Protistology 2 (2), 96-104 (2001)

Protistology

Molecular taxonomy of virus-sensitive *Chlorella* sp. – symbionts of *Paramecium bursaria*

K.V. Kvitko[1], A.V.Migunova[1], D.V. Karelov[2] and M.Ju.Prokosheva[1]

[1] *Laboratory of Microbiology, Biological Research Institute of St. Petersburg State University, St. Petersburg, Russia*

[2] *Department of molecular and radiation biophysics, Petersburg Nuclear Physics Institute, St. Petersburg, Russia*

Summary

Collection of virus-sensitive *Chlorella*-like algae isolated from a ciliate *Paramecium bursaria* is described. Differences are revealed in surface antigens and polymerase chain reaction (PCR) patterns of two groups of virus-sensitive *Chlorella* strains from «northern» and «southern» populations of hosts. A correlation between virus-sensitivity, serological characteristics, and UPPCR-patterns of the zoochlorellae strains DNA is shown. We have proposed considering this alga as the *Chlorella paramecii* group of two contrasting («northern» and «southern») ecotypes, each of them having a specific uniform serotype characteristic. This subdivision coincides with two groups of practically the same set of *Chlorella* sp. strains differing by 8 isoenzyme patterns and described in our previous paper (Linz et al., 1999).

Key words: zoochlorellae, *Chlorella vulgaris/Ch. sorokiniana, Chlorella paramecii*, molecular taxonomy, PBCV-virus, virus-sensitivity of *Paramecium bursaria* symbionts, isoenzymes, serological characterization, agglutination, precipitation, immunoblotting, surface antigen specificity, UP-PCR-patterns, stability of symbionts, DNA base composition, GC% DNA, DNA homology.

Introduction

Symbiotic *Chlorella*-like alga, zoochlorellae from the triple symbiosis *Paramecium bursaria* - *Chlorella* - PBCV-virus, are a very specific (in ecological sense) group of eukaryotic organisms. The final species and genus identification of zoochlorellae has not been accepted due to the absence of reliable species and genus criteria for this agamic alga (Huss et al., 1999).

Originally, viruses of zoochlorella were found in the *P. bursaria* cultures (Kawakami and Kawakami, 1978; Van Etten et al., 1982, 1983, 1991; Kvitko and Gromov, 1984; Kvitko et al., 1988; Migunova et al., 1996, 1999). Under natural conditions these viruses are frequently present in freshwater ponds (Van Etten et al., 1985; Reisser et al., 1986; Yamada et al., 1991, 1993; Kvitko et al., 1994, 1996; Van Etten and Meints, 1999).

In the host organism the symbiotic alga cells are protected from virus attacks by individual «perialgal» vacuoles, but become unprotected after destruction of the host cells. Apart from protection from virus, *P. bursaria* supply endosymbiont with some vitamins and nitrogen-containing organic substances, which make them «auxotrophs»; algae produce for their host

photosynthetic products - oxygen and sugars (maltose and glucose), so the symbiosis practically obligate. Therefore, the natural selection among zoochlorellae should be working in the direction of formation of clones of specialized forms with very stable combinations of signs of adaptation to host cells.

The difference in sensitivity of zoochlorella cells to two types of viruses was found for symbionts of *Paramecium bursaria* from American and European populations (Kvitko and Gromov, 1984; Reisser et al., 1986; Reisser et al., 1988a; Reisser et al., 1988b; Reisser et al., 1988c; Reisser and Widowski, 1992; Van Etten and Meits, 1999).

We have suggested that the «American» and «European» zoochlorellae (the «southern» and «northern» algae, according to our classification) represent two different ecotypes of the same species (*Chlorella paramecii*), which differ by their climate adaptations and sensitivity to different viruses, but are similar by some genome characteristics. We proposed (Kvitko et al., 1996) that these ecotypes of paramecia, zoochlorellae, and viruses should be called, correspondingly, the «southern» (not only American) and «northern» (not only European), as the «southern» ecotypes are able to grow at 32°C, whereas the «northern» ones are not (Migunova et al., 2000).

As a proof of this rule of propagation of viruses and their hosts, we are considering the following facts:

1) we have found in Canada the «European» type of viruses, whereas in the delta of the Volga, the «American» type, i.e. the same viruses that we find in Tajikistan (Voitsekhovsky et al., 1994), in Uzbekistan, and in the continental part of China (Kvitko et al., 1996);

2) the «American» type virus was found in Japan (Yamada et al., 1991, 1993) and on the Pacific coast of China (see Van Etten et al., 1991);

3) the «northern» zoochlorella strains differ from the «southern» ones by their «ts» phenotype; and finally,

4) they are sensitive only to specific «northern» or «southern» viruses.

Earlier we studied 80 symbiotic *P. bursaria* cultures (Migunova et al., 1999), 35 of them containing not only algae, but also zoochlorella viruses. In all cases of the triple symbiosis (*Paramecium bursaria* - zoochlorella - virus), ecotypes of algae and viruses coincided: the «northern» algae were sensitive to the «northern» (Pbi type) viruses, while the «southern» algae, to the «southern» (NC-64-A type) virus (Van Etten et al., 1991; Migunova et al., 1996, 1999).

We consider correlation between the sensitivity of the alga to two types of viruses and the term «zoochlorella ecotype» as an empirical rule. In the current work we are examining the application sphere of this rule.

