APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1995, p. 234–238 Vol. 61, No. 1
0099-2240/95/$04.00+0

Use of Filamentous Cyanobacteria for Biodegradation of Organic Pollutants

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Received 6 September 1994/Accepted 14 October 1994

Biodegradation is increasingly being considered as a less expensive alternative to physical and chemical means of decomposing organic pollutants. Pathways of biodegradation have been characterized for a number of heterotrophic microorganisms, mostly soil isolates, some of which have been used for remediation of water. Because cyanobacteria are photoautotrophic and some can fix atmospheric nitrogen, their use for bioremediation of surface waters would circumvent the need to supply biodegradative heterotrophs with organic nutrients. This paper demonstrates that two filamentous cyanobacteria have a natural ability to degrade a highly chlorinated aliphatic pesticide, lindane (γ-hexachlorocyclohexane); presents quantitative evidence that this ability can be enhanced by genetic engineering; and provides qualitative evidence that those two strains can be genetically engineered to degrade another chlorinated pollutant, 4-chlorobenzoate.

Cyanobacteria, free-living photoautotrophic microorganisms, can derive energy from sunlight and carbon from the air (4, 5). Some cyanobacteria are also able to fix atmospheric nitrogen (4, 5) and are therefore especially inexpensive to maintain. Filamentous cyanobacteria, including nitrogen-fixing strains that combine aerobic metabolism in their vegetative cells with anaerobic metabolism in their differentiated cells called heterocysts (39), are widespread in many ecosystems, including polluted ones (15, 19, 34). The viability and metabolic activity of these cyanobacteria, unlike those of heterotrophic microorganisms, are not subject to reduction by the decrease in the concentration of the pollutants that they may break down. Cyanobacteria have been shown to degrade both naturally occurring aromatic hydrocarbons (6–8, 13, 30) and xenobiotics (28). This paper presents evidence of a natural ability of cyanobacteria to degrade lindane. This is also the first report that cyanobacteria can be genetically engineered to enhance their degradation of organic pollutants.

Current systems for introducing organisms for bioremediation of polluted areas are restricted to the implementation of biodegradative microorganisms from soil (26); in general, surface waters contaminated with synthetic chemicals remain largely untreated by remediation programs. On the basis of our results and those cited above, we propose that the use of cyanobacteria be considered for low-cost, low-maintenance remediation of pollutants in surface waters.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Anabaena* sp. strain PCC 7120 and *Nostoc ellipsosporum* (an axenic culture derived from strain B1453-7 of the University of Göttingen algal culture collection) were grown in the light in liquid medium AA/8 with or without nitrate (22). Their growth was monitored by measurement of chlorophyll (27). The cultures were tested for the presence of contaminating heterotrophs microscopically and by plating on L agar and incubating for 1 week at 30°C. All cultures used were axenic. For determination of biodegradation, the basal medium was supplemented with lindane (γ-hexachlorocyclohexane [Sigma Chemical Co., St. Louis, Mo.]; 1% [wt/vol] stock solution in dimethyl sulfoxide [Mallinckrodt, Paris, Ky.]) at 0.5 μg ml^{-1} (1.7 μM), 0.6 mM 4-chlorobenzoate (4CB; *p*-chlorobenzoate), or 0.6 mM 4-iodobenzoate (4IB; *p*-iodobenzoate) (all from Sigma Chemical Co.). 4-Hydroxybenzoate (4HB; *p*-hydroxybenzoate) was also obtained from Sigma. Benzoate derivatives were dissolved in water upon titration with 10 N NaOH. 1,2,3-Trichlorobenzene and 1,2,4-trichlorobenzene were obtained from Aldrich Chemical Co., Milwaukee, Wis.

