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Contributions on Lindane Degradation by *Microcystis aeruginosa* PCC 7806

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Abstract: Cyanobacteria are able to tolerate, and even metabolize, moderate doses of organochlorine pesticides, such as lindane (γ-hexachlorocyclohexane), one of the most persistent and widely used in recent decades. Previous work showed that *Microcystis aeruginosa* PCC 7806 degrades lindane and that, in the presence of the pesticide, microcystin synthesis is enhanced. In this work, using in silico approaches, we have identified in *M. aeruginosa* putative homologues of the *lin* genes, involved in lindane degradation in *Sphingobium japonicum* UT26S. Real-time RT-PCR assays showed that the putative *linC* gene was induced in the presence of 7 mg/L of lindane. Additionally, *prxA*, encoding a peroxiredoxin, and involved in oxidative stress response, was also induced when lindane was present. Taking into account these results, *M. aeruginosa* PCC 7806 may degrade lindane through a metabolic pathway involving a putative 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase encoded by a *linC* homologue. However, the low similarity of the other potential *lin* homologues suggest the existence of an alternative pathway different to that of heterotrophic microorganisms such as *S. japonicum*.

Keywords: *Microcystis*; lindane; *linC*; pesticides; bioremediation

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1. Introduction

Cyanobacteria are organisms with an outstanding capacity to adapt and survive in extreme or highly degraded environments. Their metabolic plasticity includes the synthesis of a high level of potentially toxic secondary metabolites, such as cyanotoxins, that cause serious health and environmental problems. Cyanobacteria are able to tolerate and even metabolize moderate doses of organochlorine pesticides, such as lindane (γ-hexachlorocyclohexane) [1–5], one of the most persistent and widely used in recent decades. Previous work showed that *Microcystis aeruginosa* PCC 7806 degrades lindane, and that in the presence of lindane, microcystin synthesis is enhanced [6] and oxidative stress responses are induced [6]. However, there is not yet information concerning the degradative pathway in cyanobacteria.

The aerobic degradation pathway of lindane was described in *Sphingobium japonicum* UT26S, which is able to use lindane as a unique carbon and energy source [7]. The main reactions that occur during the aerobic degradation of lindane include dehydrogenation, dechlorination, hydroxylation, dehydrochlorination, and mineralization [8], and the enzymes involved in the pathway are encoded by the *lin* genes [7].

Lindane is a persistent pesticide that has been widely used in the past. Lindane is toxic and bioaccumulative and, even though its production has been eliminated in most of countries, spillages from former factories and illegal dumping of a high amount of subproducts (other hexachlorocyclohexane isomers) have given risen to serious environmental concerns. These highly polluted sites spill HCH into the surface of soils, and to the surface

and groundwater. Cyanobacteria are a good candidate to bioremediate HCH from surface waters, due to its photoautotrophic growth, plasticity, and ability to survive in a wide range of environmental conditions. Previous work suggested the presence of potential metabolic machinery to degrade HCH [1–6], which makes cyanobacteria a good candidate for the bioremediation of lindane in polluted aquatic ecosystems.

In this work we have identified genes potentially involved in the aerobic degradation of lindane by *M. aeruginosa*, and we have studied the transcriptional response in the presence of lindane of a putative *linC* homologue, the gene showing the highest similarity with the *lin* genes of *S. japonicum* UT26S. Before now, nothing was known about the degradative pathway of this organochlorine in cyanobacteria, and this is the first work that tries to decipher the metabolic transformations that occur in those photosynthetic cells.

2. Materials and Methods

2.1. Growth Conditions and Cell Visualization

The axenic strain *Microcystis aeruginosa* PCC 7806 was provided by the Pasteur Culture Collection (Paris, France), and grown in BG11 media [9] with 2 mM of $NaNO_3$, as indicated by the Pasteur Institute. Cells were grown in batch conditions with continuous agitation at 25 °C (150 rpm). The cyanobacteria were grown using a light intensity of 40 µmol of photons m^{-2} s^{-1}, unless indicated. Light was measured using a quantum sensor photometer (Skye Instruments, SKP 200). Every culture, control, and lindane stressed, was started with equal aliquots of 0.3 OD (700 nm). Three different experiments were performed in duplicate, using 250 mL of medium with cells in 500 mL Erlenmeyer flasks to avoid substantial volume changes during the sampling. Three different absorbance measurements at 700 nm (OD) were taken for each sample. Cells after 4 h of treatment were measured in a Nikon Eclipse 50i epi-fluorescent, provided with a micrometer scale. Fifty cells of each culture (with and without lindane) were measured. *Microcystis* tends to aggregate, so 0.5 M of KOH was used to individualize each cell. It was tested that the treatment had no effects on size and appearance with a light microscope.

