VOL. 54 NO. 7, JULY 2001 THE JOURNAL OF ANTIBIOTICS pp. 582 — 587

An Efficient Screening Approach for Anti-*Microcystis* Compounds Based on Knowledge of Aquatic Microbial Ecosystem

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(Received for publication February 6, 2001)

To improve the efficiency of screening for anti-*Microcystis* compounds, we planned to use algae-lysing bacteria that kill the organisms of water blooms. A two step-screening process was carried out, *i.e.*, the screening of algae-lysing bacteria and the selection of anti-*Microcystis* producers from the bacteria. Sources for the isolation of the bacteria were a co-cultivated fluid of a water sample with axenic *Microcystis viridis*, a water sample collected in a water bloom season, and a water bloom sample. The water bloom sample was the best source for the isolation of the algae-lysing bacteria and such bacteria were shown to exhibit potent activity. Seventeen strains out of 20 isolated algae-lysing bacteria produced anti-*Microcystis* activities, and one of the principles was the previously reported argimicin A. These results indicate that algae-lysing bacteria in water blooms may be good sources for potent and selective anti-cyanobacterial compounds.

Cyanobacterial water blooms occur frequently and create problems all over the world. *Microcystis* spp. producing hepatoxin microcystins have been reported to cause the death of livestock drinking the water containing dense blooms in various parts of the world[1,2]. The victims of the hepatoxin were not only cows and sheep. Over 50 human patients died in a haemodialysis unit in Brazil, who were treated with water contaminating with microcystins[3]. Since the toxin has been also known as a tumor promoter, it been identified as a risk for human health in the countries with poor water supplies[4]. Inexpensive and efficient methods to control water blooms or to decrease nutrient salts in eutrophied areas have not been developed and the problems of blooms have become increasingly serious.

In recent years, many aquatic ecological microbiologists have suggested that algae-lysing bacteria caused in the decline of a water bloom[5~7], since these bacteria kill the organisms of blooms and their increase coincides with ebb of a bloom. Therefore, algae-lysing bacteria are likely to be useful in controlling water blooms. The mechanisms of lysis of the algae are presently unclear, but excretion of algaecide has been suggested to be one possible mode of action[7,8]. We therefore considered that algae-lysing bacteria should be good sources of anti-*Microcystis* compounds. A difficult step is the isolation of algae-lysing bacteria, since they are reported frequently in environmental water but in low abundance ratio except during the decline of a water bloom[6]. In order to obtain algae-lysing bacteria efficiently, we carried out bacterial isolations from the following three sources *i.e.*, co-cultured fluid of a water sample with axenic *Microcystis* sp. to promote the ratio of active bacteria, a sample collected in the water bloom season, and a water bloom. In this paper, we describe the results of screening based on the use algae-lysing bacteria; subsequent phylogenetic analyses of the algae-lysing bacteria confirmed the results allowed comparison of the sources.

Materials and Methods

Microcystis viridis and Its Cultivation

Microcystis viridis NIES-102 was purchased from

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National Institute for Environmental Science in Japan, and grown in MA medium[9] at 25°C under fluorescent illumination (23 μE/m^2/second 12L-12D cycle).

Water Sample Collection

Lake Biwa is situated in the middle of Honshu Island of Japan and its southern part is mesotrophic. Surface water samples at the same three sites in the south basin of Lake Biwa were collected eight times from September 1996 to January 1998 (Sep. 1996, Apr., May, July, Aug., Sep., and Oct. 1997 and Jan. 1998). One of the sites faces a water channel between a small island (Kihan-tou) and the shore; the others were facing the lake. A water bloom sample, containing visible colonies of *Microcystis* sp., was obtained at a site facing the water channel in July 1997.

Co-cultivation of Bacteria with *M. viridis*

One hundred μl of the sample was added to 10 ml of a subculturing fluid (1×10^6 cells/ml) of axenic *M. viridis*, and incubated for a week. The growth of *M. viridis* in the mixed culture was compared with that of an axenic culture. The decolorized co-culture was used for the isolation of algae-lysing bacteria.

Isolation of Bacteria

The co-cultured fluid was diluted 10-fold with a MA medium and 100 μl of diluent was spread on 1/10 nutrient broth (1/10 NB: polypeptone 1 g, meat extract 1 g, NaCl 5 g, distilled water 1 liter, pH 7.2, agar 1.5 g) agar and incubated for 2 days at 30°C. Colonies were purified on 1/10 NB agar plates by repeated streaking. Each bacterial isolate was stored on the agar medium. One hundred μl of nine water samples collected in a water bloom season (July, Aug., and Sep. 1997) including a water bloom sample were sampled directly.