To transform the *Anabaena* sp. with *linA*, the *Pseudomonas paucimobilis* gene that controls the first step of lindane degradation (23), a *Bam*HI-*Eco*RI fragment from plasmid pIMA2 (23) was transferred between the corresponding sites of pRL25 (38), producing plasmid pRL634. For degradation of halobenzoates, the *Arthrobacter globiformis* operon *fcbABC* was added from plasmid pCH1 (36) as an *Eco*RI fragment in two orientations at the unique *Eco*RI site of pRL25 (38), yielding plasmids pRL1408a (with the *Bam*HI site in *fcbA* proximal to the pDU1 portion of pRL25) and pRL1408b. Plasmid pRL25 and its derivatives can replicate autonomously in *Anabaena* and *Nostoc* strains (38). Recombinant *Anabaena* strains were generated by triparental matings with *Escherichia coli* by using methylating helper plasmid pRL623 (12), which consists of pRL528 (11) supplemented with M.*Eco*T22I from pEC22 (41). Recombinant cyanobacteria bearing pRL634, pRL1408a, or pRL1408b were grown in liquid media supplemented with 5 μg of neomycin ml^{-1} (5.5 μM). *E. coli* HB101 and DH5α containing plasmids were grown on L agar or in L broth supplemented with 25 μg of chloramphenicol ml^{-1} or 50 μg of kanamycin ml^{-1}, as appropriate. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs, Beverly, Mass., and Life Technologies, Inc., Bethesda, Md.

Analysis of degradation of lindane. Lindane was extracted from cyanobacterial suspensions by vortexing for 20 to 25 s with an equal volume of hexanes ("generally a mixture of several isomers of hexane, predominantly *n*-hexane, and methylcyclopentane" [Mallinckrodt]). To measure the intracellular concentration of lindane (at intervals between 10 min and 72 h after addition of that substance), 1 ml of culture was sedimented in a bench top Eppendorf microcentrifuge, washed with 1 ml of TE (10 mM Tris, 1 mM EDTA [pH 8.0]), resuspended in 500 μl of TE, and subjected to cavitation with a model W185 Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) until no intact cells were observable by light microscopy. The resulting suspension was then lyophilized and extracted with 1 ml of hexanes.

A 1-μl volume of each hexane extract was subjected to gas chromatography in a Perkin-Elmer Autosampler gas chromatograph, with an electron capture detector, on a DB-5 fused-silica capillary column (30 m by 0.32 mm [inner diameter] with a 0.25-μm film coating) (J&W Scientific, Folsom, Calif.) in a linear temperature gradient from 110 to 190°C over 8.5 min. Chromatographic patterns were analyzed with Turbochrom software (P. E. Nelson, Cupertino, Calif.; adjusted by P. R. Loconto, Department of Civil and Environmental Engineering, Michigan State University). Degradation was assessed by measurement of the concentration of lindane in triplicate culture flasks. Control experiments were performed with cyanobacterial cultures that had been boiled for 10 min prior to supplementation with lindane and flasks with uninoculated culture medium.

Mass-spectral analysis. Analysis by gas chromatography-mass spectrometry was carried out on a JEOL AX-505H double-focusing mass spectrometer coupled to a Hewlett-Packard 5890J gas chromatograph via a heated interface. Gas-chromatographic separation was performed on a DB-5 column (see above). Direct (splitless) injection was used. The helium gas flow was approximately 1 ml min^{-1}. The temperature of the gas chromatograph increased at 10°C min^{-1} from 80 to 200°C or from 100 to 300°C. The conditions of the mass spectrometer were

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as follows: interface temperature, 280°C; ion source temperature, ca. 200°C; electron energy, 100 μA; and scan rate, 1 s scan^{-1} over the $m\,z^{-1}$ range 45 to 600.

