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Determination and significance of nitrate reductase in marine fungi

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Abstract

A previous investigation has shown that many terrestrial fungi do not produce nitrate reductase (Na-R). Recently, an examination of marine fungi revealed that all strains tested produce Na-R. Other authors, however, showed absence of this enzyme in a few marine strains. Our reinvestigation of an even broader spectrum of over 80 strains of marine fungi showed the presence of Na-R with only a few exceptions. Since the test was based on determination of nitrite, the first metabolite of Na-R activity, it cannot be excluded that the apparent absence of Na-R in these few cases was due only to a rapid turnover of nitrite by a very active nitrite reductase (Ni-R). Therefore, in all cases in which the presence of Na-R in this test was uncertain, the experiments were repeated by a method inhibiting Ni-R by fluoride and therefore accumulating nitrite which could then be detected. Using this method, it could be shown that in fact all marine fungi tested produce Na-R. Nitrogen often constitutes a limiting factor for growth of fungi. Natural seawater contains considerable amounts of nitrogen in the form of nitrate (up to 600 µg/l). If, however, marine fungi possess Na-R, they could use nitrate as a nitrogen source. Possession of Na-R therefore would constitute an important selective advantage explaining our results that all marine fungi investigated so far contain this enzyme.

Introduction

In 1961 JOHNSON and SPARROW pointed out in their comprehensive review, "Fungi in oceans and estuaries", that the investigation of the physiology of marine fungi is a prerequisite to the understanding of their role in the marine ecosystem. Since, however, only relative little physiological work has been done with marine fungi, most of this work has been carried out on individual enzymes or fungi, and since the methods were often not comparable, we set up a screening program to investigate characteristic enzymes of representative species of all systematic and ecological groups of marine and terrestrial fungi for comparison (MOLITORIS and SCHAUMANN 1986). More than 20 enzymes from several important metabolic pathways (redox-, fat-, carbohydrate-, nitrogen-metabolism) were investigated.

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One of the objectives of our screening program was also to find common properties of marine fungi contrasting with other groups of fungi. Presence or absence of nitrate reductase (Na-R) turned out to be such a differentiating property. A previous investigation by BRESINSKY and SCHNEIDER 1975, had shown that many terrestrial fungi, in particular Basidiomycetes, do not produce Na-R. Recently, our investigation of obligate marine fungi of different systematical and ecological groups (MOLITORIS and SCHAUMANN 1986) revealed that all marine fungal strains tested are able to produce Na-R. Other authors using the same test, e.g. SCHAUMANN et al. 1986, however, showed absence of this enzyme for a few marine strains, such as the marine Ascomycete *Lulworthia*. The objective of the present paper is to clarify this discrepancy by an reinvestigation using a still wider selection of strains and employing additional methods.

Material and methods

Biological material: Fungal strains, systematic position and origins are given in Table 1.

Media, incubation and growth: The strains were grown in test tubes at 22 °C with a periodicity of twelve hours light and twelve hours dark. The GPY-medium consisted of glucose (1 g/l), peptone (0.5 g/l), yeast extract (0.1 g/l) and agar (16 g/l), prepared with synthetic seawater (RILA products, Teaneck, N. J., USA), adjusted to pH 6.0 and autoclaved for 20 min at 121 °C.

Nitrate reductase (Na-R-F), normal test: Tests for the presence of Na-R were performed in the test tubes after 3 weeks of incubation by addition of the Griess-Ilosvaye reagents A and B to both, the test medium containing 15 g/l Na-nitrate, and a control without nitrate following the method of BRESINSKY and SCHNEIDER 1975. The development of a red colour indicating the presence of nitrite was observed after 60 min. The activity is given in arbitrary units from 0 (no activity) to 4 (very active) as the difference between the readings with and without nitrate (control).

Nitrate reductase test with inhibition of nitrite reductase by the addition of fluoride (Na-R+F) following in principle MORTON 1956: The test was conducted in the same way as above with the following changes: The medium was made up with deionized water and contained 8.4 g/l NaF, 1.0 mg/l Na_2MoO_4 (as in RILA) but no other RILA salts because their Ca^{++} ions would precipitate the fluoride. Since fluoride would inhibit the detection of nitrite by the Griess-Ilosvaye reagents, we used a liquid medium without agar, which allowed the removal of fluoride by precipitation with 20 % aqueous $CaCl_2$.