Selection of Algae-lysing Bacteria

A suspension of cells of isolated bacteria in MA medium (approximately 1×10^5 cells/ml, 50 μl) was added to the subcultured fluid (1×10^6 cells/ml, 200 μl) of axenic *M. viridis* in a well of a 96-well microplate and incubated for a week. Duplicate experiments were carried out and strains causing a colorless culture fluid were algae-lysing bacteria.

Screening Using Selected Algae-lysing Bacteria

An algae-lysing bacterium was inoculated into each of 6 ml of 3 media, 1/10 NB, 1/10 Trypto-soy (1/10 TS: tryptone 1.5 g, soypeptone 1.5 g, NaCl 5 g, distilled water 1 liter, pH 7.3), and 1/10 L-broth (1/10 LB: Bacto tryptone 1.0 g, Yeast extract 0.5 g, NaCl 5 g, distilled water 1 liter, pH 7.2). These were incubated at 30°C with rotary shaking until stationary phase of growth. Cultured broth (400 μl) was evaporated *in vacuo* in a centrifugal evaporator and the residue was extracted with 400 μl of 70% ethanol. Twenty μl of the extract was added a well of a 96-well microplate and dried *in vacuo*. The subcultured fluid (1×10^6 cells/ml, 200 μl) of axenic *M. viridis* was added to the well, and the microplate was incubated for a week. Duplicate experiments were carried out and activity of the extract was evaluated by visual observation. A strain judged to have ability to produce algicides when at least one of three extracts exhibited activity. The extract was diluted 4-fold with 70% ethanol, and activity of the diluent measured in the same manner.

PCR Amplification and Sequencing of 16S rDNA

From the stock cell suspensions, crude lysates were prepared by protease digestion, heat treatment, and centrifugation. 16S rDNA fragments were amplified by PCR [DNA Thermal cycler (Takara)] directly from the crude lysate using eubacterial consensus primers, 20f and 1510r (Pharmacia). PCR products were purified using the Quiaquick PCR purification kit (Qiagen), and sequenced a Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham) and primers [M13RV (5′-CAG GAA ACA GCT ATG AC), r4L(5′-ACG GGC GGT GTG TAC AAG, 1406~1389bp), r3L(5′-TTG CGC TCG TTG CGG GAC T, 1111~1093bp), r2L(5′-GAC TAC CAG GGT ACT TAA TC, 805~786bp), r1L(5′-GTA TTA CCG CGG CTG CTG G, 536~518bp); Pharmacia], followed by analysis in an auto-sequencer (model 373A; Applied Biosystems).

Phylogenetic Analysis

Sequence data were compiled with the DNASIS-Mac computer program (HITACHI Software Engineering) and compared with sequences available from the DDBJ, EMBL, and GenBank nucleotide sequence databases using the BLAST (Basic Local Alignment Search Tool) search system. Nucleotide substitution rates were determined using KIMURA's two-parameter model[10], and distance matrix trees were constructed by the neighbor-joining method[11] with the CLUSTAL X program[12]. Alignment positions with gaps and unidentified bases were not taken into consideration for the calculations. The topology of the phylogenetic tree was built by bootstrap analysis[13] with 1,000 bootstrapped trials.

Table 1. Algae-lysing bacteria and activity of extracts toward *Microcystis viridis*.

Strain No.	Sampling date	Isolated from	Activity of extract		
			1/10NB	1/10LB	1/10-TS
Group A					
96J	Sep-96	coculture	+	+	+
96B	Sep-96	coculture	+	+	+
96C	Sep-96	coculture	+	+	+
9704-07	Apr-97	coculture	−	+	−
9704-20	Apr-97	coculture	−	+	−
9704-40	Apr-97	coculture	+	−	−
9704-59	Apr-97	coculture	−	−	−
9707-06	Jul-97	coculture	−	−	−
9707-08	Jul-97	coculture	+	−	−
9707-12	Jul-97	coculture	+	−	−
9707-14	Jul-97	coculture	+	++	−
9708-02	Aug-97	coculture	+	++	−
9708-17	Aug-97	coculture	++	++	+
Group B					
M-94	Sep-97	water sample	−	−	−
Group C					
M-06	Jul-97	water bloom	++	+	+
M-17	Jul-97	water bloom	+	+	++
M-19	Jul-97	water bloom	+	+	+
M-24	Jul-97	water bloom	++	+	+
M-43	Jul-97	water bloom	++	+	+
M-50	Jul-97	water bloom	++	−	+

Activity + and + + : The extract and its fourfold dilution showed activity, respectively.