Chromatographic separation of products of dehalogenation of 4CB. Centrifugal supernatant solutions derived from cyanobacterial cultures were subjected to chromatography on thin-layer plates of silica with a fluorescent indicator (Fisher Scientific, Pittsburgh, Pa.). With one indicated exception, each lane contained the equivalent of 40 μl of culture. Separations were effected by a solvent system containing chloroform, ethyl methyl ketone, and formic acid (90:10:2.5, vol/vol/vol; all from Mallinckrodt) (36). Benzoate derivatives were detected by illumination of the chromatograms at 300 nm (Fotodyne, Inc., Hartland, Wis.), a wavelength at which the extinction coefficient of 4HB greatly exceeds that of 4CB and of 4IB (32).

Starch reaction. Recombinant and wild-type *Anabaena* sp. strain PCC 7120 were grown in the presence of 5 mM nitrate and 0.6 mM either 4CB or 4IB with shaking at 30°C for 30 days, by which time they were strongly light limited and therefore no longer growing actively; they were then incubated without aeration at room temperature for an additional 15 days. Cultures of the recombinant *Anabaena* sp. also contained neomycin. To 150 μl of a suspension of cells (use of centrifugal supernatant solutions gave similar results [data not shown]) that had been incubated with 4IB were added 150 μl of freshly boiled 2% starch (Sigma Chemical Co.) or starch indicator (Ricca, Arlington, Tex.) and then 60 μl of 1% $FeCl_3$ in 50% HCl. Iodide ions released by deiodination would thereupon be oxidized to iodine, whose presence would be detected by formation of a purple complex (25).

RESULTS

Degradation of lindane by wild-type and genetically altered cultures of *Anabaena* sp. In the presence of 5 mM nitrate, the wild-type *Anabaena* sp. growing with a doubling time of ca. 2 days (Fig. 1a; initial culture density, 1 to 3 μg of chlorophyll *a* ml^{-1}) or at a late phase of growth (initial culture density, 8 to 10 μg of chlorophyll *a* ml^{-1} [data not shown]) was able to degrade lindane from a concentration of 0.5 μg ml^{-1} to ca. 1.0 ng ml^{-1} within 1.5 to 3 days, depending on the initial cell density. In the absence of nitrate, lindane was degraded much more slowly (Fig. 1b). Changes in the concentration of lindane were negligible in the absence of cells or in the presence of boiled cells (Fig. 1). The presence of pRL634 in the *Anabaena* sp. was visualized by extraction and electrophoresis and confirmed by transformation of extracted plasmid DNA to *E. coli* and its subsequent restriction analysis (data not shown). In the absence of nitrate, the *Anabaena* sp. bearing pRL634 degraded lindane more rapidly than did the wild-type *Anabaena* sp. (Fig. 1b). Approximate initial rates of degradation of lindane with and without nitrate in Fig. 1 were 25 ± 6 and 1.5 ± 1.5 ng of lindane μg of chlorophyll^{-1} h^{-1}, respectively, for wild-type cells and 40 ± 8 and 10.1 ± 3.5 ng of lindane μg of chlorophyll^{-1} h^{-1}, respectively, for cells bearing pRL634. Over a 72-h experimental period, intracellular lindane was not detected (data not shown).

Gas chromatography showed the presence of several degradation products of lindane by wild-type *Anabaena* sp. A major peak (Fig. 2, top inset) was identified as γ-pentachlorocyclohexene by comparison of its mass spectrum with that of the authentic compound (shown in reference 24) and by its gas-chromatographic retention time, 4.10 ± 0.02 min. The substances produced by wild-type *Anabaena* sp. strain PCC 7120 in the presence of nitrate and by the strain bearing pRL634 in the absence of nitrate and that have that retention time cochromatographed. A minor peak (Fig. 2, lower inset) was identified as 1,2,4-trichlorobenzene by comparison of its mass spectrum with that of authentic 1,2,4-trichlorobenzene (shown in reference 23) and its retention time, 1.90 ± 0.02 min, in the gas chromatograph. A ca. fourfold-less-abundant product with a retention time of 2.09 ± 0.02 min was similarly identified as 1,2,3-trichlorobenzene. The kinetics of the appearance and disappearance of the first two of these products are shown in Fig. 2. This figure presents actual concentrations of lindane and of 1,2,4-trichlorobenzene, but because pentachlorocyclohexene is not commercially available, the figure shows the peak area of that substance as a fraction of the initial peak area of lindane.