Any organism can be subjected to genomic analysis, the «genomic dactyloscopy», using universal primers (UP-PCR). The method of UP-PCR was specifically developed and successfully applied to study differences both inside and between species (Sambrook et al., 1989; Bulat et al., 1991, 1992, 1998, 2000). The goal of this paper was to analyze variability of such zoochlorella molecular markers, as specificity of surface antigens, including receptors to viruses, and, hence, sensitivity to two known virus ecotypes, PCR-patterns (and correlation between these characteristics as the basis for their taxonomy).

Materials and Methods

Zoochlorellae strains and culture conditions

The following strains were used:

Isolates from the «northern» ecotype of *P. bursaria*: 241-80 (Goettingen), Pbi (Goettingen and Reisser, 1984.), OCH, OC (Karelia). Isolates from the «southern» (USA) ecotype of *P. bursaria*: 211-6 (Loefer, 1936), NC-64-A, N-1-A.

As a control, we used mutants of NC-64-A: As-18, As120, As-21, resistant to streptomycin, As-1 (=Ac-1), resistant to streptomycin, and two toxic amino acids canavanin and b-alanine; As21DDG1, As21DDG4, 2-deoxy-D-glucose-resistant, AZ4 (=A-3-4) and A-23, a fast growing subclone of NC-64-A (Migunova et al., 1992). Mutants of strain OCH-OCHg (hexamethylenetetramine-resistant), OCHk (canavanin-resistant). Virus-sensitive subclones of NC-64-A, tested with 3 antisera, were designated as H-Act-R, H-3, H-3-1, H-4, H-7.

As the second set of controls, we used typical strains of the free-living *Chlorella*: *Ch. sorokiniana* - 211-8k; *Ch. protothecoides* - 211-7a, *Ch. vulgaris* 211-11b (obtained from the Goettingen collection: Schlosser, 1982), a variant of *Ch. vulgaris* - CALU-157 (from the Algae Collection of St. Petersburg University: Gromov and Titova, 1988), and our strains resistant to virus isolates: Ac-2, 7/19, 6/29(3), A-23-1, OCH/4-2A, As-18-2, and As120-1 (Migunova et al., 1992).

All strains were cultured on the Bold Base Media (BBM) added with various organic substances and tested for sensitivity to virus, as described earlier (Migunova et al., 1992, Linz et al., 1999).

Serological characteristics of algae strains

Rabbit antisera were obtained for all serological reactions. We used 5 antisera: the 5/9 antiserum obtained as a result of 9 subcutaneous injections of living cells of the «southern» strain As-1; the 6/9 antiserum to living cells of the «southern» strain NC-64-A after 9 immunizations; the NC-64-A AS antiserum to living cells of this strain (gift of J.L. Van Etten); the preimmune serum 7/0 (blood of rabbit drawn 7 days

before immunization); the antiserum 7/8 to cells of the «northern» strain OCH after 8 injections of living cells of these algae.

To characterize the tested alga strains, we used the Ouchterlony «double diffusion» method (Migunova et al., 1992) and a modified Western blot (polyacrylamide gel electrophoresis, transfer to nitrocellulose membrane, and incubation with the obtained antisera and specific stains) (Catty and Rikundalia, 1991).

As a source of antigens in these tests, we used homogenates of algal cells and preparations of virus proteins, while in test for agglutination, intact cells (Migunova et al., 1992). In the agglutination test, inverted titers of antisera were determined.

Genotyping of algal strains

For PCR amplification, we used universal primers HE45, L15/AS20a2, A6M13, A2M13, AS2, 19-2M13, AA 2, and L-15 (designed by S.A.Bulat at Eukaryotic Genetics Laboratory, St. Petersburg Nuclear Physics Institute). The PCR amplification was performed in 3 stages, using an Astec PC-700 amplifier (Japan) with a programmed temsperature control system, as described by Sambrook at al. (1989):

1. Denaturation (95°C, for 4 min; 85°C, for 0.01 min) of the mixture 1: DNA (1 ml), primer (1 ml), H_2O (2 ml), vaseline oil (30 ml). After denaturation, the mixture 2 was added: dNTP x 10 (1.5 ml), Taq-DNA-polymerase buffer (SibEnzym) x 10 with Mg (1.5 mkl), DMCO (0.75 ml), H_2O (6.5 ml), Taq-DNA-polymerase (SibEnzym) 2 ml at 5 units each.

2. 35-37 denaturation cycles (95°C, for 1 min); annealing (52°C, for 1 min); synthesis (72°C, for 1 min).

3. Synthesis (72°C, for 10 min; 12°C, for 0.01 min). The resulted amplicons were separated in agarose (1.4%) or polyacrylamide (5%) gel, stained with ethidium bromide, viewed, photographed using an UV transilluminator, and compared with the size standard (PstI digested l phage DNA).

The results obtained were documented using a Kodak digital camera. Statistical analysis was performed as described by Glotov et al. (1992).

To construct dendrograms, we calculated the degree of serological and genetical relationship of algal strains from the presence and absence of bands corresponding to antigens of the studied algae as well as of bands in the gel, which correspond to PCR-fragments of DNA with the definite number of base pairs for each algal strain. The degree of the relationship was expressed as fractions of one.