2.2. In Silico Studies of lin Genes

All the in silico analyses were performed in *M. aeruginosa* NIES-843, since the genome of the strain PCC 7806 is not available. In order to identify putative *lin* genes in *M. aeruginosa*, the sequences of the proteins codified by genes *linA*, *linB*, *linC*, *linD*, *linE*, and *linR* from *S. japonicum* UT26S were retrieved from UniProt [10]. Using these sequences as queries and an expectancy value threshold of 0.005, a protein BLAST (Basic Local Alignment Search Tool) using the CyanoBase similarity search [11] with default parameters was performed on the *M. aeruginosa* NIES-843 genome. In all cases, the sequence with the lowest expectation value was selected and a pairwise global alignment using EMBOSS Needle [12] with default settings was performed to obtain the percentages of identity and similarity. Following the criteria proposed by Rost [13], which establish that 30% of identity is an appropriate cut-off to determine whether two protein sequences are homologous, only the protein sequences of *M. aeruginosa* showing more than 30% of identity, with respect to the corresponding sequences of *S. japonicum* UT26S, were considered as *lin* genes homologues.

The LinC phylogenetic tree was generated via maximum likelihood analysis using Phylogeny.fr. [14,15] Sequences of proteins codified by putative *linC* genes in other cyanobacteria were obtained performing a protein BLAST restricted to cyanobacteria (taxid: 117), using the LinC protein sequence from *S. japonicum* UT26S as query and the NIH BLASTp suite [16], and then selecting in each case the sequence with the lowest expectation value. Multiple sequence alignment of these sequences was performed using MUSCLE [17], while sequences were curated using G-blocks, with options for a less stringent selection [18]. A maximum likelihood phylogenetic tree was built using PhyML with default settings [19]. To assure statistical significance, 100 bootstraps were used in the computation of the tree.

2.3. RNA Isolation and RealTime RT-PCR

Sampling was performed very carefully to avoid RNA degradation during manipulation. Aliquots of 25 mL of the cultures were harvested by centrifugation at 4000× *g* for 4 min at 4 °C. After removing the supernatant, each cell pellet was resuspended in 600 µL of 50 mM Tris-HCl (pH 8), 100 mM EDTA, and 130 µL of chloroform, and incubated in ice for 3 min to eliminate external RNases. The buffer was removed by centrifugation at 13,000× *g* for 5 min at 4 °C. Finally, the cell pellets were frozen in liquid nitrogen and kept at –80 °C until RNA isolation was achieved. Cells were lysed using TRIZOL (Invitrogen) according to the manufacturer's instructions. After the chloroform extraction, RNA was collected from the aqueous layer, and precipitated in isopropanol and liquid nitrogen. The RNA pellet was washed twice with 75% ethanol.

Prior to retrotranscription, the total RNA was treated with 40 units of DNase I (Pharmacia) in a volume of 100 µL, using a buffer containing 4 mL of 1 M Tris-HCl (pH 7.5) and 0.6 mL 1 M of $MgCl_2$ in DEPC-H_2O. The sample was incubated at 37 °C for 1 h. After digestion, the enzyme was inactivated by phenol acid and chloroform extraction, and RNA was precipitated with absolute ethanol. The successful digestion of DNA was assessed via PCR, with primers targeting the 16S rRNA gene (Table 1). RNA integrity was checked using a 1% agarose gel, and the concentration was determined by measuring the absorbance at 260 nm. Its purity was assessed by the ratio A_{260nm}/A_{280nm}. For reverse transcription, 1 µg of total RNA was mixed with 150 ng of random hexamer primers (Invitrogen) and diluted with the annealing buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA, 150 mM KCl) to a final volume of 10 µL. The mixture was heated at 85 °C for 10 min and then incubated at 50 °C for 1 h. After this, the RNA was reverse-transcribed with 200 U of SuperScript™ (GibcoBRL) in the presence of 2 µL of deoxyribonucleoside triphosphate mixture (2.5 mM each), 2 µL of dithiothreitol (100 mM), and 4 µL of the 5× buffer provided by the manufacturer with the reverse transcriptase enzyme kit. The volume was adjusted to 20 µL in DEPC-H_2O. The mixture was incubated at 47 °C for 1 h and finally heated at 75 °C for 15 min.