Results

Isolation of Algae-lysing Bacteria

The isolated bacteria were divided into following three groups for convenience. Three-hundred and eleven strains from 16 decolorized fluids among 24 co-cultures of lake water with axenic *M. viridis* (group A), 117 strains from 8 water samples collected in a water bloom season (group B), and 38 strains from a water bloom sample (group C) were obtained. Thirteen strains of group A, one of group B, and 6 strains of group C suppressed the growth of *M. viridis*, when were co-cultivated in MA medium, and were selected as algae-lysing bacteria.

The Results of Screening Using Algae-lysing Bacteria

The 70% ethanol extracts of the cultured broths of 11 strains of group A, none of group B, and 6 strains of group C showed activity against *M. viridis*. Some exhibited the activity at fourfold dilution and many of them were the extracts of strains of group C. Algae-lysing bacterial strains and extracts activity are listed in Table 1.

Phylogenetic Analyses Based on 16S rDNA Sequences

Nineteen algae-lysing (one strain M-06 of group C could not be re-cultivated) and a non-algae-lysing strain 9710-05 were submitted to phylogenetic analysis based on 16S rDNA sequences. The determined sequences of bacteria

Table 2. Similarity of 16S rDNA sequence of isolated algae-lysing bacteria to their closest phylogenetic relatives.

Strain No.	Closest relative and accession number of 16S rDNA	Sequence similarity (%)
96J	*Pseudomonas putida* U70977	99.9
96B	*Pseudomonas plecoglossicida* AB009457	99.7
96C	*Pseudomonas plecoglossicida* AB009457	99.9
9704-07	*Pseudomonas* sp. AJ002813	99.8
9704-20	*Pseudomonas* sp. J1 AF195877	98.8
9704-40	*Flavobacteriaceae* str. 2 AB024308	96.3
9704-59	*Pseudomonas* sp. 'FSL D1-024' AF205135	99.9
9707-06	*Pseudomonas putida* U70977	99.8
9707-08	*Pseudomonas* sp. AJ007004	99.1
9707-12	*Acinetobacter* sp. X89709	98.9
9707-14	*Chryseobacterium* sp. FR2 AF217562	98.8
9708-02	*Flavobacteriaceae* str. 2 AB024308	95.0
9708-17	*Flavobacteriaceae* str. 2 AB024308	96.4
M-94	*Matsuebacter chitosanotabidus* AB006851	97.7
M-17	*Sphingomonas trueperi* X97776	99.6
M-19	Uncultured bacterium VC2.1 Bac29 AF068803	98.9
M-24	*Xanthomonas campestris* X99298	96.6
M-43	*Xanthomonas campestris* X99298	96.6
M-50	*Xanthomonas campestris* X99298	96.6
9710-05*	*Vogesella indigofera* AB021385	97.6

