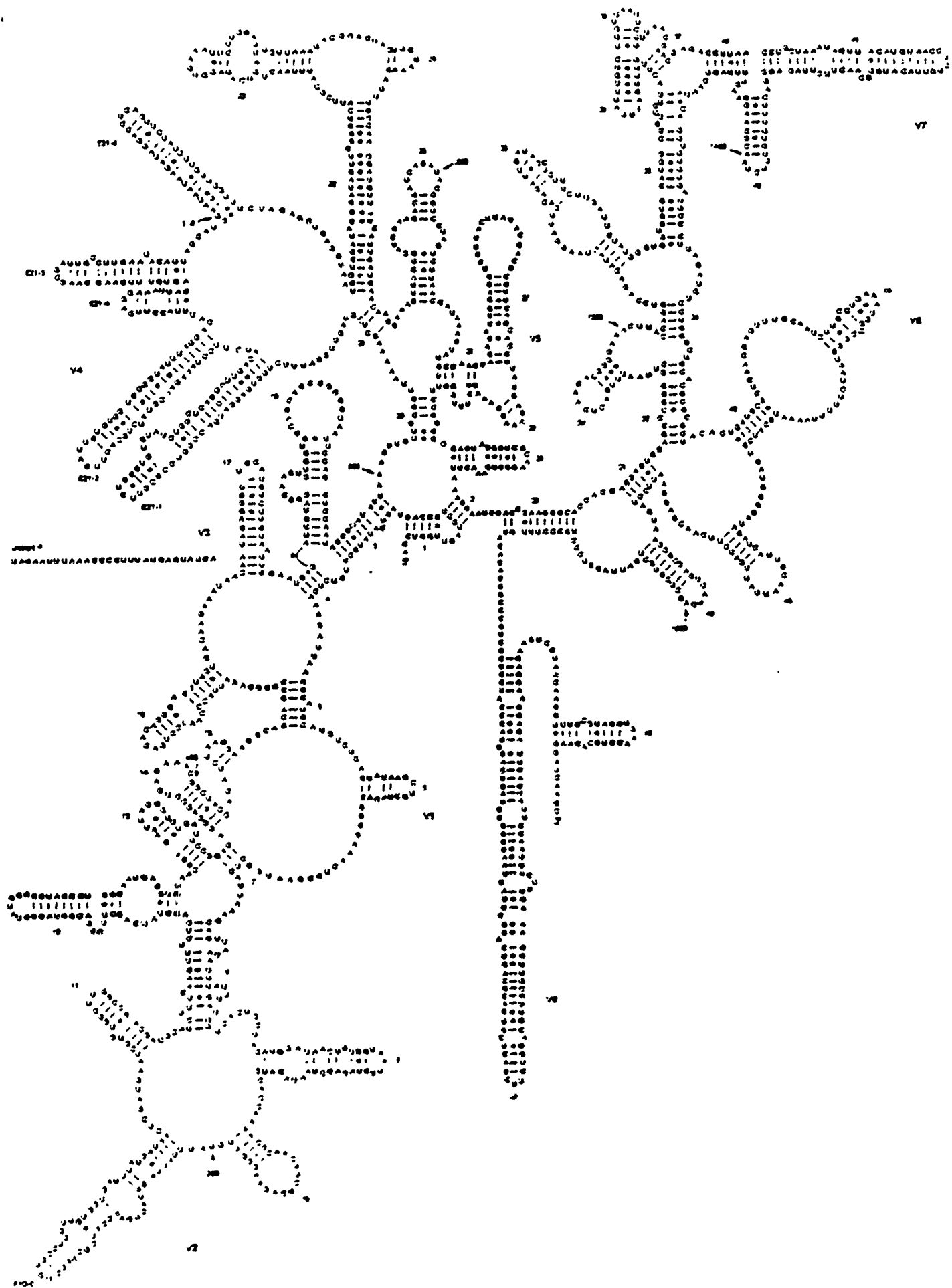


Fig. 2



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S.p. -----TTAATTGAAATATGAAATAATAAATAGGAACTC-AGTTCTA-----TT
P.H. ....T.....G.....
C.C. ....T.....G.A..T.AG..T.T.G.A..T.
S.H. ....T.....A.....C.....T.....G.....
T.A. ....T.....A.....AG.....T.T.G.....
P.B. TGAGAGCCSAATGATT.....TATCACTGCGTTTTCG.....T.....T.....C.A..T.A.....A.A.....TTTTGTC
P.L. TTTTAAATTCCTTTATTCATTTAATGTCATGCAATTTTCG.....T.....T.....G.A..T.A.....A..CTA.AA.T..TTTTGTC
P.F. TTTTAAATTCCTTCATTTTCTTTTTCGTTTTCGAAATTTTCG.....T.....T.....C.A..T.A.....A..CTA.AA.T..TTTTGTC
O.N. ....T.....A.....JA.....T.T.....CC.

S.p. TTCTTCTTTCTAGAGCT-GAGGTAATGTCGATAGCGATAC-TTGGGGCCATTCGTATTTAALTC-TGAGAGGTAATTCCTTCTAGATTTCTTAAAGAGC
P.H. ....A.TA.....C.....
C.C. ..A.....G...A.C.ATC.C..C.A.T.....C.A.....T.....A
S.H. ....GA.....A.....A.TA.....C.....A.....
T.A. ....CTACCA.A.....TA.....A.C.....N.....A.....
P.B. ..T..TC..AT.TTC.....TA..T.C.A.TA.....AG...C.....T.....C.JA.....A.....TC.JG.....A
P.L. ..T..TC..AT.TTC.....TA..T.C.A.TA.....AG...T.....A.....C.JA.....A.....TC.JG.....