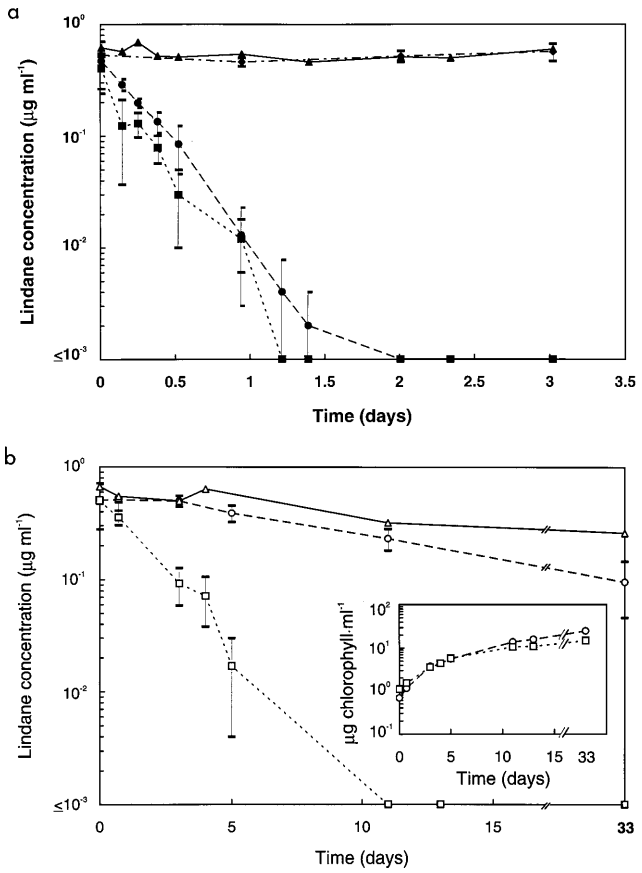

FIG. 1. Degradation of lindane by wild-type *Anabaena* sp. strain PCC 7120 (●, ○), by a derivative strain bearing pRL634 (■, □), by boiled cells (◆), and by uninoculated medium (▲, △; single samples). The medium contained (a) or lacked (b) 2.5 mM KNO_3 and 2.5 mM $NaNO_3$; the difference in the timescales should be noted. In the experiment in Fig. 1a, the wild-type culture increased from 2.81 ± 0.13 to 5.18 ± 0.11 μg of chlorophyll ml^{-1} and the recombinant culture increased from 2.66 ± 0.26 to 3.92 ± 0.54 μg of chlorophyll ml^{-1} from 0 to 48 h. The inset in Fig. 1b shows the time courses of the chlorophyll concentrations of the wild-type and recombinant cultures whose degradation of lindane is presented in that figure.

Wild-type *N. ellipsosporum* degraded lindane at about the same rate as did the *Anabaena* sp. in the presence of nitrate, and like the *Anabaena* sp., it degraded lindane much more slowly in the absence of nitrate than in its presence. As in *Anabaena* sp., the rate at which *N. ellipsosporum* degraded lindane in the absence of nitrate was increased by transformation with pRL634 (data not shown).

