

Correlation between virus-sensitivity and isoenzyme spectrum in symbiotic *Chlorella*-like algae

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Summary

The collection of virus-sensitivity *Chlorella*-like algae, symbionts of *Paramecium bursaria* and some others resistant to viruses *Chlorella* strains is described. Specific agglutination by antisera according to the groups of virus-sensitivity algae is observed. The difference of the isoenzyme patterns of two virus-sensitive *Chlorella* strains groups from European and American populations are shown. Into groups this patterns were identical. This characteristics of each free-living, resistant to virus *Chlorella* strains are individual. The correlation between virus-sensitivity, isoenzyme spectrum and serological characterization of *Chlorella* strains are exposed.

Key words: zoochlorellae, PBCV-virus, symbionts, virus-sensitivity, isoenzyme, serological characterization, agglutination, surface antigen specificity

Introduction

Symbiotic *Chlorella*-like algae, zoochlorella are a very specific (in the ecological sense) group of eukaryotic organisms. The sensitivity of zoochlorella cells to viruses was found as a frequent situation for *Hydra viridis* (Meints et al., 1981, Van Etten et al., 1981, 1991) and *Paramecium bursaria* symbionts (Kawakami and Kawakami, 1978; Van Etten et al., 1981, 1983, 1991; Kvitko and Gromov, 1984; Reisser et al., 1986, 1991; Yamada et al., 1991). In both host organisms the symbiotic algae cells are defended against virus attack in individual, host derived "perialgal" vacuoles and become defenseless after distraction of the host cells. The natural selection among zoochlorellae should be working in the direction of forming clones of specialized forms with very stable combinations of characters. We consider this correlation between virus-sensitivity and a zoochlorella status as an empirical rule, the application sphere for this rule we have to investigate.

We studied *Chlorella* strains isolated from *Paramecium bursaria* and specific to them viruses (*Phycodnaviridae*). The variability of such *Chlorella* markers as isoenzyme spectrum, surface antigen specificity and sensitivity for two known ecotypes of viruses is the subject of this paper.

Material and Methods

***Chlorella* strains and culture conditions.** The *Chlorella* strains studied were: a) Isolates from European populations of *Paramecium bursaria*: *Ch. spec.* 241.80 (from the Sammlung of Algenkulturen at Göttingen (SAG), FRG; cf. Schlösser, 1982); *Ch. spec.* strains Pbi, PbAm and PbBS (all from W.Reisser, Göttingen; cf. Reisser et al., 1988). *Ch. spec.* strain OCh (isolated from *P. bursaria* at lake Cherlivoye in Karelia, Russia, by E.S.Krayeva; cf. Kvitko et al., 1988). Isolates from American populations of *P. bursaria*, known as *Ch. spec.* 211-6 (= "*Ch. paramecii*" Loefer, from SAG; cf. Schlösser, 1982); *Ch. spec.* strain NC64A (from J.Van Etten, Lincoln, Nebraska; cf. Van Etten et al., 1983). b) Mutants of strain NC64A (Migunova et al., 1992): As-21-skb-1 – resistant to streptomycin and two toxic aminoacids: kanavanin and b-Alanin; As-21-2Du – resistant to diuron, an inhibitor of photosynthesis; Az4 (k) – fast growth phenotype. c) Type strains of free-living *Chlorella* serving for comparison: *Ch. protothecoides* Krüger 211-7a (SAG); *Ch. vulgaris* Beijerinck 211-11b (SAG); and *Ch. sorokiniana* 211-8k (SAG, all are obtained from the Collection of Algae of St.Petersburg State University; cf. Gromov and Titova, 1988). *Chlorella* strains OCh, PbAm, PbBS, and 241.80 were cultured in FES medium as described by