Table 1. Results of the identification of putative *lin* genes in *Microcystis aeruginosa* NIES-843. For each of the *lin* genes of *Sphingobium japonicum* UT26S, the gene of *M. aeruginosa* NIES-843 that presented the lowest expectancy value according to the protein BLAST is annotated, and the parameters of the pairwise alignment between these two sequences are indicated (sequence alignments are included in Supplementary Material, Figures S1–S3). In the case of *linA*, *linD*, and *linE* no sequences presenting an expectancy value lower than the threshold (0.005) were found.

***Sphingobium japonicum* UT26S**		***Microcystis aeruginosa* NIES-843**		**Pairwise Alignment**			
Gene	**Anotation**	**Gene**	**Anotation**	**Identity**	**Similarity**	**Gaps**	**Score**
linA	Gamma-hexachlorocyclohexane dehydrochlorinase	-	-	-	-	-	-
linB	Haloalkane dehalogenase	*MAE_40040*	putative hydrolase	26.2%	41.2%	17.2%	171.0
linC	2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase	*MAE_33900*	3-oxoacyl-[acyl-carrier protein] reductase	34.4%	50.0%	7.6%	346.0
linD	2,5-dichlorohydroquinone reductive dechlorinase	-	-	-	-	-	-
linE	Chlorohydroquinone/hydroquinone 1,2-dioxygenase	-	-	-	-	-	-
linR	HTH-type transcriptional regulator LinR	*MAE_62070*	transcriptional regulator	15.2%	27.8%	46.2%	71.0

Real-time PCR was performed using the ViiA™ 7 Real-Time PCR System (Applied Biosystems). Each reaction was set up by mixing 12.5 µL of SYBR Green PCR Master Mix with 0.4 µL of 25 µM primer mixture and 10 ng of cDNA template in a final volume of 30 µL. Amplification was performed at 60 °C. Negative controls with no cDNA were included.

Transcript levels of target genes were normalized to those of the housekeeping 16S rRNA. The sequences of the specific primers are shown in Table S1. Relative quantification of gene expression was performed according to the comparative Ct method (the ΔΔCt method) [20]. The minimum fold change threshold was set to ±1.5 fold.

3. Results

3.1. In Silico Identification of lin Gene Homologues

In silico studies allowed the identification of genes homologous to the *lin* genes of *S. japonicum* UT26S in *M. aeruginosa* NIES-843 genome. The results of these in silico studies are summarized in Table 1.

As can be seen in Table 1, only the protein encoded by the gene *MAE_33900* of *M. aeruginosa* NIES-843, which corresponds to the possible *linC* homologue, presented more than 30% of identity with respect to the corresponding sequence of *S. japonicum* UT26S, whereas the rest of the sequences presented identities lower than 30%. For this reason, following the criteria proposed by Rost [13], which established that 30% of identity is an appropriate cut-off to determine whether two protein sequences are homologous, the gene *MAE_33900* was proposed as a putative *linC* gene of *M. aeruginosa*. Additionally, bidirectional BLAST showed that the protein encoded by the *linC* gene of *S. japonicum* UT26S, despite not being the best hit, also appears as a putative homologue of the protein encoded by the gene *MAE_33900* of *Microcystis aeruginosa* NIES-843 with an expectancy value of $1{\cdot}10^{-37}$.

3.2. M. aeruginosa PCC 7806 Growth in the Presence of Lindane

Lindane was used at its water solubility limit of 7 mg/L to avoid the undesired effects of the solvents. Visual observation after 4 h of treatment using an optical microscope with an oil-immersion objective lens (total magnification 1000×) indicates that lindane-treated *M. aeruginosa* shows a larger size (6 ± 0.5 mm diameter versus 4 ± 0.3 mm of the control culture of cells grown without the pesticide) (data not shown). Additionally, thylakoids seem to be less packed, and gas vesicles were absent when lindane was present in the culture media. Figure 1 shows the small differences in growth in both *M. aeruginosa* PCC 7806 cultures, control versus 7 mg/L lindane.