Results and Discussion

Serological characteristics of zoochlorella strains

Using the agglutination method for evaluation of the antiserum titer, a specific agglutination of algal cells was observed, depending on the strain of algae and the used antiserum (Table 1). The strains resistant to viruses of both the «northern» and «southern» ecotype showed a very poor agglutination and precipitation, or their complete absence, with all used antisera.

Strains OCH and 241-80 (sensitive to «northern» viruses) had a high inverted titer of agglutination (28) with the antiserum 7/8. However, these strains did not demonstrate any reaction with the antiserum 6/9 against the strain NC-64-A. Similarly, the strain

NC-64-A, its mutants, and the strain 211-6 showed a high inverted titer of agglutination with the antiserum 6/9 and did not agglutinate with any antiserum against the «northern» zoochlorella strains.

By the Ouchterlony method, we observed two precipitation bands only in two fast-growing strains, As-21 and AZ4, when the antiserum 6/9 was used. The zoochlorella strains sensitive to the «southern» type viruses showed bands of precipitation with the antiserum 6/9 against zoochlorellae of the «southern» ecotype and a high inverted titer of agglutination (27-29) with this antiserum. Strains of «northern» ecotypes did not agglutinate with this antiserum, nor did they produce precipitation bands.

Hence, our data indicate specific agglutination and precipitation in accordance with the groups of zoochlorellae differing by sensitivity to virus.

Fig. 1a is a diagram of immunoblotting with the serum 5/9 against surface antigens of the «southern» zoochlorella strain, As-1. We tested cell homogenates of 7 zoochlorella strains and one control strain (*Chlorella vulgaris*, CALU157) from our collection. As a result of immunoblotting, we observed 9 bands with the molecular mass from 52 to 146 kDa. The band 79 kDa was common to all 8 strains, while the 52 kDa band, only to zoochlorellae. Two «northern» zoochlorella strains (OCH, 241-80) produced bands of about 146, 123, and 86 kDa. The «southern» zoochlorella strains (NC-64-A, N-1-A, 211-6, and mutants of NC-64-A, A-3-4; As-1) differed from the «northern» strains by the presence of the bands of 126 and 108 kDa. The strain of the free-living *Chlorella* CALU-157 produced a triple band of 132, 126, and 117 kDa, which was absent in zoochlorellae.

In another similar experiment, immunoblots were obtained of 14 protein samples, including 12 algal strains and two viruses, which were tested with 3 antisera.

Immunoblots of the same 14 protein preparations with the serum 7/8 (against the «northern» zoochlorella

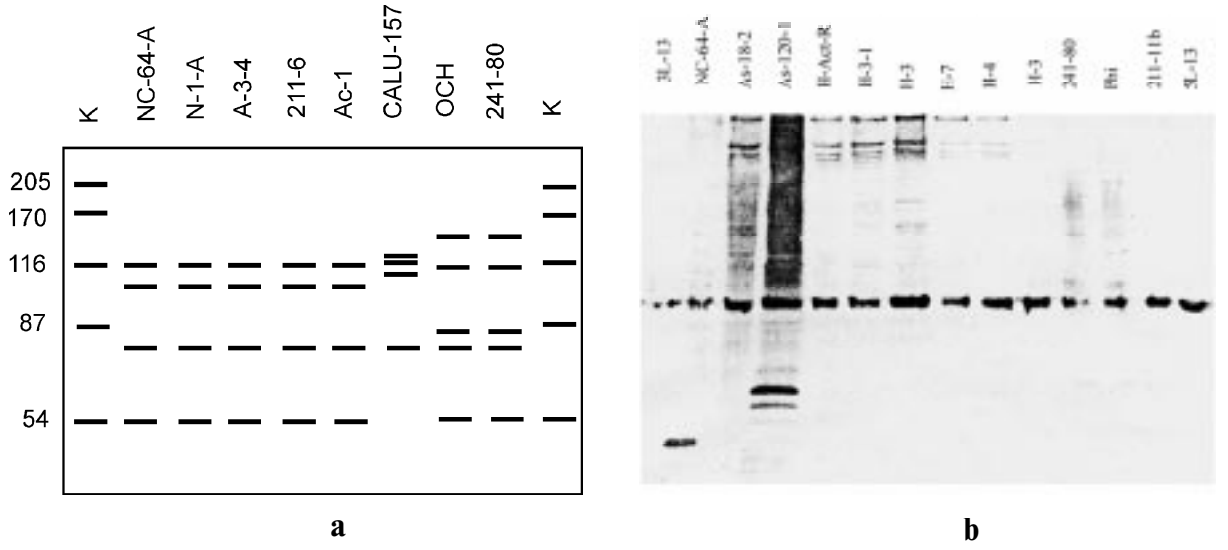

Fig. 1. Immunoblots with rabbit antisera against the «southern» and «northern» zoochlorella strains: **a** - results of immunoloblotting with serum against cells of the «southern» zoochlorella type NC64A, As-1; **b** - data with serum against the «northern» zoochlorella OCH.

OCH) were obtained (not illustrated here). Two of them (the first and the last) contained proteins of the «southern» and «northern» viruses. No specific positive reactions of the 7/8 antiserum to antigens of the both viruses were revealed. Only one band (common to all 14 samples) was visible in lanes with viral proteins. This is the so-called «preimmune effect»; it is due to the fact that blood of rabbit No.7, even prior to immunization, contained unspecific antibodies with a prominent positive reaction. This was proved by the test with the 7/0 antiserum, in which only these «preimmune» bands could be seen.