P.F. ..T..TC..AT.TTC.....TA..T.C.A.TA.....AG...C.....A.....C.JA.....A.....TC.JG.....
O.N. ..JAG.GA.....A.....A.TA.....A.....A.....C.....T.....A.....C.....T.....

S.p. GACTACTCGGAAAG-ATTTJCCAAGGATTTTTTCATTTGATCAAGAACGAAAGTTAGGGATCGAAJACGATTCACATACCGTCTCTAGCTTTAACCATAAAC
P.H. ....C.....T.....T.....T.....
C.C. C...A.....C.....T.....T.....
S.H. A.....C.....A.....A.....C.....G.....
T.A. A.....C.....A.....A.....G.....C.....
P.B. A..A.....C.....T.AA..AC..C..A.....A.....GT.....G..A.....
P.L. A..A.....C.....T.T.AA..AC..C..A.....A.....GT.....G..A.....
P.F. A..A.....C.....T.T.AA..AC..C..A.....A.....GT.....G..A.....
O.N. A..TA.....C.....A.....A.....

S.p. TATGCCAACTAGAGATTGGAGCTCCTTACT-----CATAGGACTCCTTCAGCACCTTATGAGAAA
P.H. ..G.....TC.....
C.C. C.....GAG.GTC.....ATTATT.....TC.....
S.H. ....G.....A.....AAAA..C.TC.....T.....C.T.....
T.A. ....G.....C.G.....T.....GA.....
P.B. ....G.....GTT.....T.AAAA..TT.TAAA..TAAGATTTCGCTT.....CGGGG..ATTTCCTAGATT..CT.....T.....
P.L. ....G.....GTC.....T.AAAG..GT.AAAA.AAATAAAGATTTCTAATCGAAATTAGAATATCTTTAGCTT..CT.....T.....
P.F. ....G.....GTC.....T.AAAG..GT.AAAA.AAATAAAGATTCATCTT.....TCGAGCTGACTTTTACAGATT..CT.....T.....
O.N. ....G.....G..C.....CGCT.....-TAT.CG--.....G.....

S.p. TCAAAGTCCTTTGGCTTCGGGGGGGAGTATGCTCCCAAGGCTGAAACTTAAAGGAATTGACCGAAGGGCACCACCAGGAGCTGGAGCCCTCGCGCTTAAATTTG
P.H. ....T.....
C.C. ....T.....
S.H. ....C.....
T.A. ....C.....
P.B. ....C.....TCC.....CGA.....J.....A.....C.....T.....
P.L. ....C.....TCC.....CGA.....J.....A.....C.....T.....
P.F. ....C.....TCC.....CGA.....J.....A.....C.....T.....
O.N. ....T.....C.....

P.p. ACTCFACACCGGAAAC TTACCAGTTTCAGACATAGTAAUGATTCACAGATTCATAGCTTTTCGTTGATTTATA TGGGTTGTTGTCATGGCCCTTTCTTAC
P.H. ....A.....C.....
C.C. ....A.....C.....
S.H. ....C.....A.....J.....
T.A. ....C.....T.....T.TA.....AGAGT.....A.....T.....A.....A.....T.....
P.B. ....A.....C.....T.....T.TA.....AGAGT.....A.....T.....A.....A.....T.....
P.L. ....A.....C.....T.....T.TA.....AGAGT.....A.....T.....A.....A.....T.....
P.F. ....A.....C.....T.....T.TA.....AGAGT.....A.....T.....A.....A.....T.....
O.N. ....A.....C.....G.....

S.p. TTGGTGGG-CTGATTTGTTGTTTAAATTTGCTTAAAGCAACGAGACTTAACTTCTAATATGTT-ACATGTAAC
P.H. ....T.....
C.C. ....T.....A.....JA..G.....
S.H. ....T.....A.....JA..T.....T.....
T.A. ....T.....G.....JA..T.....T.....T.....G.....JA..T.....T.....T.....G.....JA..T.....T.....T.....G.....
P.B. ..C...A.TA.....A.....T.....T.....G.....JA..T.....T.....T.....G.....JA..T.....T.....T.....G.....JA..T.....T.....T.....G.....
P.L. ..C...A.TA.....A.....T.....T.....G.....JA..T.....T.....T.....G.....JA..T.....T.....T.....G.....JA..T.....T.....T.....G.....
P.F. ..C...A.TA.....A.....T.....T.....G.....JA..T.....T.....T.....G.....JA..T.....T.....T.....G.....JA..T.....T.....T.....G.....
O.N. ....T.....A.....C.....G.....

```

Fig. 2 (2)



S.p. -----CTCCTTACATDXXCAA-CTT TTAGADTAATTTTDS--TGTATAACJCAAGCAAGTTTGGCAATAACAGGTTCTGTGATCC  
P.H. ....T.....G.....C.....  
C.C. ....A..C..TC...T..G.....A...TC...T.....C.....T.....  
S.H. ....CG.T.T..TATC.....C.....  
T.A. ....ACTG.C.CGTTATCG.....C.DT.A.AA.T.....A.....  
P.b. TTTCTAGTAAGGATGTA.TC.C.TT..T-TA.TG.....A..GA.....A.....A.....C.....  
P.l. TTTTGTGCAAGAATGTA.TTAC.TT..T.TA..G.....A.....A.....A.....C.....  
P.f. TTTTGTGCAAGAATGTA.TTAC.TT..T.TA..G.....A.....A.....A.....C.....  