Table 1. Isoenzyme visualization

Enzymes	Abbreviation	Gel concentration (%)	Visualisation methods (references)
Aspartataminotransferase	AAT	7,5	Braun et al., 1978
Aconitase	ACO	6,0	Wehling, 1986
Diaphorase	DIA	6,0	Wehling, 1986
Malatdehydrogenase	MDH	5,0	Adams, Joly, 1980
NAD-dependent Aromatic Alcohol Dehydrogenase	AADH-NAD	6,0	Jaaska, 1978
NADP-dependent Aromatic Alcohol Dehydrogenase	AADH-NADP	6,0	Schmidt et al., 1984
Superoxide Dismutase	SOD	7,5	Beauchamp, Fridovich, 1971

Reisser (Reisser, 1984). All the other strains studied were cultured in a modified Bold's basal medium (MBBM) as described by Van Etten (Van Etten et al., 1983).

Test for virus-sensitivity. 200 ml of mid-log phase growing algae at a concentration of 1×10^8 to 2×10^8 cells per ml were mixed with 3 ml of 0,75% soft agar MBBM and overlaid on Petri plates containing 15 ml of MBBM plus 1,5% agar. Algal lawns were inoculated by stabbing a needle with diluted suspensions of the NC64A-virus PBCV-1 (kindly given by J. Van Etten) and the Pbi-virus 101G-2, isolated by the plaque assay method described by Van Etten (Van Etten et al., 1983) from a pond near St. Petersburg in 1991. Algal plates were incubated at 23°C in continuous light and observed after 3 days.

Serological characterization of *Chlorella* strains. Antisera were raised against *Chlorella* strains OCh, 241.80, and NC64A, respectively, and assayed by the agglutination test as described (Migunova et al., 1992).

Analysis of isoenzymes. *Chlorella* cells were disrupted in test tubes with glass beads (0,65 mm in diameter) in a 5mM Na-phosphate buffer, pH 6.0, containing 10mM NaCl, 50mM $\text{Na}_2\text{S}_2\text{O}_3$, 2mM dithiothreitol, and 15% sucrose. Homogenization using a Vortex mechanical homogenizer was carried out on ice. The crude cell extract was centrifuged (5000g for 25min) and the supernatant was used for isoenzyme analysis. The samples were layered onto a vertical polyacrylamide gel of various concentration (Table 1) and electrophoresed under native conditions. Isoenzymes were visualized by enzyme-specific reactions (Table 1) using corresponding dyes (see references).

Results

Sensitivity of *Chlorella* strains to viruses

Studied *Chlorella* strains can be divided into 3 groups by sensitivity to viruses (Table 2): 1) sensitive to Pbi-viruses, resistant to NC64A-viruses; 2) sensitive to NC64A-viruses, resistant to Pbi-viruses; 3) resistant both to Pbi-viruses and NC64A-viruses. To the first group belong *Chlorella* strains OCh, PbAm, PbBS, and 241.80, to

the second group belong *Chlorella* strain 211-6, strain NC64A and its mutants Az4 (k), As-21-skb₂-1 and As-21-2Du. The type strains of free-living *Chlorella* studied represent the third group. All virus-sensitive *Chlorella* strains were isolated from European (OCh, PbAm, PbBS, 241.80) and American (211-6 and NC64A) strains of *Paramecium bursaria*, respectively, or are mutants (subclones) of *Chlorella* strain NC64A.

Serological characterization of *Chlorella* strains

Specific agglutination of algal cells depending on *Chlorella* strain and used antiserum is observed in analysis of antiserum titer with the agglutination method. The *Chlorella* strains sensitive to Pbi-viruses show a high inverted titre (2⁸) of agglutination reaction with antisera raised against *Chlorella* strains OCh and 241.80, respectively (Table 2), but they do not show any reaction with the antiserum raised against strain NC64A. Conversely, *Chlorella* strain NC64A and its mutants, and strain 211-6 show a high inverted titre of agglutination with the antiserum raised against *Chlorella* NC64A and do not agglutinate with any antiserum against European *Chlorella* strains.