Proteins of two «northern» zoochlorella strains show a weak positive reaction with the antiserum 7/8. It is also possible to see an unspecific response to this antiserum of two virus-resistant strains and the appearance of a new band of a low molecular mass for homogenates of NC-64-A. Several large proteins of all six NC-64-A subclones showed a cross-reaction with the antiserum 7/8. Thus, we observed the complete correlation between antiserum titers, precipitation reactions, immunoblotting results, and sensitivity to virus in two zoochlorella groups.

The results of immunoblotting (Fig. 1a) and a reflection of these data on dendrogram (Fig. 3a) indicate the complete (1.0) similarity within the «northern» group of zoochlorellae. The same values (1.0) were obtained for all strains of the «southern» zoochlorella. As to similarity between the «southern» and «northern» groups, it was estimated as 0.6, as compared to 0.48 for similarity between the strains sensitive and resistant to viruses.

Hence, our data indicate the presence of two alternative types of specific surface antigens for zoochlorellae of the «southern» and «northern» types corresponding to their virus-specificity. Use of other antisera did not reveal any additional zoochlorella serotypes.

Genotyping of zoochlorella strains, using the polymerase chain reaction (PCR) with universal primers

In this work, we studied ability of 8 primers to amplify DNA sequences of zoochlorella strains. The amplification occurred only when two primers, AA 2 and L-15, were used. The results of separation of PCR-patterns in 5% polyacrylamide gel electrophoresis are presented in Fig. 2.

Amplification with the primer L-15 produced similar PCR-patterns inside each of the 3 groups corresponding to the «northern» and «southern» zoochlorella ecotypes, as well as to two free-living, virus-resistant *Chlorella* sp. strains. The primer L-15 behaves as a conservative one: it reveals stable genome elements (Fig. 2a). Not only bright bands but also intermediate and weak ones were identical inside the groups. The level of identity in the groups corresponded to 0.95-0.85 (Fig. 3c).

A more complex set of PCR patterns (32 fragments) was observed after amplification with the primer AA 2 and a subsequent separation of fragments in the polyacrylamide gel (Fig. 2b). When this primer was used, a great diversity of the PCR patterns was observed, and, as seen on dendrogram (Fig. 3b), the level of identity was 0.9-0.73 inside the groups. It is possible that the primer AA 2 is a variable one.

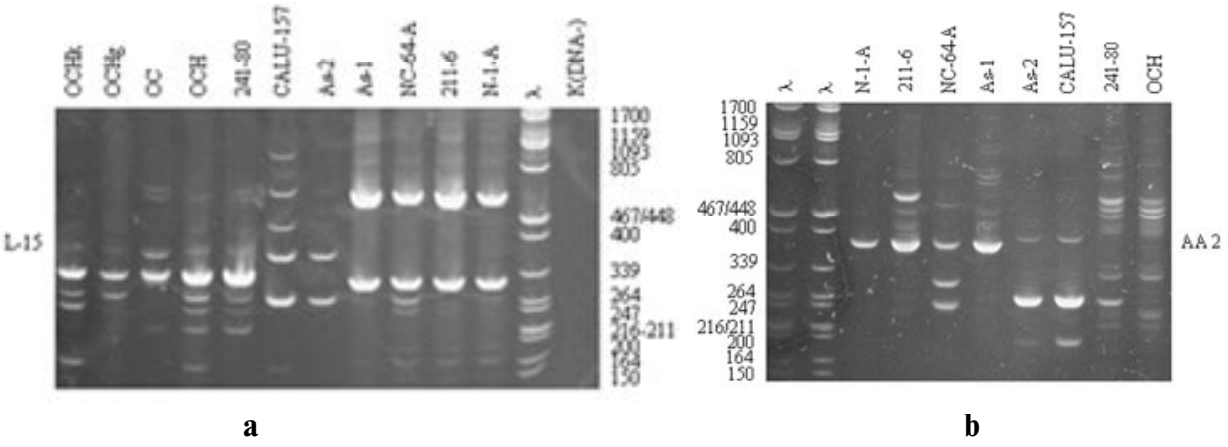

Fig. 2. Results of electrophoretic separation of PCR fragments of DNA of the «southern» (N-1-A, 211-6, NC-64-A, As-1) and «northern» (241-80, OCH, OCHg, OCHk, OC) zoochlorellae (**a**), and virus-resistant strains (CALU-157, As-2) of *Chlorella* in 5% polyacrylamide gel (**b**).

The conservative primer L-15 can be used for a strong PCR identification at the level of ecotypes: of the «northern» (241-80, OCH, OCHg, OCHk, OC) to be different from the «southern» (N-1-A, 211-6, NC-64-A, As-1) and from the free-living, virus-resistant ecotypes (CALU 157, As-2), whereas the variable primer AA 2 allows comparing strains within these ecotypes. Thus, the «southern» ecotype strain N-1-A has only one, ~400 bp band (common to all «southern» strains), whereas the strains 211-6, NC-64-A, and As-1 have from 4 to 6 additional bands. The «northern» strains, 241-80 and OCH, are peculiar to have bands in the region of 264-247 bp.

We observed a similarity between dendrograms constructed on the basis of results of the serology test (immunoblotting) and PCR genotyping (Fig. 3). Comparison of the dendrograms has allowed us to divide the tested *Chlorella* strains into 3 different groups: virus-sensitive «northern» and «southern» zoochlorella strains and more markedly differing from them, virus-resistant *Chlorella* sp.