O.N. ....TAAAG.GAACC.G.....A..A.....A.....A.....C.....  
-----ACTA..A.....

S.p. CCTTAGATGTCCTGGCTGCACCGCCGCTACACTGATGCCCTCACCAGTT-----  
P.H. ....T.....T.....  
C.C. ....T.....AT.....G.....  
S.H. ....T.....ATC.....  
T.A. ....T.....T.....  
P.b. ....A..A..A.....T.....AT.TAA.....CCTTAAATTTATATCTGTGCTTACGTT...TAAAGCCTATGTTTCAGTA  
P.l. ....AA..A.....T.....ATATAT.....ACTAAAAATATATCATTTGTATATGTTAT.ACATATACATTT...GC  
P.f. ....AA..A.....T.....ATATAT.....TTTAAAAATATG...TATATTTGTATCTTTGATGCTTATATTT...TC  
O.N. ....C.....T.....C..ATA..G.....

S.p. TG---CAATCTTCCCTGAAATGCCC-GGGTAATCTTT-TTAAATGCTGATCGGATCGGGATAGATCATTGCCAATTATGATCTTCAACGAGGAATTCGTA  
P.H. .A..TG.C....C..T.G..TTT.....TC.....T.....A.....  
C.C. .A..TTTC....G..G..TT.....C.....T.....CTC.....AG.....  
S.H. .AT..G..C....G.C..C.G.-T.TA.....G.GT.....T.....A.....G.....  
T.A. .AT...-C...G.C..G.G.-.T.....GT.C.....C..T.....G..A..G.G.....G...  
P.b. .ATATTTT..C.C.ACTG..AA.TGTA.....A.C..T.CAT.....T.....A...G.....G...  
P.l. .ATAATTT..C.C.ACTG..AA.TGTA.....A.C..T..AT.....T.....A...G.....G...  
P.f. .CATACTTT..C.C.GCCG..A..GTA.....A.C..T..AT.....T.....A...G.....G...  
O.N. .A..CTTCC.AGCT.C..G.G.CAGCT.....A-GC-.T...G.....T..G.....A...G.....

S.p. GTAAGCCGAGTCATCAGCTCTGCTGATTACGTCCTGCCCTTGTACACACCGCCCTCGGCTCCTACCGA--TTGAGTGATCCGTTGAATTTTCGGA  
P.H. ....A.....  
C.C. ....A..A.....A..T.....AT.....T...C.T.....A.....  
S.H. ....G...A....G...T.C.C.....  
T.A. ....T...A.....T...A.....C.....  
P.b. ....AT..T.....A..T.....C.....AA..AT.A...G..T...  
P.l. ....AT..T.....A..T.....C.....AA..AT.A...G..T...  
P.f. ....AT..T.....A..T.....C.....AA..AT.A...G..T...  
O.N. ....A.....T.C..T.C.....A.....TT.C.....CC..T...

S.p. CTGACCCAGTGCCTCAGCTCTGGA----CGTTGCCCTGGAAAGTTTCATGAACCTTATCACTTAGAGGAAGGAGAGTGGTAACAAGGTTTCCGTAGGT  
P.H. ...CA.....T...T.C...A.....AGC.....AGTGA.....  
C.C. .C.TTC.TCATAAG..TC...CTTT.....GA.G.G.....GG.....AC.G...A.....  
S.H. .C.TTC...TGTG..G.T.C--..GTGTGC.GAA..G.....TG.....A.....  
T.A. .C.T..A--..T-TCC.G..A--..GGAAC.TC.A..G.....TG.....A.....  
P.b. .AAGAAA.TA.AAATTT-----ATTTTTA..TTT.....G.ACC-G.A..T.C...TT..A.....  
P.l. .AAGAAA.AATAAATTTA-----TATTTT...TTT.....AACC-G.A..T.C...TT..A.....  
P.f. .AAGAAA.A.TAAATTA-----ATTTTTA..TTT.....AACC-G.A..T.C...TT..A.....  
O.N. .C.CS.A..CTGOTAT.....TG.....A.C.AG.A..A.....

S.p. GAACCTGCAGAAGGATCAGC-  
P.H. ....G.....  
C.C. ....  
S.H. ....  
T.A. ....  