Chlorella strains, resistant to both the Pbi-virus and NC64A-virus agglutinate very poor with all used antisera, or do not show any reaction (Table 2). Thus, specific agglutination according to the groups of virus-sensitivity is observed.

Isoenzyme analysis

The used antibodies bind to antigens located on the surface of the *Chlorella* cells. In our next experiment we analyzed several isoenzymes which are involved in the metabolism in the cell. We adapted methods that are successfully used for the visualization of isoenzymes in higher plants (Table 1). We have got patterns for 7 isoenzymes (see below).

We began our study with comparing isoenzyme patterns of the analyzed virus-sensitive strains (Fig. 2). The European virus-sensitive strains OCh, 241.80, PbAm and PbBS showed identical electrophoretic mobility of the isoenzymes NAD-dependent Aromatic Alcohol Dehydrogenase (AADH-NAD), NADP-dependent Aromatic

Table 2. Sensitivity of *Chlorella* strains to viruses and serological characterisation of the strains using the agglutination method

<i>Chlorella</i> strains	Sensitivity to		Inverted antiserum titers*		
	Pbi-virus 101G-2	NC64A-virus PBCV-1	Antisera raised against strains Och	241.80	NC64A
European zoochlorella					
Och	+	–	2 ⁸	2 ⁸	2 ⁰
241.80	+	–	2 ⁸	2 ⁸	2 ⁰
PbBS	+	–	2 ⁸	2 ⁸	2 ⁰
PbAm	+	–	2 ⁸	2 ⁸	2 ⁰
American zoochlorella					
211-6	–	+	2 ¹	2 ⁰	2 ⁸
NC64A	–	+	2 ¹	2 ¹	2 ⁸
As-21-skb-1	–	+	2 ⁰	2 ⁰	2 ⁸
As-21-2Du	–	+	2 ¹	2 ¹	2 ⁷
Az 4 (k)	–	+	2 ⁰	2 ⁰	2 ⁸
Type strains					
<i>Ch. protothecoides</i>					
211-7a	–	–	2 ¹	2 ¹	2 ⁰
<i>Ch. sorokiniana</i>					
211-8k	–	–	2 ²	2 ³	2 ³
<i>Ch. vulgaris</i>					
211-11b	–	–	2 ¹	2 ⁰	2 ³

* Inverted antiserum titers were identified using the agglutination method in microtiter plates.

Alcohol Dehydrogenase (AADH-NADP) and Aconitase (ACO). We have obtained similar patterns for AADH-NADP and ACO between the American *Chlorella* strains 211-6 and NC64A and the mutants of NC64A. These patterns are different to the patterns of the European *Chlorella* strains. We did not find any patterns of AADH-NADP for the American isolates.

To address the question of taxonomic grouping of virus-sensitive *Chlorella* strains we included cell extracts of the 3 type strains *Chlorella protothecoides* 211-7a, *Chlorella sorokiniana* 211-8k and *Chlorella vulgaris* 211-11b for the analyses of the isoenzymes Diaphorase (DIA), Aspartataminotransferase (AAT), Superoxide Dismutase (SOD) and Malatdehydrogenase (MDH). Again, the European virus-sensitive *Chlorella* strains OCh, 241.80, PbAm, and PbBS show identical electrophoretic mobility of isozymes of all analysed isoenzymes (Fig. 1). The patterns are different to the isoenzyme patterns of both the American virus-sensitive *Chlorella* strains and type strains. However, the AAT isoenzyme patterns of *Chlorella* sp. strains OCh, 241.80, PbAm, and PbBS and *Ch. vulgaris*