The *Anabaena* sp. bearing *fcbABC* dehalogenates 4-halobenzoates. Bacterial strains that are able to dechlorinate can often, more generally, dehalogenate (17, 31). We therefore assessed the ability of cyanobacterial strains to deiodinate 4IB as well as to dechlorinate 4CB. The presence of pRL1408a and pRL1408b in the *Anabaena* sp. was directly demonstrated by extraction, restriction, and electrophoresis and confirmed by similar procedures after transformation of plasmid DNA to *E. coli* (data not shown). After 2 weeks of incubation of cultures of the *Anabaena* sp. bearing pRL1408a and pRL1408b in the presence of 4IB, no color developed in the reaction with starch; however, a colorimetric reaction was observed after 30 days (data not shown) and 45 days (Fig. 3b). Analysis of the

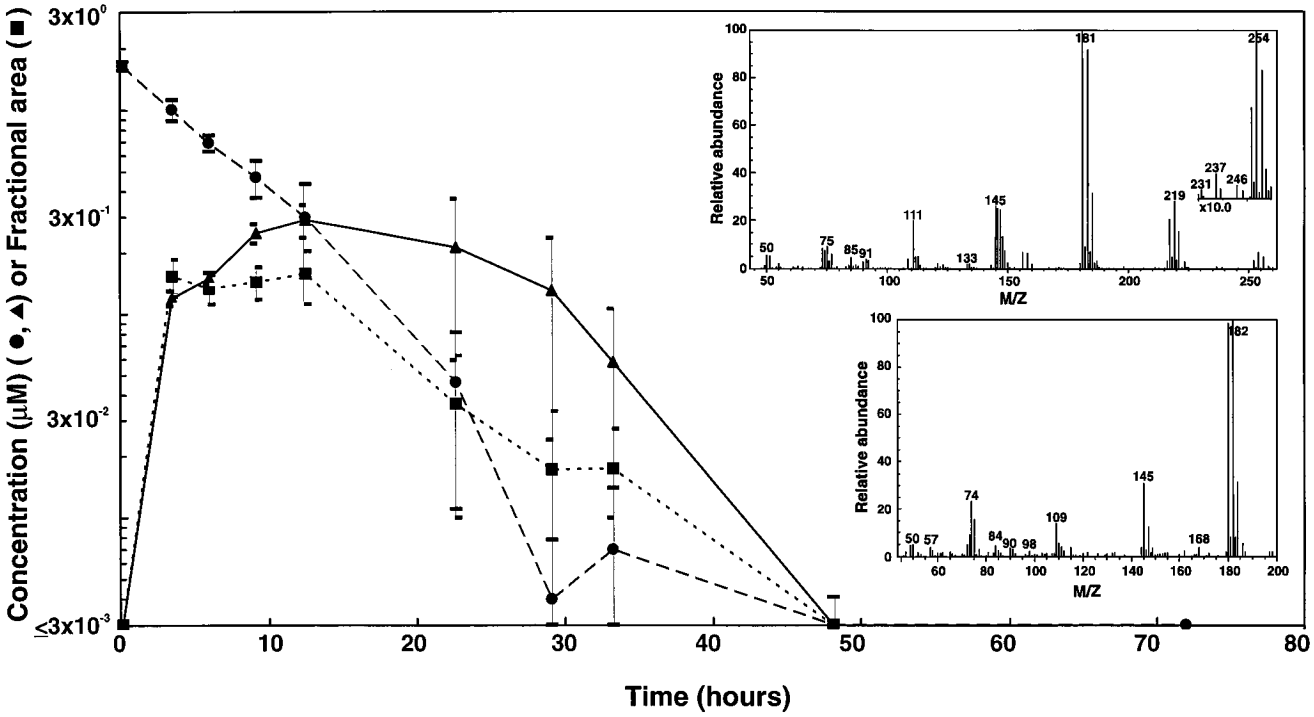

FIG. 2. Time course of production of the substances identified as γ-pentachlorocyclohexene (■; for units, see the text; the mass spectrum of the cyanobacterial product is shown in the upper inset) and 1,2,4-trichlorobenzene (▲; the mass spectrum of the cyanobacterial product is shown in the lower inset) upon degradation of lindane (●) by wild type *Anabaena* sp. strain PCC 7120 grown in the presence of nitrate.

culture supernatant solutions by thin-layer chromatography after 45 days showed that 4CB and 4IB had been degraded by the *Anabaena* sp. bearing pRL1408a to levels undetectable by UV irradiation and had been partially degraded by the *Anabaena* sp. bearing pRL1408b (Fig. 3a). The wild-type *Anabaena* sp. had degraded neither substrate detectably (Fig. 3). Similar results were obtained with *N. ellipsosporum* (data not shown). The principal product of dehalogenation by the *Anabaena* sp. had the R_f of 4HB. Some chromatographs showed, in addition, the presence of a band of unknown composition at an R_f between those of 4CB (or 4IB) and 4HB.