P.b. ....G.....TT-A  
P.l. ....G.....TT-A  
P.f. ....G.....TT-A  
O.N. ....G.....TT-A

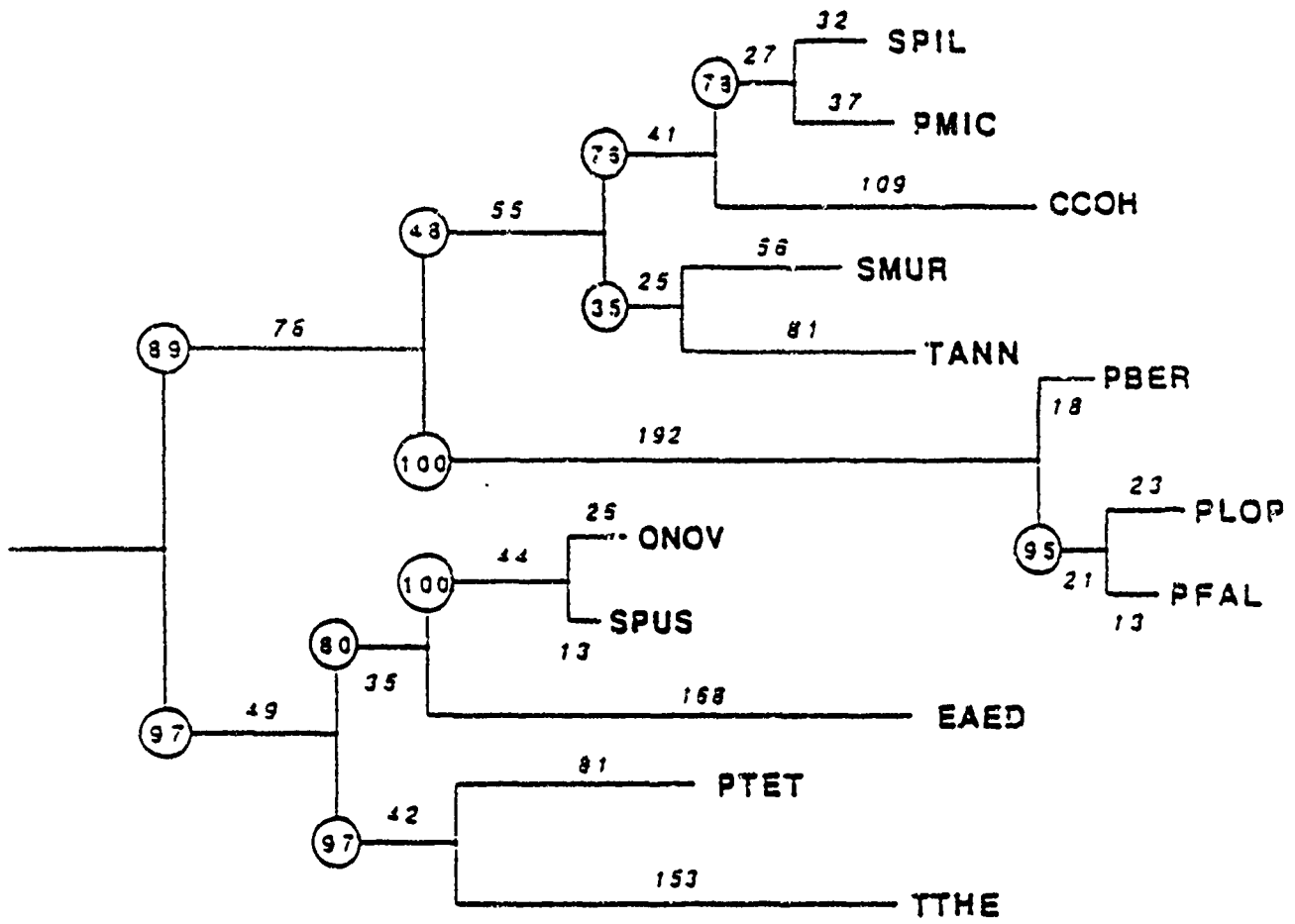


Fig. 3

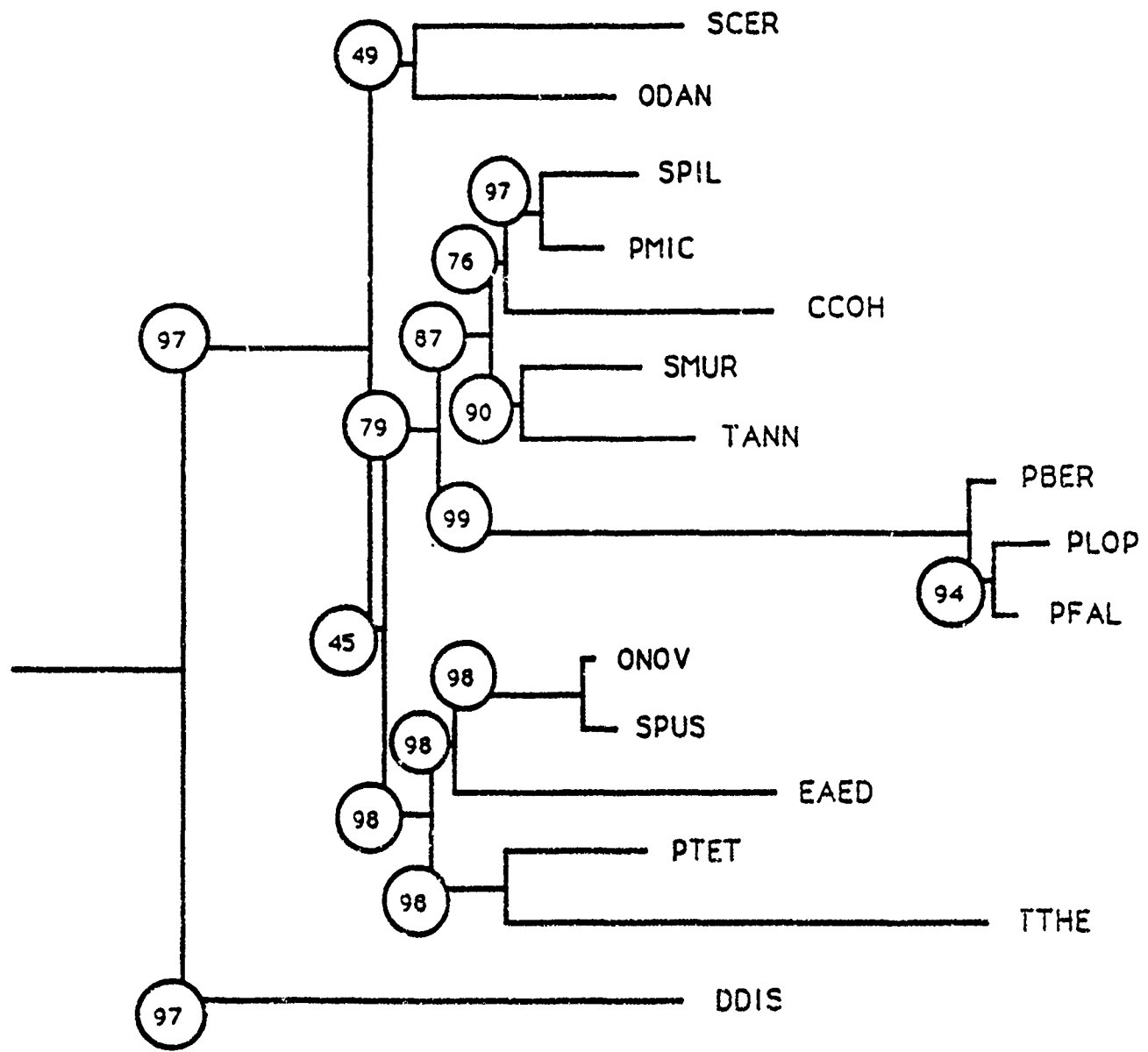


Fig. 4

POLYCLONAL ANTIBODIES AGAINST IRON-SUPEROXIDE DISMUTASES FROM  
*E. COLI* B CROSS-REACT WITH SUPEROXIDE DISMUTASES FROM  
*SYMBIODINIUM MICROADRIATICUM* (DINOPHYCEAE)<sup>1</sup>

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ABSTRACT

Assays for superoxide dismutases (SODs) were performed using cell-free extracts of the symbiotic dinoflagellate *Symbiodinium microadriaticum*, after separation in undenatured polyacrylamide gels, and appropriate inhibitors (KCN and H<sub>2</sub>O<sub>2</sub>). The results indicate the presence of Cu/Zn-, Mn-, and Fe-SODs. In immunoblot assays, polyclonal antibodies against Fe-SOD from *E. coli* B cross-reacted with two major polypeptides in the water-soluble fraction, and one polypeptide in the Triton X-100-solubilized pellet fraction. The polypeptide common to both fractions, with a relative molecular mass of 43.5 kDa, was identified as Mn-SOD. In *S. microadriaticum*, Fe-SOD, found only in the water-soluble fraction, appears to be monomeric, with a relative molecular mass of 49.5 kDa.

*Key index words: Superoxide dismutases; ferrisuperoxide dismutase; immunoblot assays; Symbiodinium microadriaticum; Dinophyceae.*

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Superoxide dismutases (SODs) catalyze the disproportionation of the superoxide radical (Fridovich 1975, Sawyer and Valentine 1981). In conjunction with catalase and various peroxidases, they protect aerobic cells from the deleterious effects of highly reactive oxygen free-radicals produced through the reaction of superoxide with hydrogen peroxide (Asada and Takahashi 1987, Afans'ev 1989). With little difference in their catalytic properties, the SODs are distinguishable by their relative sensitivities to  $\text{CN}^-$  and  $\text{H}_2\text{O}_2$ , and the associated prosthetic