The same 3 groups of strains were also described in our previous paper (Linz et al., 1999) reporting isoenzyme spectra of symbiotic zoochlorellae. As a control, 3 typical strains were taken: 211-7a (*Ch. protothecoides*), 211-8k (*Ch. sorokiniana*), и 211-11b (*Ch. vulgaris*). A complete similarity was shown within the group of 4 «northern» zoochlorella strains: OCH, 241-80, PbBS, and PbAm.

The same was seen for 5 «southern» forms: two «southern» strains (NC-64-A and 211-6), as well as a fast-growing subclone NC-64-A - AZ4, were identical in all groups of isoenzymes (1.0). A marked mutant of NC-64-A - As-21-skb-1 (=As-1) resistant to streptomycin, canavanin, and b-alanine had small differences (0.98), whereas the mutant As21DDG1 (resistant to 2-deoxy-d-glucose) had the similarity level 0.9.

Similarity between the «southern» and «northern» groups was estimated as 0.47. Similarity of the strain *Ch. vulgaris* 211-11b with strains of the «southern» group was 0.56, while with the «northern» strains it reached 0.66. For comparison, similarity of the strain 211-8k with the «southern» zoochlorellae was 0.48, while with the «northern» type, 0.58. The strain 211-7a showed similarity with the «southern» (0.56) and «northern» (0.63) groups of zoochlorellae. Such is the estimation of similarity, based on the «isoenzyme patterns», between the «southern» and «northern» strains as well as with the *Chlorella* typical strains.

Are the two zoochlorella ecotypes parts of the same species? According to the chemotaxonomic criterion, such as the cell wall composition (Kapaun et al., 1992) and other parameters (Reisser et al., 1988c; Reisser & Widowski, 1992), the «northern» strains of zoochlorellae (in this case, isolates from German populations of *Paramecium bursaria*), like the «southern» strains, were determined as forms close to free-living algae of the group *Chlorella vulgaris/sorokiniana*. However, there is a difference: the cell wall of the «northern» zoochlorella strains contains glucosamine (7-16.6%) and fucose (3.8-6.7%), whereas the «southern» strains contain glucosamine, but only traces of fucose (NC-64-A has only 0.7 M% fucose; Meints et al., 1988). By ability to excrete sugars they also are similar, but differ by spectra of the excretes: the «northern» strains (Pbi and 241-80) produce maltose and glucose, while the «southern» (NC-64-A and 211-6), only maltose (Kessler et al., 1991).

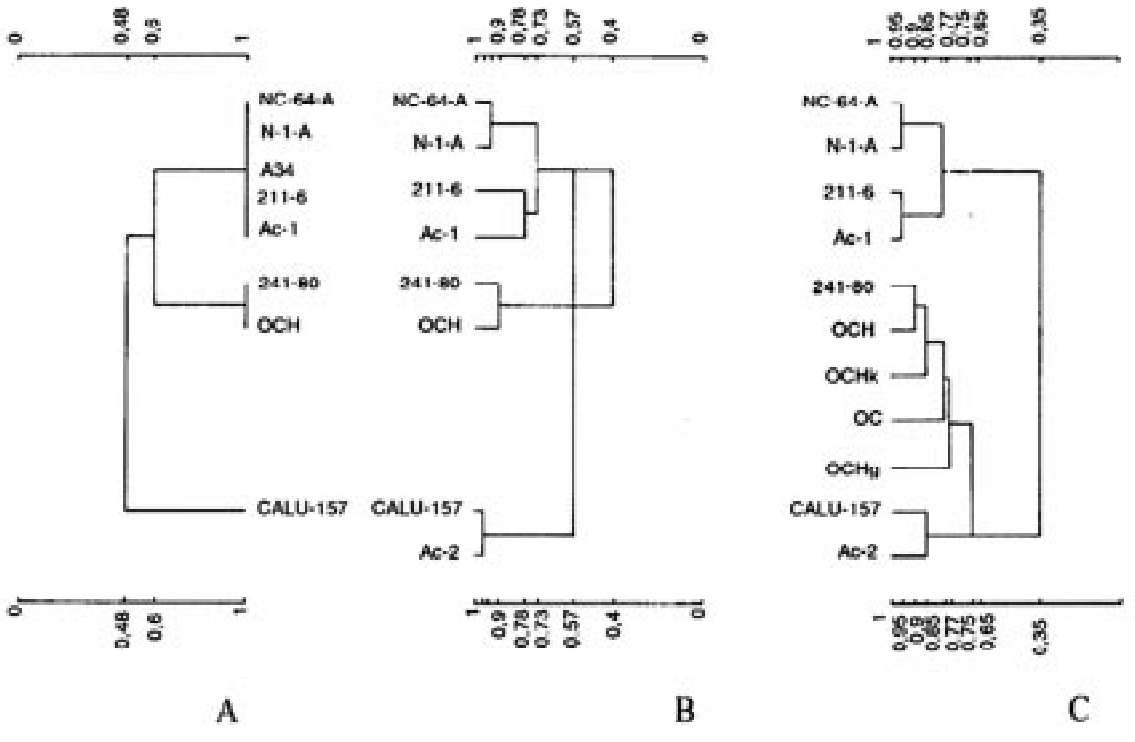

Fig. 3. Comparison of dendrograms plotted on the basis of results of immunoblotting (a) and PCR genotyping using primers AA 2 (b) and L-15 (c).s

We have revealed a similarity of surface antigens of the typical strain *Ch. vulgaris* 211-11b and of the strain NC-64-A. However, this is only phenotypic similarity.