The comparability of the nitrate reductase tests without and with addition of fluoride was established by control experiments in which the medium did not contain fluoride, seawater salts (RILA) or molybdenum salts.

Results and discussion

More than 80 strains of marine fungi comprising all major systematic groups with altogether 54 species (1 Phycomycete, 15 Ascomycetes, 1 Basidiomycete, 21 Deuteromycetes, 6 ascosporous yeasts, 8 basidiosporous yeasts and 3 asporogenous yeasts) were tested for the presence of Na-R by nitrite detection using the Griess-Ilosvaye reagent (Table 1 and Fig. 1).

Principle of the test	a)	b)	c)	d)
Nitrate NO_3 → (Nitrate-Reductase (Na-R)) → Nitrite NO_2 + Griess Ilosvaye Reagent → red colour; → (Nitrite-Reductase (Ni-R)) → Nitric oxide NO	NO_3 … no NO_2	NO_3 … Na-R inhib. … no NO_2	NO_3 … Na-R … NO_2 … Ni-R … NO	NO_3 … Na-R … NO_2 … Ni-R inhibited by fluoride … NO
Colour:	colourless	colourless	colourless	red colour
Na-R activity present:	none	yes	yes	yes
Na-R activity measured:	none	none	none	yes
Explanation:	no Na-R present	Na-R present but inhibited	Na-R present, NO_2 not detectable because metabolized by very active Ni-R	Na-R present and detectable because NO_2 degradation inhibited by fluoride

Fig. 1. The Griess-Ilosvaye test for nitrate reductase. Principle and possible interpretations of results (a, b, c and d).

Table 1 (Part 1). Marine fungal strains, their origin and nitrate reductase activity without (Na-R-F) and with inhibition of nitrite reductase activity by addition of fluoride (Na-R+F).

Number	Strain	Cl.[1]	Orig.[2]	Nitrate reduct.[3] Na-R-F		Na-R+F
M 039	*Acremonium furcatum*	D	KMPB	2.5		4.0
M 037	*Acremonium potronii*	D	KMPB	2.0		4.0
M 038	*Acremonium* sp.	D	KMPB	2.5		4.0
M 141	*Amylocarpus encephaloides*	A	pp	1.5		
M 001	*Asteromyces cruciatus*	D	KMPB	2.5		1.0
M 048	*Asteromyces cruciatus*	D	KMPB	2.5		2.0
M 118	*Candida guilliermondii*	YI	SC	u	*	0.5
M 119	*Candida guilliermondii*	YI	SC	u	*	0.5
M 120	*Candida guilliermondii*	YI	SC	0	*	0.5
M 121	*Candida guilliermondii*	YI	SC	u	*	0.5
M 122	*Candida guilliermondii*	YI	SC	u	*	0.5
M 123	*Candida guilliermondii*	YI	SC	0	*	0.5
M 148	*Candida guilliermondii*	YI	SC	0.5		0.5
M 124	*Candida tropicalis*	YI	SC	1.25	*	0.5
M 040	*Cephalosporium sclerotigenum*	D	KMPB	2.5		2.0
M 139	*Cirrenalia tropicalis*	D	pp	3.0		
M 056	*Corollospora lacera*	A	KMPB	2.5		
M 058	*Corollospora lacera*	A	JK	1.0		
M 015	*Corollospora maritima*	A	KMPB	2.5		
M 059	*Corollospora maritima*	A	JK	2.5		
M 150	*Corollospora maritima*	A	M	3.0		
SR 13	*Corollospora maritima*	A	SR	2.0		
M 087	*Corollospora trifurcata*	A	JK	2.0		
M 103	*Cryptococcus albidus*	YI	JF	2.5		
M 061	*Cytospora rhizophorae*	D	JK	1.5		
M 111	*Debaryomyces hansenii*	YA	SC	u	*	0.5
M 112	*Debaryomyces hansenii*	YA	SC	0	*	0.5
M 113	*Debaryomyces hansenii*	YA	SC	u	*	0.5
M 114	*Debaryomyces hansenii*	YA	SC	0	*	0.5
M 115	*Debaryomyces hansenii*	YA	SC	0	*	0.5
M 116	*Debaryomyces hansenii*	YA	SC	1.0	*	0.5
M 003	*Dendryphiella salina*	D	KMPB	2.5		1.5
M 094	*Digitatispora marina*	B	JK	0.5		0.5
M 125	*Digitatispora marina*	B	pp	0.5		0.5
M 126	*Digitatispora marina*	B	pp	0.5		0.5
M 131	*Digitatispora marina*	B	pp	1.0		0.5
M 147	*Digitatispora marina*	B	pp	1.0		
M 062	*Drechslera halodes*	D	JK	0.5		0.5
M 050	*Doratomyces* sp.	D	KMPB	1.5		2.0
M 045	*Fusarium sambucinum*	D	KMPB	1.0	*	1.0
M 065	*Leptosphaeria australiensis*	A	JK	1.5		
M 090	*Leptosphaeria obiones*	A	JK	3.5		0.5
M 066	*Leptosphaeria oraemaris*	A	JK	1.0		
M 142	*Lulworthia lignoarenaria*	A	pp	2.0		
M 068	*Lulworthia* sp. I	A	JK	u	*	0.5
M 017	*Lulworthia* sp. II	A	KMPB	0.75	*	1.0