metal. In prokaryotes, these enzymes occur as Fe-SOD and/or Mn-SOD (Asada and Takahashi 1987), while in fungi and most "higher" eukaryotes the Cu/Zn-SOD predominates (Asada et al. 1977), but may coexist with Mn- or Fe-SOD, the latter usually associated with mitochondria or chloroplasts. Among the protists and algae, the Mn- and Fe-SODs are most widespread, except in the Charophyceae where the Cu/Zn-SOD predominates (de Jesus et al. 1989). One protist group that has not been studied in this regard are the dinoflagellates. We have employed a combination of enzyme assays in undenatured polyacrylamide gels (with the use of appropriate inhibitors), and polyclonal antibodies against Fe-SOD from *E. coli* B to demonstrate the existence of Fe-SOD in the symbiotic dinoflagellate *Symbiodinium microadriaticum*. An unusual observation is that this organism also shows evidence for Mn-SOD and Cu/Zn-SOD.

*Symbiodinium microadriaticum* cells were grown in ASP-8A as previously described (Matta and Trench 1991). Cells were harvested by centrifugation (9 000 xg) at 4 °C, resuspended in 10mM phosphate buffer (pH 7.8) with 10% (v/v) glycerol and 0.1mM dithiothreitol, and frozen at -70 °C. After thawing, the cells were ruptured by three passages through a French pressure cell at 83 MPa., and the resulting suspension centrifuged for 2 h at 100 000 xg at 4 °C. The recovered supernatant was fractionated with  $(\text{NH}_4)_2\text{SO}_4$  at 20%, 60% and 100% saturation, and the precipitates were dissolved in 50 mM phosphate buffer (pH 7.8), extensively dialyzed at 10 °C against 10 mM Tris-HCl (pH

7.8), and concentrated by vacuum centrifugation (Speedvac). The pellet was washed twice in 10mM phosphate buffer (pH 7.8), and solubilized with 0.1% (v/v) Triton X-100.

*Escheria coli* B was obtained as a paste from Grain Corp., Muscatine, Iowa. Extracts were prepared by digesting 1 g paste for 30 min. at 24 °C with 3.0 μM lysozyme in 250 mM Tris-HCl (pH 8.0), 50 mM glucose and 10mM Na<sub>2</sub>EDTA.