The strains of *P. bursaria* endosymbionts (Pbi, 241-80, 211-6, and NC-64-A) were analyzed for percentage of GC in DNA and degree of homology (DNA hybridization methods) and were found to be rather similar. This parameters of zoochlorellae were studied repeatedly (Duglas and Huss, 1986; Kessler et al., 1988; Huss et al., 1989). In 1986, A.E.Douglas and V. A.R.Huss showed DNA of two strains (211-6 and NC-64-A) to give 79% of hybridization. The same level of homology (79-74%) of these two zoochlorella strains was initially established at hybridization with DNA of the typical strain of *Ch. vulgaris* 211-11b. Later on, Huss et al. (1989) confirmed homology between the «southern» strain 211-6 and the «northern» strains 241-80 and Pbi. The homology level was high, but varied markedly (53% or even 65-99% under rigid conditions and 60-68% under mild reassociation conditions). The level of DNA homology of endosymbiotic strains with the *Ch. vulgaris* (211-11b) was low (from 16-20 to 23-28% under rigid and 27-30 to 52-53%, under relaxed reassociation conditions). Similarly low was this parameter at hybridization with DNA of the *Ch. sorokiniana* strain 211-8k (from 17 to 31% under rigid and 50-54% under mild reassociation conditions).

Kessler with coauthors described the base composition of DNA (in % GC) for symbionts of *P. bursaria*: for NC-64-A 61.7%, for 211-6, 62.5%, whereas two free-living strains of *Ch. vulgaris* had 62.1-62.3%, while those of *Ch. sorokiniana*, 64.8% (Kessler et al., 1988). A year later, Huss et al. (1989) reported a higher content of zoochlorella DNA GC: for 241-80 66.4%, for Pbi and 211-6, 67.1%, while larger limits were established for *Ch. sorokiniana*: from 61.2 to 66.6 GC%, the zoochlorellae being considered *Chlorella* sp. and related to *Ch. vulgaris/Ch. sorokiniana* group. Reisser et al. (1988) indicated even higher % GC values: 74.4 and 73.3% for Pbi and 241-80, respectively. Such diversity of data for zoochlorella strains reported by different authors makes the GC content not too valuable base for taxonomic investigations; the DNA hybridization criterion provides more reliable information about relations of the strains of the «southern» and «northern» ecotypes.

The idea of reality of the existence of the reliable species of the endosymbiotic zoochlorellae, which is called *Chlorella paramecii* (Kessler and Huss, 1990), seems to us interesting; however, it has to be confirmed by ribotyping. So far, this was performed only for one isolate from *P. bursaria* and 4 strains from *Hydra viridis* (Huss et al., 1999). This allowed the authors to identify two types of the virus-sensitive *Chlorella* sp.: the first

Table 1. Serological characterization of zoochlorella using rabbit antisera to "northern" and "southern" zoochlorella strains and their sensitivity to viruses of both types.

Strain	Presence of precipitaion bands by using antisera to "southern" type		Agglutination titer for antisera to		Sensitivity to virus		Correlation of serology characters and reaction on virus
	slow	fast	NC64A 6/9	OCH 7/8	PBCV-1 southern	PBCV-2L northern	
NC-64-A	+	-	2^9	0	+	-	+
	+	-	2^9	0	+	-	+
	+	-	2^9	0	+	-	+
	+	+	2^9	0	+	-	+
	+	-	2^7	0	+	-	+
	+	-	2^7	0	+	-	+
	+	-	2^9	0	+	-	+
	-	-	2	0	-	-	+
	+	+	2^8	0	+	-	+
	+	-	2^9	0	+	-	+
	-	-	2	0	-	-	+
7/19	-	-	2^3	0	-	-	+
6/29(3)	-	-	2	0	-	-	+
OCH	-	-	0	2^8	-	+	+
OCH/4-2	-	-	0	0	-	-	+
241-80	-	-	0	2^8	-	+	+
Total	9+, 7-	2+, 14-	9+, 7-	2+, 12-	9+, 7-	2+, 14-	16+, 0-

isolate (from *P. bursaria*) was closer to *Ch. sorokiniana*, while the second group (*Hydra viridis* symbionts, their viruses are different: see Van Etten et al., 1982, 1991) was closer to *Ch. protothecoides*, its cells differing by morphology of chloroplasts and by the absence of pyrenoid. We hope to perform ribotyping of our collection in future. At present, we have only data for two ecotypes that were genotyped using the PCR priming method.

If to take into consideration the data about a high level of DNA homology between representatives of the «southern» and «northern» strains (Huss et al., 1989), as well as our data about their similarity by criteria of genomic dactyloscopy, we may agree with the opinion (Kessler and Huss, 1990) that symbionts of *Paramecium bursaria* could belong to the *Ch. paramecii* species called «nomen nudum» by Shihira and Kraus (1965). This species still is to be characterized, and it should be subdivided as a minimum into two zoochlorella ecotypes that differ by their sensitivity to 32°C, by surface antigens, and by sensitivity to two types of viruses.