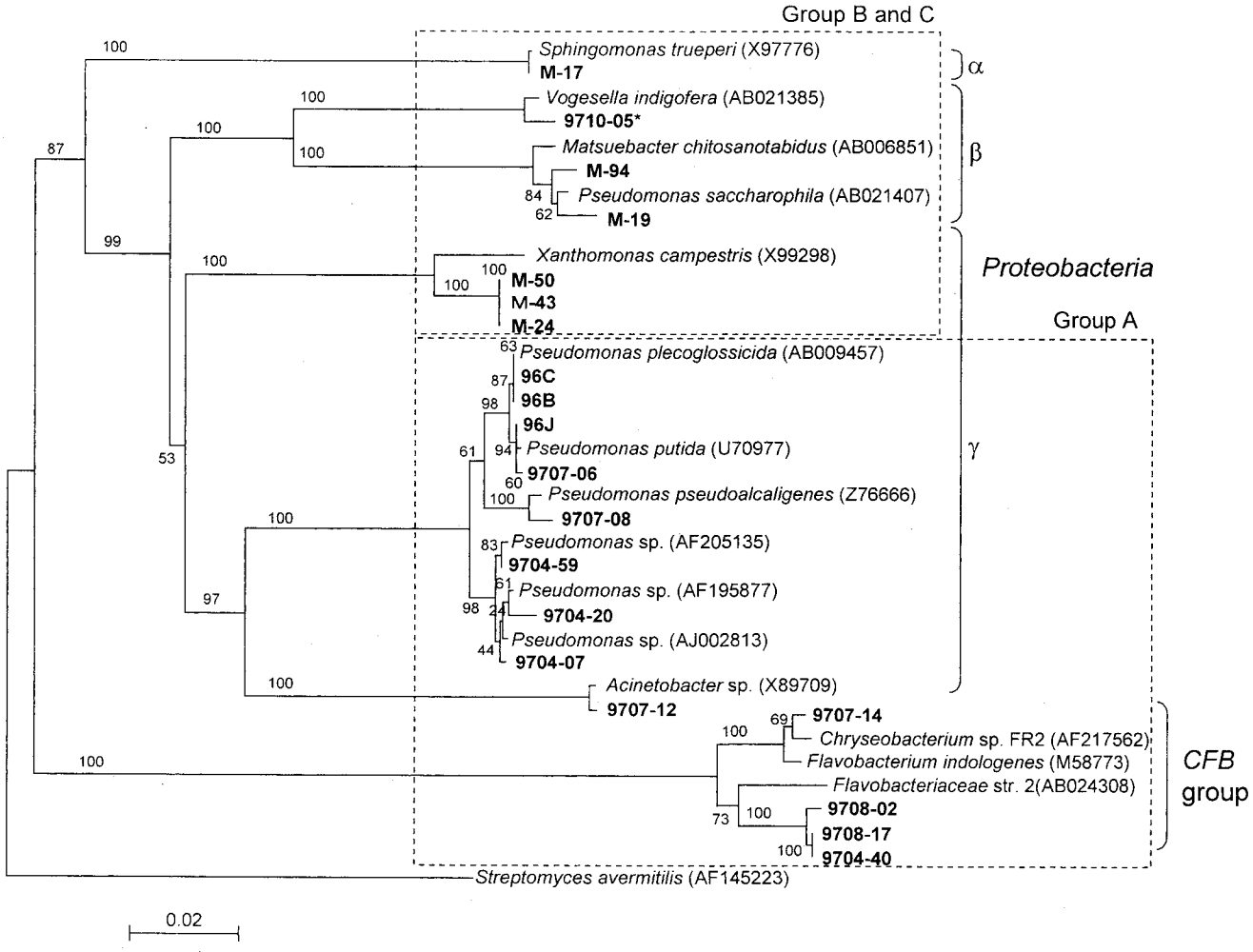
* Non algae-lysing bacterium.

ranged from 1,047 to 1,076 residues corresponding to the 335 to 1405 positions in the *Escherichia coli* numbering system. The BLAST homology search of the determined 16S rDNA sequences showed 95.0 to 99.9% identity to known sequences of relatives (Table 2). Figure 1 shows a neighbor-joining phylogenetic tree constructed on the distance matrix data for algae-lysing bacteria and related bacteria. Algae-lysing bacteria were classified into various phylogenetic clusters, *i.e.*, α, β, and γ subdivisions of *Proteobacteria* and *Cytophaga-Flavobacterium-Bacteroides* (*CFB*) group. The nearest phylogenetic neighbors of group A bacteria were *Pseudomonas* spp. of γ subdivision of *Proteobacteria* and the *CFB* group. On the other hand, those of group C were classified into β and γ subdivisions of the *Proteobacteria*, excepting *Pseudomonas* spp. Three strains of group C, *i.e.*, M-24, M-43, and M-50, appeared to be identical.

Discussion

A random screening method for anti-cyanobacterial compounds from marine bacteria has been reported[14]; the distinction between this method and the one we describe herein are the sources from which bacteria were isolated. Thirty-seven producing organisms were obtained from 2,594 marine bacteria isolated from water and macro algae samples collected at Yap, Palau, and Okinawa; β-cyanoalanine was identified as the active compound from a strain that showed the highest activity[14]. In our procedure, seventeen producers were identified in 510 strains isolated in the two-step screening procedure we describe. After the first step, we found that the group B (water samples) was a poor source with only one strain being selected as an algae-lysing bacterium from 117 strains of this group. This result

Fig. 1. Distance matrix tree showing phylogenetic positions of representative algae-lysing bacteria.



The bacteria analyzed are shown in bold type (*non-algae-lysing strain). The sequence of *Streptomyces avermitilis* was used to root the tree. Numbers above the branches are percent of bootstrap values based on 1,000 replications. The scale bar indicates 2 nucleotide substitutions per 100 nucleotides.

indicated that the algae-lysing bacteria were rare even in the water bloom season. On the other hand, 13 strains were selected from 355 isolates of the group A (co-cultures); co-cultivation was considered to increase the ratio of algae-lysing bacteria to higher levels than group B. It was noteworthy that six producers were selected from only 38 strains of group C (a water bloom sample). Of these six strains, at least half were identified as different genera. The results of phylogenetic analyses indicated the difference between algae-lysing bacteria of group A and group C. Therefore, although the proportion of algae-lysing bacteria could be increased by co-cultivation, the inhibitor-producing bacteria isolated from co-cultivated fluid and a water bloom were different. The best source of algae-lysing bacteria identified in this study was a water bloom, which would be expected from existing knowledge of aquatic microbial ecology.