Separation of water-soluble proteins was accomplished by electrophoresis in 10% polyacrylamide gels (PAGE) under non-denaturing conditions. Assays for SOD were conducted as described in Harris and Hopkinson (1978). In addition to the extracts from *S. microadriaticum* and *E. coli*, Fe- and Mn-SOD standards from *E. coli*, and Cu/Zn-SOD from bovine liver (Sigma) were assayed simultaneously. Protein content of samples loaded onto gels was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

The water-soluble extract, and the Triton X-100 solubilized pellet fraction from *S. microadriaticum*, the crude extract from *E. coli* and the standard *E. coli* Fe- and Mn-SOD were rendered 1.5% (v/v) with sodium dodecyl sulphate (SDS), heated at 60 °C for 15 min. Polypeptides were separated by SDS-PAGE in a gradient 5-20% polyacrylamide, using BioRad's standards for the estimation of molecular size. The separated polypeptides were electroblotted onto nitrocellulose and probed with the anti-*E. coli* Fe-SOD serum (1:200 dilution) as described by Roman et al. (1988). Control assays were conducted using pre-immune sera.

Assays for SOD in aqueous extracts of *S. microadriaticum* separated in native PAGE show (Fig 1, track 1) that four zones of activity are resolved (A - D). Treatment of gels with 10mM KCN, a known inhibitor of Cu/Zn-SOD (Beauchamp and Fridovich 1973, Giannopolitis and Ries 1977) prior to the assay for SOD, eliminated band C, suggesting that it represents Cu/Zn-SOD. We observed that 1mM CN<sup>-</sup> did not inhibit Cu/Zn-SOD in extracts of *S. microadriaticum*, but did inhibit Cu/Zn-SOD from bovine liver (data not

presented). We were unable to resolve enzyme activity in gels of the separated Triton X-100-solubilized pellet fraction, but SOD activity has been demonstrated in this fraction (Matta and Trench 1991).

Treatment of gels with 5mM H<sub>2</sub>O<sub>2</sub> (a known inhibitor of Fe- and Cu/Zn-SOD [Lavelle et al. 1977; Dougherty et al. 1978; Kanamatsu and Asada 1979; Asada and Takahashi, 1987]) prior to the assay for SOD eliminated bands A and C. Since CN<sup>-</sup> eliminated only band C, this observation suggests that band A represents Fe-SOD. The remaining bands, B and D, represent Mn-SOD. Treatment of extracts of *E. coli* with 5mM H<sub>2</sub>O<sub>2</sub> also eliminated the two lower bands shown in Fig. 1 (track 2). The lowest band is Fe-SOD (track 3); the higher band could be either another isoenzyme of Fe-SOD or the hybrid Mn/Fe-SOD (Dougherty et al. 1978). The remaining upper-most band represents Mn-SOD (track 4).

Figure 2 (track 1) shows that in immunoblot assays, the anti-*E. coli* Fe-SOD antibodies cross-reacted with two major (and one minor) polypeptides in the soluble fraction from *S. microadriaticum*, with M<sub>r</sub> values of 43.5, 49.5kDa (and 66kDa) respectively. The Triton X-100 solubilized pellet fraction demonstrated a single cross-reactive polypeptide with a M<sub>r</sub> value 43.5 kDa. In extracts of *E. coli*, a single major polypeptide of M<sub>r</sub> 20kDa cross-reacted with the anti-*E. coli* Fe-SOD antibodies. In none of these assays did control pre-immune serum demonstrate any cross-reactivity.

The anti-*E. coli* Fe-SOD antibodies cross-reacted strongly with standard (Sigma) Fe-SOD and less strongly with Mn-SOD from *E. coli*. This observation is not surprising in view of the high primary sequence and structural homology demonstrated by these two proteins (Asada et al. 1977; Steinman and Hill 1973; Takao et al. 1990; Grace, 1990). Based on the observation that in *Euglena gracilis* Fe-SOD is located in the soluble fraction, while Mn-SOD is thylakoid-bound (Dougherty et al. 1978) in the chloroplast, we conclude that the weakly reactive 43.5 kDa polypeptides in the soluble and pellet fractions of *S. microadriaticum* are Mn-SOD. The strongly reactive 49.5 kDa polypeptide found in the

soluble fraction represents Fe-SOD, which in *S. microadriaticum*, may be either monomeric or homodimeric. Ferrisuperoxide dismutases from bacteria, cyanobacteria and *Euglena* are homodimeric, the subunits having  $M_r$  values of 19 - 22 kDa (Slykhouse and Fee, 1976; Dougherty et al. 1978; Kanematsu and Asada, 1979). Our observation of an apparent homodimeric Fe-SOD of 20k Da in *E. coli* B is therefore consistent with these reports. If the polypeptide in *S. microadriaticum* is homodimeric, its holoprotein would be more similar in size to that of *Tetrahymena pyriformis* than to *E. coli* or *Euglena*. Fe-SOD from *T. pyriformis* is a tetramer with subunits of 22.7 kDa (Barra et al. 1990).

The role of the superoxide dismutases in protecting aerobic organisms from the potentially deleterious effects of oxygen free radicals is well known (Elstner, 1982; Halliwell and Gutteridge 1989). In symbiotic associations between dinoflagellates and marine invertebrates, the combined activities of the superoxide dismutases in the symbionts have been found to show a positive correlation with oxygen tension (Dyken and Shick 1982; Matta and Trench 1991), but the precise form of SOD that responds to elevated oxygen has not been resolved. Although evidence for Cu/Zn-SOD in symbiotic dinoflagellates has been reported (Lesser and Shick 1989), and confirmed in this paper, the presence of Fe-SOD and Mn-SOD in dinoflagellates has not been demonstrated previously. Our observations would suggest that Mn-SOD is probably responsible for the increased activity (on a protein-specific basis) reported (Matta and Trench 1991) in the Triton X-100-soluble pellet fraction from *S. microadriaticum*, when the cells were grown under hyperoxic conditions.