Thus, taxonomy of symbiotic *Chlorell*a-like algae is a complex problem that we attempted to solve in this study, using immunological methods in combination with the molecular method of PCR genotyping.

Acknowledgments

The work was supported by the Russian Foundation for Basic Research (project 98-04-49842). The authors thank Prof. W.Reisser (Goettingen, Germany) for kindly providing strains Pbi, PbAm, and PbBS, and Prof. J.Van Etten (Lincoln, Nebraska, USA) for strain NC-64-A, virus PBCV-1, and for sample of NC-64-A antiserum. We are grateful to Dr. S.A.Bulat (Department of Molecular and Radiation Biophysics, St. Petersburg Nuclear Physics Institute, Gatchina, Russia) for supplying us the primers and for discussion of the results. The authors are grateful to Dr. L.Z.Pevzner for editing the English version of the manuscript.

References

Bulat, S.A., Mikhailov, N.V. and Korolyuk A.M. 1991. Gene identification of bacterial species and serovars by the method of polymerase chain reaction with universal oligonucleotides: redentification of previously isolated *Yersinia pseudotuberculosis* strains. J. of Microbiol., Epidemiol. and Immunobiol. 12, 2-7 (in Russian).

Bulat S.A., Kaboev O.K., Mironenko N.V., Ibatullyn

F.M., Luchcina L.A., and Suslov A.V. 1992. Polymerase chain reaction with universal primers for genomic study of genomes. J. Soviet Genetics. 28, 549-557 (in Russian with English summary).

Bulat S.A., Lubeck M., Mironenko N.V., Jensen D.F., and Lubeck P.S. 1998.UP-PCR analisis and ITS1 ribotyping of strains of *Trichoderma* and *Gliocladium* Mycol. Res.102, 933-943.

Bulat S., Lübeck A.M, Alekhina I.A., Jensen D.F., Knudsen I.M.B and Lübeck P S. 2000. Identification of an UP-PCR derived SCAR marker for an antagonistic strain of *Clonostachys rosea* and development of a strain-specific PCR detection assay. Applied and Environmental Microbiology. 66, 4758-4763.

Catty D. and Rikundalia. 1991. Western-blotting and immunochemical dyeing of antigen bands. In: Antibodies. A Practical approach. (Ed. Catty D.). Mir, Moscow. pp. 230-237 (in Russian).

Douglas A.E. and Huss V.A.R. 1986. On the characteristics and taxonomic position of symbiotic *Chlorella*. Arch. Microbiol. 145, 80-84.

Glotov N.V., Jyvotovsky L..A., Hovanov N.V. and Hromov-Borisov N.N. 1982. Biometry. Leningrad University Press. (in Russian).

Gromov B.V. and Titova N.N. 1988. Collection of Algae at Biological Institute of Leningrad University (CALU). In: Algae Collections in the USSR. Pusshino. pp.52-91 (in Russian with English summary).

Huss V.A.R., Huss G., and Kessler E. 1989. Deoxyribonucleic acid reassociation and interspecies relationships of the genus *Chlorella* (*Chlorophyceae*). Plant Syst. Evol. 168, 71-82.

Huss V.A.R., Frank C., Hartman E.C., Hirmer M., Kloboucek A., Seidel B. M., Wenzeler P., and Kessler E. 1999. Biochemical taxonomy and molecular phylogeny of the genus *Chlorella* sensu lato *(Chlorophyta*). J. Phycol. 35, 587-598.

Kapaun E., Loos E. and Reisser W. 1992. Cell wall composition of virus-sensitive symbiotic *Chlorella* species. Phytochemestry. 31, 3103-3104.

Kawakami H., and Kawakami N. 1978. Behavior of a virus in a symbiotic system *Paramecium bursaria – zoochlorella*. J. Protozool. 25, 217-225.

Kessler E., Huss V. A. R. and Rahat M. 1988. Species-specific ability of *Chlorella* strains *(Chlorophyceae)* to form stable symbioses with *Hydra viridis.* Pl. Syst. Evol. 160, 241-246.

Kessler E. and Huss V.A.R. 1990. Biochemical taxonomy of symbiotic *Chlorella* strains from *Paramecium* and *Acanthocystis*. Bot. Acta. 103, 140-142.

Kessler E., Kauer G. and Rahat M. 1991. Excretion of the sugar by *Chlorella* species capable and incapable of symbiosis with *Hydra viridis*. Bot. Acta. 104, 58-63.

Kvitko K.V. and Gromov B. V. 1984. New findings of a titratable infective zoochlorella virus. Doklady AN USSR 279, 998-999 (in Russian with English summary).

Kvitko K.V., Krayeva E.S. and Mamkaeva K.A. 1988. Interactions of a virus and symbiotic *Chlorella* from Karelian strains of «Green Ciliate» *Paramecium bursaria*. Abstracts of All Union Symp. Plant Ultrastructure.Kiev, p.208.

Kvitko K.V., Voicechovskij E.V., Migunova A.V., Pioch D., and Linz B. 1994. Symbiotic index as a valuation of the conditions in water ecosystems, an example of poligon "Krasny Bor" basins and its sanitary zone. Problems of ecology and natural preservation. 4, 47-57.

Kvitko K.V., Zelennikova O., Migunova A. V., Voitsekhovsky E., Litvinov D. and Safonova E.Th.1996. Ecological and climatic rules for PBCV-virus circulation. Abstracts of X-th International Congress of Virology. Jerusalem. Israel, p.250.