DISCUSSION

Many chemicals are released into surface waters either as a method of disposal or as a consequence of the technology of their utilization. In particular, the use of pesticides, many of which are toxic or contain toxic contaminants, is central to the high yields of modern agriculture. In the United States, pesticides are used annually at a rate of more than 4 lb (1.8 kg) per capita (3). Lindane is a toxic compound with potential long-term persistence (2, 29). In 1976, 530,000 lb of lindane were applied in the United States on crops and for treatment of seeds (9). In California alone, 41,500 lb (18,800 kg) of lindane were sold in 1990 (35), whereas in 1991, U.S. usage of lindane on crops as a whole decreased to 65,500 lb (29,700 kg) (18). Of the total amount of lindane used, 50% has been applied directly to crops or used for treatment of seeds and 20% has been used for treatment of hardwood lumber (9).

Soil bacteria (23, 37, 42), fungi (37), and an *E. coli* strain from rat feces (16) that use lindane as a primary carbon source degrade it to γ-2,3,4,5,6-pentachlorocyclohexene, α-, β-, and γ-tetrachlorocyclohexene, or pentachlorobenzene, depending on the strain. Only *P. paucimobilis* UT26 degraded γ-pentachlorocyclohexene further, leading to the accumulation of 1,2,4-trichlorobenzene (23). The loss of lindane from rapidly (Fig. 1) and slowly (data not shown) growing cultures of two filamentous nitrogen-fixing cyanobacteria cannot be attributed

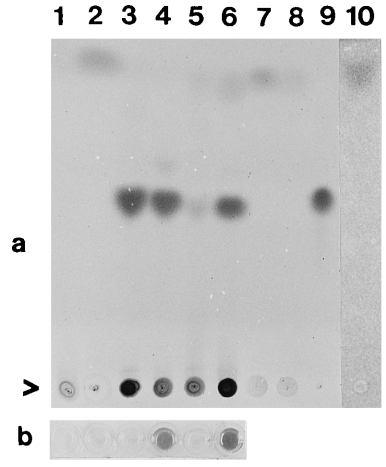

FIG. 3. Degradation of 4CB and 4IB by *Anabaena* sp. strain PCC 7120 harboring pRL1408a and pRL1408b, as of 45 days of incubation. (a) Supernatant solutions from cultures of the wild-type *Anabaena* sp. (lanes 1, 2, and 10), the *Anabaena* sp. bearing plasmid pRL1408a (lanes 3 and 4), and the *Anabaena* sp. bearing plasmid pRL1408b (lanes 5 and 6) were subjected to chromatography on thin layers of silica, and their content of halobenzoates and halobenzoate derivatives was visualized by irradiation at 300 nm. Cultures 1, 3, 5, and 10 contained 4CB; cultures 2, 4, and 6 contained 4IB. Lanes 7, 8, and 9 contained 4IB, 4CB, and 4HB, respectively. In lane 10, a 25-fold-concentrated solution (i.e., the equivalent of 1 ml rather than 40 μl of culture) has been loaded to enhance the visualization of the spot of 4CB. The origin is at the bottom. (b) Starch reaction (see text) by the cultures used in panel a, photographed with white light.