It used to be generally accepted that phylogenetically, Fe-SOD and Mn-SOD are distributed principally among the prokaryotes and some "lower" eukaryotes. Although as a gross approximation, this still holds true for Fe-SOD, Mn-SOD is found in "higher" plants. Cu/Zn-SOD is usually regarded as the dominant form of the enzyme in eukaryotes, with the Fe- and/or Mn-SOD associated with organelles (mitochondria or chloroplasts). However, Cu/Zn-SOD has recently been reported (Lesser and Stochaj 1990) in the cyanobacterium



*Prochloron*, and is known to occur in several eubacteria (Grace 1990). The inferred phylogeny of the dinoflagellates based on small subunit ribosomal RNA sequences (Johnson et al. 1990; Gajadhar et al. 1991; Barta et al. 1991; Sadler et al. 1991) indicates that they share a common ancestry with the apicomplexa and the ciliates. Although no information on the metal associated with the superoxide dismutases in the apicomplexa is available, it may be significant that both the ciliates (Barra et al. 1990) and the dinoflagellates (this paper) possess ferrisuperoxide dismutases. Studies are currently underway using immunocytochemical techniques to determine the subcellular localization of the various superoxide dismutases in *S. microadriaticum*.

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Figure legends.

Figure 1. Patterns of electrophoretic migration of superoxide dismutates from *S. microaerophilicum* (track 1, 100 µg protein); *E. coli* B, crude extract (track 2, 100 µg protein); and commercial (Sigma) standard *E. coli* Fe-SOD (track 3, 15 U or 5 µg protein) and Mn-SOD (track 4, 17 U or 5 µg protein). Track 1 shows the 60 - 100%  $(\text{NH}_4)_2\text{SO}_4$  fraction.

Figure 2. Immunoblot (Western) analyses of a water-soluble extract (track 1) and a Triton X-100-solubilized pellet fraction (track 2) from *S. microaerophilicum*, and a water-soluble extract of *E. coli* B (track 3). Molecular sizes are indicated on the left. On immunoblots where equivalent quantities of proteins were analysed (data not shown), the anti-*E. coli* Fe-SOD serum showed a strong reaction with standard (Sigma) *E. coli* Fe-SOD, and only a weak reaction with standard *E. coli* Mn-SOD.

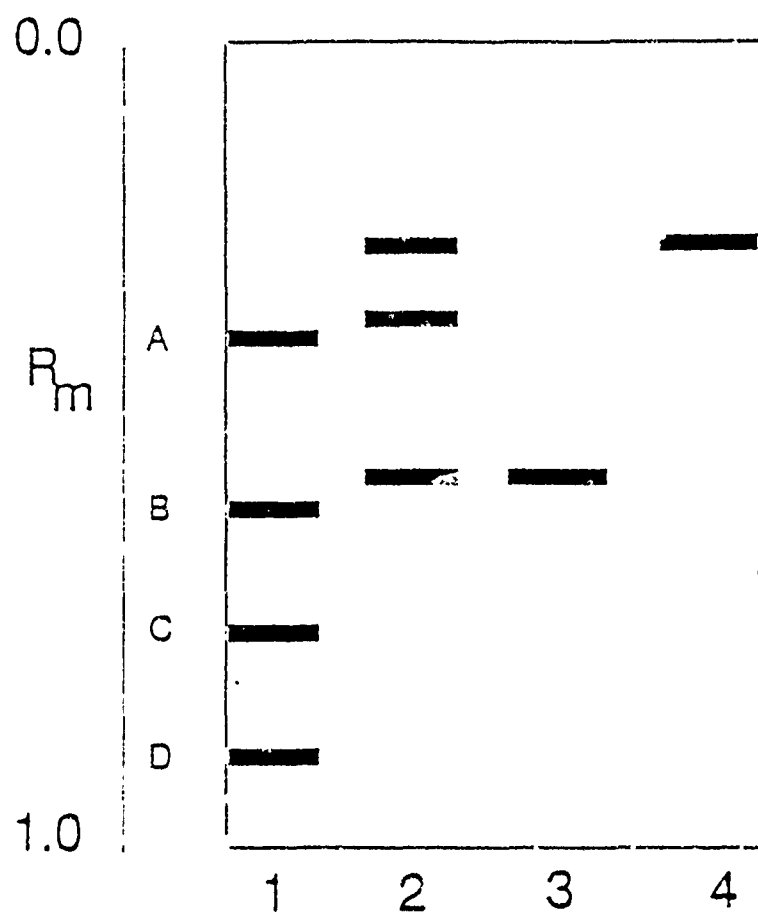


Figure 1

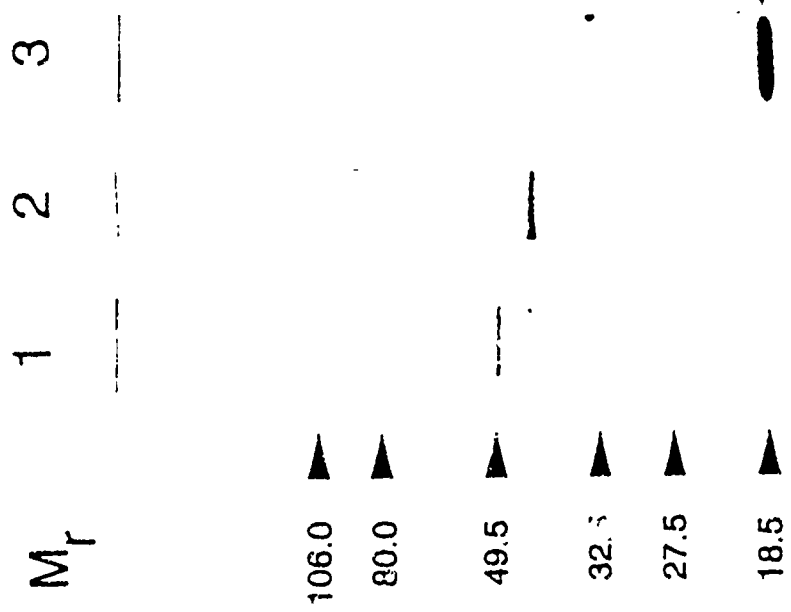


FIGURE 4

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ABSTRACTS

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**CARBONIC ANHYDRASE IN THE HYDROTHERMAL VENT TUBE-WORM *RIFTIA PACHYPTILA*** Jones, R.E. Kochevar\*, J.J. Childress and N.S. Govind.