Table 1 (Part 2). Marine fungal strains, their origin and nitrate reductase activity without (Na-R-F) and with inhibition of nitrite reductase activity by addition of fluoride (Na-R+F).

Number	Strain	Cl.[1]	Orig.[2]	Nitrate reduct.[3] Na-R-F	Na-R+F
M 018	*Lulworthia* sp. III	A	KMPB	0 *	0.5
M 092	*Lulworthia* sp. V	A	JK	1	
M 069	*Macrophoma* sp.	D	JK	1.5	
M 143	*Marinospora longissima*	A	pp	0.5	1.0
M 071	*Microascus senegalensis*	A	JK	0.75*	0.5
M 072	*Microascus senegalensis*	A	JK	0.5 *	0.5
M 019	*Microascus trigonosporus*	A	KMPB	0.5	3.0
M 006	*Monodictys pelagica*	D	KMPB	0 *	2.0
M 070	*Monodictys pelagica*	D	JK	0.75*	
M 052	*Monodictys* sp.	D	KMPB	2.5	
M 144	*Nautosphaeria cristaminuta*	A	pp	0.5	1.0
M 140	*Orbimyces spectabilis*	D	pp	1.5	
M 053	*Phoma* sp.	D	KMPB	1.0 *	0.5
M 145	*Remispora stellata*	A	pp	0.5	0.5
M 099	*Rhodosporidium bisporidiis*	YB	JF	1.5	
M 100	*Rhodosporidium capitatum*	YB	JF	u *	0.5
M 102	*Rhodosporidium dacryoidum*	YB	JF	2.5	
M 101	*Rhodosporidium malvinellum*	YB	JF	2.0	
M 080	*Rhodosporidium sphaerocarpum*	YB	JF	1.5	
M 081	*Rhodosporidium diobovatum*	YB	JF	2.0	
M 108	*Rhodosporidium toruloides*	YB	JF	3.0	
M 082	*Rhodotorula minuta*	YA	JF	1.25*	
M 104	*Rhodotorula aurantiaca*	YI	JF	1.5	
M 106	*Rhodotorula glutinis*	YI	JF	2.5	
M 107	*Rhodotorula graminis*	YI	JF	2.5	
M 083	*Rhodotorula rubra*	YA	JF	2.5	
M 073	*Savoriella paucispora*	A	JK	1.5	
M 137	*Sigmoidea marina*	D	SN	0	0.5
M 085	*Sporobolomyces salmonicolor*	YB	JF	1.5	
M 079	*Trichocladium achrasporum*	D	JK	1.25*	
M 034	*Ulkenia visurgensis*	P	KMPB	1.0 *	0.5
M 074	*Varicosporina ramulosa*	D	JK	4.0	0.5
M 054	*Verticillium lecanii*	D	KMPB	u *	1.5
M 008	*Zalerion maritimum*	D	KMPB	0.5 *	0.5
M 009	*Zalerion maritimum*	D	KMPB	2.5	

Determination of enzyme activity after 3 weeks of growth.

Cl.[1]: P = Phycomycetes, A = Ascomycetes, B = Basidiomycetes, D = Deuteromycetes, YA = ascomycetous yeast, YB = basidiomycetous yeast, YI = imperfect yeast.