211-11b are identical. Similarity of patterns of all analysed isoenzymes between *Chlorella* strains 211-6 and NC64A, and NC64A subclones As-21-skb-1, As-21-2Du and Az4 (k), is shown in Fig. 1. Isoenzyme patterns are specific for these strains and are not similar to the patterns of any other *Chlorella* strain. However, the Diaphorase patterns of *Chlorella protothecoides* 211-7a are almost similar to these of the American strains. It should be stressed, unlike strain NC64A, its mutants show lower activity or absence of some patterns, and also formation of isoenzyme patterns *de novo*. It is known that every *Chlorella* mutant can show some minor changes in its own specific intensity and quantity of isoenzyme patterns (Bers et al., 1971). Moreover, forms of isoenzymes can change when cells are exposed to the effect of protein synthesis inhibitors (Bers, 1973). We suppose that insignificant changes in isoenzyme spectrum of strain As-21-skb-1 are caused by the effect of protein synthesis inhibitors during the selection of this strain.

Thus, *Chlorella*-like algae strains OCh, 241.80, PbAm, and PbBS isolated from *Paramecium bursaria* of various places in Europe are sensitive to one group of viruses (Pbi-

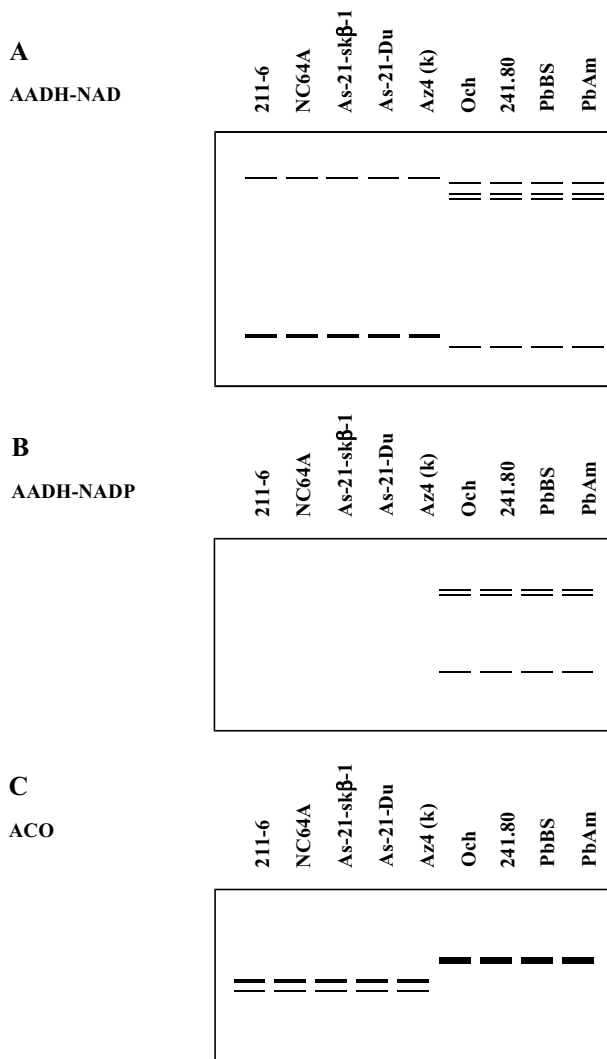


Fig. 1. The electrophoretic mobility of the isoenzymes (A – AADH-NAD, B – AADH-NADP, C – ACO) of the *Chlorella* strains. The European virus-sensitive *Chlorella* strains (OCh, 241-80, PbBS, PbAm), the American *Chlorella* strains (211-6, NC64A, As-21-skb-1, Az4(k), As-21-Du). The difference of the isoenzyme patterns of two virus-sensitive *Chlorella* strains groups from European and American populations are shown. Into groups this patterns were identical.

viruses), they have got similar surface antigenes and they are characterized by identical isoenzyme patterns of analysed isoenzymes: aconitase (ACO), aspartataminotransferase (AAT), diaphorase (DIA), malat dehydrogenase (MDH), NAD-dependent aromatic alcohol dehydrogenase (AADH-NAD), NADP-dependent aromatic alcohol dehydrogenase (AADH-NADP), and superoxide dismutase (SOD). On the other hand, American *Chlorella* strains 211-6 and NC64A isolated from *Paramecium bursaria* of various geographical origin, and the analysed subclones of strain NC64A: As-21-skb₂-1, As-21-2Du, Az4 (k) are sensitive to an other group of viruses (NC64A-viruses), they are characterized by similar