At the second step of our screening, the extracts of 17 strains out of 20 algae-lysing bacteria showed anti-*Microcystis* activity which indicated that algae-lysing bacteria were good sources of anti-*Microcystis* compounds. The potencies of the strains of group C were higher than those of group A as shown in Table 1. These results suggested that many of the algae-lysing bacteria depressed water blooms by producing algicides in aquatic environments, and that the algae-lysing agents probably inhibit the growth of cyanobacteria at low concentrations because the compounds are diluted in a natural

environment. Thus, we anticipated that the active compounds produced by algae-lysing bacteria would be potent. Strain M-17 isolated from a water bloom showed the highest activity; the strain produced a novel pentapeptide, argimicin A, which was active against *M. viridis* at a concentration of 12 ng/ml (16 nM)[15], more active than a well known herbicide DCMU. Argimicin A is a potent, selective anti-cyanobacterial compound. Algae-lysing bacteria in a water bloom might be selected to maintain the ecosystem balance in nature and it seems logical that a potent and selective active inhibitor of water bloom microbes would be produced. Our results indicate that the efficiency of inhibitor screening might be improved by adequate consideration of the relationships between microorganisms in natural circumstances and that the search for potent and selective biological active agents could be improved by appropriate ecological studies.

Acknowledgments

The present work was financially supported in part by Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Science, Sports and Culture, Japan.

References

1) PUSCHNER, B.; F. D. GALEY, B. JOHNSON, C. W. DICKIE, M. VONDY, T. FRANCIS & D. M. HOLSTEGE: Blue-green algae toxicosis in cattle. J. Am. Vet. Med. Assoc. 213: 1605~1607, 1998
2) VAN HALDEREN, A.; W. R. HARDING, J. C. WESSELS, D. J. SCHNEIDER, E. W. HEINE, J. VAN DER MERWE & J. M. FOURIE: Cyanobacterial (blue-green algae) poisoning of livestock in the western Cape Province of South Africa. J. S. Afr. Vet. Assoc. 66: 260~264, 1995
3) POURIA, S.; A.DE ANDRADE, J. BARBOSA, R. L. CAVALCANTI, V. T. S. BARRETO, C. J. WARD, W. PREISER, K. GRACE POON, G. H. NEILD & G. A. CODD: Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. Lancet 352: 21~26, 1998
4) FALCONER, I. R.: Effects on jumanhealth of some toxic cyanobacteria (blue-green algae) in reservoirs, lakes and ivers. Toxicity Assessment 4: 175~184, 1989
5) SHILO, M.: Lysis of blue-green algae by Myxobacter. J. Bacteriol. 104: 453~461, 1970
6) YAMAMOTO, Y. & K. SUZUKI: Distribution and algal-lysing activity of fruiting myxobacteria in Lake Suwa. J. Phycol. 26: 457~462, 1990
7) REDHEAD, K. & S. J. L. WRIGHT: Lysis of the cyanobacterium Anabaena flos-aquae by antibiotic-producing fungi. J. Gen. Microbiol. 119: 95~101, 1980
8) YAMAMOTO, Y.; T. KOUCHIWA, Y. HODOKI, K. HOTTA, H. UCHIDA & K.-I. HARADA: Distribution and identification of actinomycetes lysing cyanobacteria in a eutrophic lake. J. App. Phycology 10: 391~397, 1998
9) WATANABE, M. M. & H. MIKIYA: Media, p32. *In* NIES-collection list of strains, 5th ed. Microalgae and protozoa. National Institute of Environmental Studies, Tsukuba, Japan. 1997
10) KIMURA, M.: A simple method for estimation evolutionary rates of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111~120, 1980
11) Saitou, N. & M. Nei: The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406~425, 1987
12) THOMPSON, J. D.; T. J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN & D. G. HIGGINS: The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876~4882, 1997
13) FELSENSTEIN, J.: Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783~791, 1985
14) YOSHIKAWA, K.; K. ADACHI, M. NISHIJIMA, T. TAKADERA, S. TAMAKI, K.-I. HARADA, K. MOCHIDA & H. SANO: β-Cyanoalanene production by marine bacteria on cyanide-free medium and its specific inhibitory activity toward cyanobacteria. Appl. Environ. Microbiol. 66: 718~722, 2000
15) IMAMURA, N.; I. MOTOIKE, N. NODA, K. ADACHI, A. KONNO, & H. FUKAMI: Argimicin A, a novel anti-cyanobacterial compound produced by an algae-lysing bacterium. J. Antibiotics 53: 1317~1319, 2000