to sequestration, because lindane was not lost from suspensions of boiled cells and could not be recovered from disrupted, lyophilized cells. Moreover, intermediate products of its degradation were observed (Fig. 2), namely, γ-2,3,4,5,6-pentachlorocyclohexene and (in a molar ratio of approximately 4:1) both 1,2,4- and 1,2,3-trichlorobenzene (data not shown). The kinetics of the appearance and disappearance of these substances is consistent with the idea that the γ-pentachlorocyclohexene is an intermediate in the production of 1,2,4- and 1,2,3-trichlorobenzene. Because trichlorobenzenes did not accumulate, they were either further degraded or volatilized. It merits emphasis that *Anabaena* sp. and *N. ellipsosporum* cometabolize lindane; i.e., they degrade it but are not dependent on it for their growth and viability.

Many bacteria can denitrify (40), and some can couple denitrification to biodegradation (21). Nitrate supports the anaerobic biodegradation of halogenated aromatics by *Pseudomonas* sp. (33), and anaerobic catabolism of phthalic acid was described for a *Bacillus* sp. that respires nitrate both aerobically and anaerobically (1). Although heterocysts provide microaerobic sites in O_2-producing cyanobacteria (39), the presence of nitrate blocks the formation of heterocysts by the *Anabaena* sp. and by *N. ellipsosporum*, so that low-pO_2 sites for metabolism are presumably absent during continuous illumination. The activation, by nitrate, of anaerobic degradation of 4CB by a coryneform bacterium, NTB-1, was explained by the existence of a nitrate-dependent transport system specific for 4CB; that system increased the intracellular concentration of 4CB in the presence of nitrate (20). Because we did not detect intracellular lindane, we could not determine whether nitrate enhanced the rate of uptake of lindane. However, the stimulation of the rate of degradation of lindane by nitrate that we observed may be attributable to increased availability of nitrogen to nitrate-supplemented cultures. The idea that vegetative cells grown on N_2-derived nitrogen may differ metabolically from vegetative cells grown on nitrate, in ways other than nitrate-metabolizing enzymes per se, is supported by the finding (14) that at least eight proteins are synthesized by nitrate-grown cultures of *Anabaena* sp. strain PCC 7120 that are absent from N_2-grown cultures. Alternatively, perhaps some protein involved in the transport or reduction of nitrate is also required for the transport or metabolism of lindane. Why the *Anabaena* sp. and *N. ellipsosporum* have the capacity to degrade lindane can currently be the subject only of conjecture.

Anabaena sp. strain PCC 7120 and *N. ellipsosporum*, supplied with the *fcbABC* operon from *Arthrobacter globiformis* (36), gained the capacity to dechlorinate 4CB; higher concentrations of 4CB (2 to 5 mM), used in the original study (36), inhibited growth of the cultures. The orientation of the *fcbABC* sequence relative to the nearby pDU1 portion of pRL1408a and pRL1408b affected the extent of dechlorination, possibly because of the influence of a promoter in the pDU1 portion (10), although other explanations are not excluded. Also, genetic engineering by addition of the *linA* gene enhanced the degradation of lindane by the two cyanobacterial strains, at least when they were grown with N_2. It appears likely that other biodegradative operons will also be expressed in cyanobacteria and that cyanobacteria will prove useful for biodegradative applications in surface waters.

ACKNOWLEDGMENTS

We thank T. V. Tsoi for pCH1 and for unpublished sequence data, Y. Nagata for pIMA2, and Paul R. Loconto for initial assistance with gas chromatography. Mass-spectral data were obtained at the Michigan State University Mass Spectrometry Facility. We thank Douglas Gage and Beverly Chamberlain of that facility for help with the identification of the products of breakdown of lindane.

This work was supported by Research Excellence Funds through the Microbial Ecology/Hazardous Waste Management Center, Michigan State University, and by U.S. Department of Energy grant DE-FG01-90ER20021. The Michigan State University Mass Spectrometry Facility is supported in part by grant DRR-00480 from the Biotechnology Research Technology Program, National Center for Research Resources, National Institutes of Health.

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