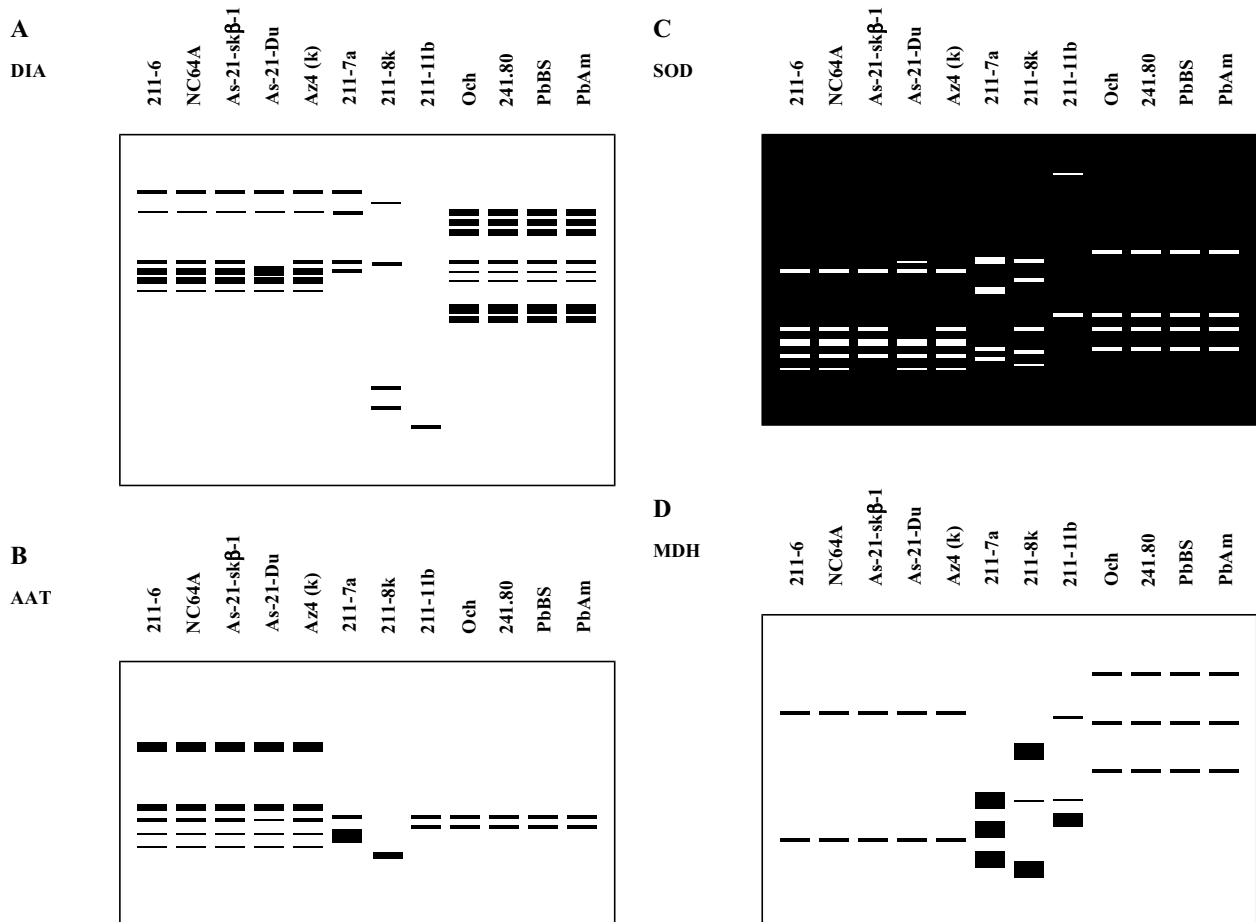
surface antigenes and show similar patterns of all analysed isoenzymes, with the exception of mutants As-21-skb-1 and As-21-2Du showing minor changes for some of the enzymes.