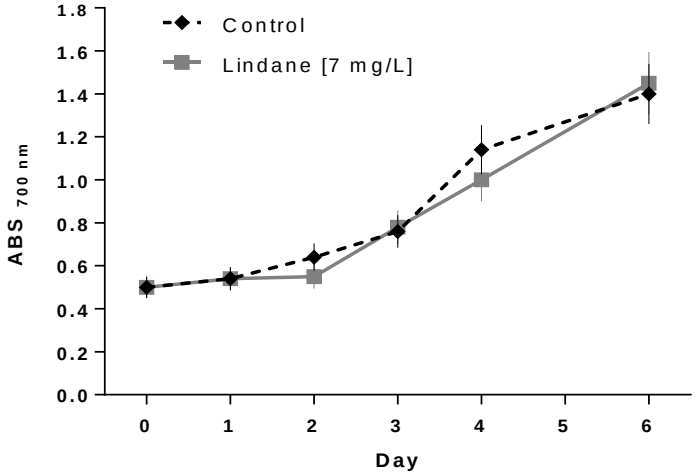

Figure 1. *M. aeruginosa* PCC 7806 growth curve. ♦ Control: cells grown in BG 11 with 2 mM $NaNO_3$, ■ Cells grown in the same conditions in the presence of 7 mg/L of lindane.

3.3. linC and prxA Are Induced in the Presence of Lindane in M. aeruginosa PCC 7806

Cells from the cultures shown in Figure 1, at day 3 and day 6, were used to perform transcriptional analysis. RNA from *M. aeruginosa* PCC 7806 cell cultures performed in the presence and in absence of lindane was isolated to carry out the transcriptional response of the proposed *linC* gene (MAE_33900) for *M. aeruginosa* as well as the gene MAE_35830,

hereafter *prxA*, that encode the 2-cys type peroxiredoxin PrxA orthologue (87% identity to PrxA from *Anabaena* sp. PCC 7120). The fold change of those genes expression after 3 and 6 days of lindane exposure is reported in Figure 2.

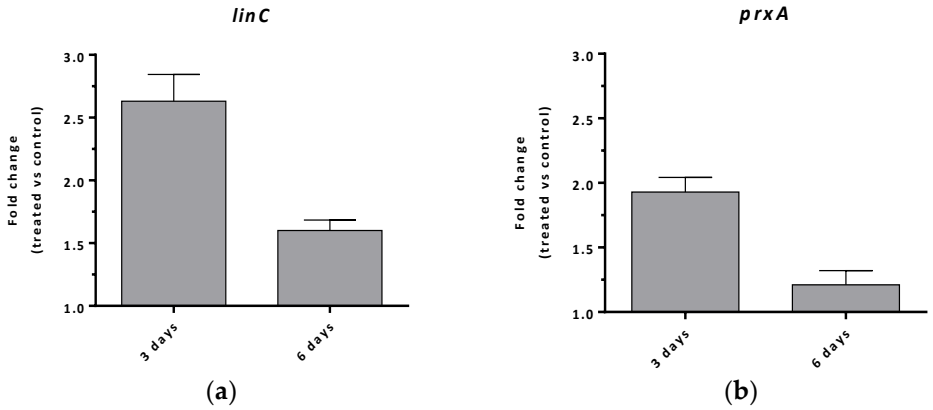

Figure 2. Relative transcription in *M. aeruginosa* PCC 7806 as a consequence of 7 mg/L lindane treatment of the (**a**) putative *linC* and (**b**) *prxA* genes. Values are expressed as fold change and correspond to the average of three biological and two technical replicates; the standard deviation is indicated.

As seen in Figure 2a, the proposed *linC* gene for *M. aeruginosa* PCC 7806 increased its transcription after 3 and 6 days of treatment with lindane (7 mg/L). The value obtained at time 3 days was 2.6-fold, while at time 6 days it was 1.6-fold (Figure 2a). The decrease in *linC* induction at 6 days with respect to 3 days indicates that the transcriptional response to that lindane concentration begins to decline after 3 days, even though from our data it is not possible to set the time of the initiation of the decline. In the case of the peroxiredoxin *prxA*, it also displayed an increase in gene expression after 3 days of lindane exposure, up to 1.9-fold, whereas after 6 days no significant change versus control cells was observed (Figure 2b).