High levels of carbonic anhydrase activity were detected in two organs of the hydrothermal vent tube-worm *Riftia pachyptila* Jones. The plume, which functions as the site of respiratory gas exchange, exhibited levels of activity ranging from 569-1094  $\Delta\text{pH g}^{-1} \text{min}^{-1}$ . The trophosome, in which the chemoautotrophic endosymbiotic bacteria are housed, also had high levels of activity (216-720  $\Delta\text{pH g}^{-1} \text{min}^{-1}$ ). Other tissues had much lower CA activities, typical of invertebrate tissue (4-61  $\Delta\text{pH g}^{-1} \text{min}^{-1}$ ). Western blots utilizing rabbit anti-chick CAII IgG demonstrated cross reactivity with the *Riftia* carbonic anhydrase, and showed it to migrate in PAGE as a dimer, with bands at approximately 27k and 28k MW. Possible functions of carbonic anhydrase in this symbiosis will be discussed.

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**ROLE OF THYROID HORMONES IN ROTENONE TREATED TELEOST; ANABAS TESTUDINEUS**  
BLOCH : EFFECT ON OXIDATIVE ENZYME ACTIVITIES. Sukhinder P.Chugh and Sukhpal Singh\*, Punjab Agricultural University, Ludhiana, India

Injection of thyroxine ( $T_4$ ) and triiodo-thyronine ( $T_3$ ) stimulated the activities of oxidative enzymes such as cytochrome oxidase and  $\alpha\text{-G}$ -glycero-phosphate dehydrogenase ( $\alpha\text{-GPDH}$ ) in the liver of teleost. Administration of rotenone inhibited the activities of all the enzymes studied except succinate dehydrogenase and  $\alpha\text{-GPDH}$ . Simultaneous administration of  $T_3$  or  $T_4$  with rotenone stimulated the rotenone-inhibited activities of oxidative enzymes. The data clearly indicate that the rotenone-inhibited oxidative metabolism in *A. testudineus* can be reversed by the administration of thyroid hormones.

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**ULTRASTRUCTURE OF THE BODY WALL OF THE MARINE ECHIURAN WORM *URECHIS CAUPO***. J. Menon\*, and A.J. Arp. San Francisco State University, CA.

Structural features of the body wall of the marine worm, *Urechis caupo* at the light and ultrastructural level are investigated. The body wall consists of an outer rugose papillate integument covered by a thin cuticle. The three main cuticular sublayers are identified. Microvilli, cytoplasmic extensions from the epithelial surface, give rise to epicuticular projections. Surrounding the epicuticular projections there is a fibrous mucus material forming a supracuticular coat. The epidermis consists of columnar goblet-shaped cells which on histochemical examination reveal the presence of acid mucopolysaccharides and PAS positive material. Histochemically, two types of mucus secreting cells have been identified: orthochromatic and metachromatic. Ultrastructurally the cells reveal an abundance of golgi and an extensive membrane system indicative of active secretory processes. The metachromatic cells are packed with secretory vesicles containing mucus with a fibrous substructure. The fibrous nature of the mucus is the same as that forming the supracuticular mucoid coat. The orthochromatic mucus cells are characterized by large, distinctive electron dense mucus globules lying in the very large secretory cavity. At higher magnification, the globule core of orthochromatic cells reveal a fine pattern of parallel lines. The possible adaptive role of the mucus secreting integument in the sulfide rich habitat of the worm is discussed.

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**RESPIRATORY PROTEIN FUNCTION IN INVERTEBRATES FROM SULPHIDE RICH ENVIRONMENTS.** N. Sanders\* and B.R. McMahon. Bamfield Marine Station, B.C. and Univ. of Calgary, Alberta, Canada.

Oxygen equilibrium curves were generated using fresh hemolymph samples from three burrowing invertebrate species found in sulphide rich environments: the thalassinids *Callinassa californiensis* and *Upogebia pugettensis*, and the bivalve *Solemya reidi*. For comparison, 3 pandalid shrimps species were examined. High  $\text{O}_2$  affinity at physiologically relevant temperatures, a temperature effect at higher temperatures, a specific L-lactate effect and moderate Bohr shifts were found for the hemocyanin (Hc) of the thalassinids and *Solemya*. The Hc from the pandalids had lower  $\text{O}_2$  affinity, less pronounced temperature effects, a large effect of L-lactate and large Bohr shifts. Thiosulphate caused a change in the Hc- $\text{O}_2$  affinity of some species. Animals kept hypoxic, and with  $\text{H}_2\text{S}$  added, had increased hemolymph L-lactate. Data are discussed in relation to the eco-physiology of the animals. Supported by AHFMR, BMS and NSERC.