Based on these results we suppose a correlation between *Chlorella* surface antigenes including the receptor for viruses and isoenzyme patterns in virus-sensitive *Chlorella* strains. In order to check our supposition we isolated *Chlorella* viruses which should confirm or refute our grouping of *Chlorella* strains by virus-sensitivity, surface antigenes, and isoenzyme spectrums by attacking *Chlorella* strains with different isoenzyme patterns.

Viruses that infect *Chlorella* NC64A we found in water collected in Tadzhikistan (Voitsekhovskiy et al.,1994). Plaque forming viruses of *Chlorella* Pbi-type were found in water samples collected near St.Petersburg. However, several attempts to find plaque forming virus attacking both *Chlorella* NC64A and *Chlorella* Pbi have been unsuccessful. Thus, isolated by us viruses did not change our grouping of symbiotic *Chlorella* strains.

Discussion

We have analysed virus-sensitivity, surface antigenes of living cells and spectrums of 7 isoenzymes of 6 symbiotic *Chlorella* strains, 3 subclones of symbiotic *Chlorella* strain NC64A, and 3 type strains *Ch. vulgaris*, *Ch. sorokiniana*, and *Ch. protothecoides*. It is now evident, that virus-sensitive *Chlorella* strains, former endosymbionts of the protozoan *Paramecium bursaria* isolated in Europe (strains OCh, 241.80, PbAm, and PbBS) show identical isoenzyme patterns. Based on morphological, physiological and biochemical studies Reisser (1984; Reisser et al.,1988) suggests that strains PbAm, PbBS, and 241.80 are closely related to the *Chlorella vulgaris* group to which belong *Ch. vulgaris*, *Ch. sorokiniana* and *Ch. lobophora*. Our finding that aspartataminotransferase isoenzyme patterns of Pbi-virus sensitive strains and type strain *Ch. vulgaris* 211-11b are identical, supports this suggestion. On the other hand, the Diaphorase patterns of the American strains and type strain *Chlorella protothecoides* 211-7a are almost similar. However, it was not found any similarity between isoenzyme patterns of American virus-sensitive *Chlorella* strains, former endosymbionts of *P. bursaria*, and investigated type strains *Chlorella vulgaris* 211-11b and *Chlorella sorokiniana* 211-8k, although strain NC64A is closely related to free-living *Chlorella vulgaris* (Reisser et al.,1988) by morphological and physiological features, and strain 211-6 belongs to the *Chlorella vulgaris* group (Huss et al.,1989). Analysis of G+C mol % contents and deoxyribinucleic acid reassociation (Huss et al., 1989) revealed that strain 211-6 is closely related to European virus-sensitive *Chlorella* strain Pbi (99 % homology) although they are members of different systems *Paramecium bursaria* – *Chlorella* – vi-



Figs 2–3. Isoenzyme patterns (**A** – DIA; **B** – AAT; **C** – SOD; **D** – MDH) are specific for the European *Chlorella* strains (Och, 241-80, PbBS, PbAm), for the American strains (211-6, NC64A, As-21-skb-1, Az4(k), As-21-Du) and are not similar to the patterns of other virus-resistant *Chlorella* strain (*Chlorella protothecoides* 211-7a, *Chlorella sorokiniana* 211-8k and *Chlorella vulgaris* 211-11b).

rus, but strain 241.80 shows less DNA homology (65%) with strain Pbi although they belong to the same system.

Thus, taxonomy of symbiotic *Chlorella* is a complex problem to solve which we currently have begun electrocaryotyping of *Chlorella* chromosomes using pulsed field gel electrophoresis.

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