Orig.[2]: SC = S. Crow, Athens; JF = J. Fell, Miami; pp = Gareth Jones, Portsmouth; JK = J. Kohlmeyer, Morehead City; M = Collection of marine fungi, Univ. Regensburg; BSN = S. Newell, Sapeloisland; SR = S. Rohrmann, Regensburg; KMPB = K. Schaumann, Kulturensammlung Mariner Pilze, Bremerhaven.

Na-R activity[3]: activity given in arbitrary units from 0 (no activity) to 4 (very strong activity); u = uncertain activity (positive and negative results in repetitions); * = activity given as average of repetitions.

Most of the fungi showed the presence of Na-R, only a few showed low Na-R activity; in 9 strains the results were uncertain and in 8 strains no activity was found even in repeated tests (Table 1). This was especially true for the group of marine yeasts. Generally, in those cases where several strains of a given species were investigated, the Na-R activities observed were rather similar (Table 1).

The normal nitrate reductase test (Na-R-F) used is based on the detection of nitrite (Fig. 1a-d), the first (and toxic) metabolite of Na-R activity. However, it cannot be excluded, that an observed absence of red colour, indicating the absence of Na-R is due only to a rapid turnover of nitrite by a very active nitrite reductase (Ni-R) (Fig. 1c).

For this reason, all the few cases in which Na-R activity was found in the previous test to be lacking, uncertain or low and in addition some strains with relatively high Na-R activity were reinvestigated using a different method. In this subsequent method Ni-R was inhibited by the addition of fluoride, a specific inhibitor (MORTON 1956, NASON and EVANS 1953), by which nitrite accumulates which can then be detected by the Griess-Ilosvaye reagent as explained in Fig. 1d.

Using this modified version of the Na-R test (Na-R+F), it could be shown that in fact all marine fungi tested produce Na-R, even in those cases where the normal test had given negative or uncertain results (Table 1). In addition, it was shown that generally those strains which display high activity in the normal Na-R test, also exhibit high activity in the modified test (Table 1).

Nitrogen often constitutes a growth-limiting factor in fungi. Nitrogen reductase enables the organism producing this enzyme to use nitrate as a nitrogen supply.

BRESINSKY and SCHNEIDER (1975) in their investigation of nearly 200 terrestrial fungi, using the same test for Na-R as in this paper have shown that most of the Deuteromycetes tested produce this enzyme whereas most of the 135 Basidiomycetes investigated lack Na-R.

MOLITORIS and SCHAUMANN (1986) have shown the presence of Na-R in all obligate and facultative marine fungi they investigated. SCHAUMANN et al. (1986), however, using the same test did not detect this enzyme in some species of *Lulworthia*. In this paper by far the majority of the 81 marine fungal strains investigated showed Na-R activity. Only a few strains gave uncertain results or did not show this enzyme at all. This was found in particular for the marine yeasts, for a few Deuteromycetes and for a few Ascomycetes as in some of the *Lulworthia* strains investigated. Generally, in those cases where several strains of a given species were investigated, the Na-R activities observed were rather similar.

Since the nitrite produced from nitrate by Na-R can be metabolized very quickly by fungi (NICHOLAS 1965) and might therefore evade detection in the medium by our test, we inhibited Ni-R by the addition of fluoride (MORTON 1956, modified) to prevent the metabolization of nitrite and to obtain the accumulation of this metabolite (Fig. 1d).

Using this modified test all strains including those with low, uncertain or no Na-R in the normal test now produced the red colour, indicating the presence of nitrite and therefore production of Na-R.

Of all the enzymes investigated so far using the same method (MOLITORIS and SCHAUMANN 1986) nitrate reductase is the only one common to all marine fungi investigated with the same methodology. Since natural seawater contains

considerable amounts of nitrogen in the form of nitrate (up to 600 µg/l), marine fungi producing this enzyme would be able to use nitrate as a nitrogen source. Possession of Na-R therefore would constitute an important selective advantage for these organisms, explaining our result that all marine fungi investigated so far contain this enzyme.

Similarly, the lack of nitrate reductase in many terrestrial fungi, could be explained by the lack of an urgent need for nitrogen in this environment because of the availability of often high amounts of nitrogen-containing organic material. The absence of Na-R observed in particular in many terrestrial Basidiomycetes can be easily understood since they comprise a high number of mycorrhizal strains which by their symbiosis with higher plants fulfil their nitrogen requirements by direct import of organic material from these plants.

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