Linz B., Linz A., Migunova A. V. and Kvitko K. V. 1999. Correlation between virus-sensitivity and isoenzyme spectrum in symbiotic *Chlorella*-like algae. Protistology. 1, 76-81.

Loefer J.B. 1936. Isolation and growth characteristics of the zoochlorella of *Paramecium bursaria.* Am. Nat. 70, 184-188.

Meints R.H., Burbank D.E., Van Etten J.L. and Lamport D.T.A. 1988. Properties of the *Chlorella* receptor for the virus PBCV-1. J. Virology. 164, 15-21.

Migunova A. V., Kvitko K. V. and Knyaseva N.V. 1992. Variability of serological characteristics in labelled virus-sensitive zoochlorella strains symbiotic with *Paramecium bursaria*. Algologya. Kiev, 2, 43-48 (in Russian with English summary).

Migunova A.V., Skoblo I.I., Kraeva E., Litvinov D., Voitsekhovsky E., and Kvitko K.V. 1996. Zoochlorella viruses carrier status of *Paramecium bursaria*. Abstracts of X-th International Congres of Virology. Jerusalem, Israel, p.251.

Migunova A.V., Kvitko K.V., Skoblo I.I. and Prokosheva M.J. 1999. Ecology of symbiotic system:*Paramecium bursaria-Chlorella-virus*. Vestnic SPb GU. Ser.3. 3. 17, 80-83.

Migunova A.V., Kvitko K.V., Prokosheva M.J. and Litvinov D.B. 2000. The temperature influence for the reproduction of *Paramecium bursaria, Chlorella* and PBCV-viruses in the 3-th symbiosis system. Vestnic SPbGU. Ser.3. 1. 3, 65-75.

Reisser W. 1984. The taxonomy of green algae endosymbiotic in Ciliates and a Sponge. British phycological J. 19, 309-318.

Reisser W., Becker B. and Klein T. 1986. Studies on ultrastructure and host range of a *Chlorella* attacking virus. Protoplasma. 135, 162-165.

Reisser W., Burbank D.E., Meints S.M., Meints R.H., Becker B., and Van Etten J.L. 1988a. A comparison of viruses infecting two different *Chlorella*-like green alga. Virology. 167, 143-149.

Reisser W., Klein T. and Becker B 1988b Studies on phycoviruses I. On the ecology of viruses attacking *Chlorellae* exsymbiotic from an European strain of *Paramecium bursaria*. Arch. Hydrobiol. 111, 575-586

Reisser W., Vietze S. and Widowski M. 1988c. Taxonomic studies on endocytobiotic *Chlorophyceaen* algae isolated from different American and European strains of *Paramecium bursaria*. Symbiosis. 6, 253-270.

Reisser W. and Widowski M.1992. Taxonomy of eukaryotic freshwater associations. In: Algae and Symbioses. (Ed. Reisser W.). Biopress. Bristol. pp.21-40.

Sambrook G., Fritsch E. and Maniatis T. 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press. 2, pp.141-147.

Schlosser U.G. 1982. Sammlung von Algenkulturen:. Ber. Deutsch. Bot. Ges. 95, 181-276.

Shihira I. and Krauss W. 1965. *Chlorella* Physiology and Taxonomy of Forty-one Isolates. Univ. of Maryland. p.16.

Van Etten J.L., Meints R.H., Kuczmarski D., Burbank D.E., and Lee K. 1982. Viruses of symbiotic *Chlorella*-like algae isolated from *Paramecium bursaria* and *Hydra viridis*. Proc. Natl. Acad.Sci. USA. 79, 3867-3871.

Van Etten J.L., Burbank D.E., Kuczmarski D., and Meints R.H. 1983. Virus infection of culturable *Chlorella*-like algae and development of a plaque assay. Science. 219, 994-996.

Van Etten L.L., Van Etten C.H., Johnson J.K., and Burbank D.E. 1985 A survey for viruses from fresh water that infects a eukaryotic *Chlorella*-like alga. Appl. Environ. Microbiol. 49, 1326-1328.

Van Etten J.L., Lane L.C. and Meints R.H. 1991. Viruses and Viruslike Particles of Eukartyotic Algae. Microbiological Reviews. 55, 586-620.

Van Etten J.L. and Meints R.H. 1999. Giant viruses infecting algae. Annu. Rev. Microbiol. 53, 447-494.

Voitsekhovsky E.V., Linz B., Kvitko K.V. and Gavrilova O.V. 1994. The finding of an American ecotype zoochlorella virus in Tadjikistan. Vestnik Leningradskogo Univ. 3, 105-106 (in Russian with English summary).

Yamada T., Higashiyama T. and Fukuda T. 1991. Screening of natural waters for viruses which infect *Chlorella* cells. Appl. Environ. Microbiol. 57, 3433-3437.

Yamada T., Shimomae A., Furukawa S. and Takehara J. 1993. Widespread distribution of *Chlorella* viruses in Japan. Biosci. Biotech. Biochem. 57, 733-739

Address for correspondence: Konstantin V. Kvitko. Laboratory of Microbiology, Biological Research Institute of St. Petersburg State University, Oranienbaumskoye sh. 2, Stary Peterhof, 198004, St. Petersburg, Russia. E-mail: konstantin.kvitko@paloma.spbu.ru

The manuscript is presented by A.L. Yudin