4. Discussion

As previously described [6], *M. aeruginosa* PCC 7806 not only tolerates high lindane concentrations but it is also able to degrade it, similarly to other cyanobacteria [1–5]. The lindane degradative pathway is unknown in cyanobacteria, and for this reason we considered it interesting to carry out an in silico study to try to identify the presence of homologues for *lin* genes described in *Sphingomonadaceae* [7] in *M. aeruginosa* PCC 7806. The initial reason for performing the experimental work with *M. aeruginosa* PCC 7806 was because this strain is a model strain, and the literature concerning *Microcystis* and microcystins is the most abundant. The entire genome of *M. aeruginosa* PCC 7806 is not yet available and, for this reason, the genome of *M. aeruginosa* NIES-843 has been used for bioinformatic studies. The high identity of the known sequences may permit the extrapolation of results [21], but the possibility of differences between the two genomes should not be ruled out. The identification of *lin* gene homologues in the *M. aeruginosa* NIES-843 genome has only been partial and, in fact, only a potential *linC* homologue has been found (Table 1). In the case of *linA*, *linD*, and *linE*, no results were obtained when a protein BLAST was performed, suggesting that either these genes are not present in *M. aeruginosa* NIES-843, or their sequences are highly divergent with respect to those of *S. japonicum*. This is not strange, since photosynthetic organisms may have different degradative pathways than the ones present in heterotrophs. With respect to the *linB* and *linR* genes, two putative genes were found in *M. aeruginosa* NIES-843 (Table 1), but their percentages of identity (26.2% and 15.2%, respectively) were lower than 30% and, consequently, were not high enough to be considered homologues of their respective *lin* genes of *S. japonicum* according to the criteria established by Rost [13].

In the case of the putative *linC* gene, the sequence found in *M. aeruginosa* NIES-843 holds a 34.4% of identity with respect to the *linC* gene of *S. japonicum* (Table 1). Consequently, as the percentage of identity was higher than 30% [13], it can be proposed as the putative *linC* of *M. aeruginosa* NIES-843. A phylogenetic tree of proteins codified by putative *linC* genes of other cyanobacteria revealed that this gene is also present in other species of cyanobacteria (Supplementary Material, Figure S4). Interestingly, in some of these bacteria, such as *Synechococcus* sp. WH 8020, *Crocosphaera chwakensis* CCY0110, and *Microcoleus asticus* IPMA8, it is already annotated as 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase. These findings suggest that the 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase enzyme could be present in several cyanobacteria and, perhaps, could allow them to tolerate and metabolize lindane.

The transcriptional analysis of the putative *linC* for *M. aeruginosa* revealed its induction after treatment with lindane (7 mg/L) (Figure 2a). In the heterotrophic bacteria *S. japonicum* UT26, *linC* is constitutively expressed [22]. However in *M. aeruginosa* PCC 7806, *linC* increased its expression after 3 and 6 days of lindane exposure (Figure 2a), suggesting that *linC* is an inducible gene that may take part in the lindane degradative pathway in this cyanobacterium. Moreover, the decrease in *linC* induction at 6 days with respect to 3 days suggest that the lower transcriptional response could be related to the time course of the intracellular concentration of lindane [6]. This behavior has been observed with *linD* and *linE* genes in *S. japonicum* UT26, whose induction disappears after 1 to 2 h, paralleling the degradation of lindane [22].

It was previously reported that in *M. aeruginosa* PCC 7806, the degradation of lindane by this cyanobacterium is coupled to an induction of a gene related to its own degradation, *nirA*, and genes related to the nitrogen and iron metabolism, *ntcA* and *furA*, respectively [6]. Additionally, *mcy* operon gene expression, as well as microcystin production, are likely enhanced due to oxidative stress caused by the pesticide [6]. The fact that in this work we found that the *prxA* gene increases the expression (Figure 2b) in the presence of lindane supports such a hypothesis. Peroxiredoxins are thought to play an important role in the reduction of endogenously generated H_2O_2 [23]. Therefore, the fact that expression of *prxA* increases after treatment with lindane (Figure 2b) would indicate that the internalization and/or degradation of lindane by this cyanobacterium is an example of it deploying its defenses against oxidative stress.

The identification of potential gene candidates to be involved in lindane degradation is just the first step in understanding the fate of lindane when is metabolized by cyanobacteria. Putative *linC* seems to be involved in the degradative pathway, based on its induction in the presence of pesticide. Future work needs to complete the identification of more candidates, for instance obtaining information by the determination of intermediaries of the degradation pathway. The interest of this work is that cyanobacteria present ideal ecophysiology characteristics to arise as a promising strategy to eliminate lindane residues in aquatic environments.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14081219/s1, Table S1: Primers used in Real-Time PCR analyses, Figure S1: Pairwise alignment between LinB from *S. japonicum* UT26S and the protein codified by the gene *MAE_40040* of *Microcystis aeruginosa* NIES-843, Figure S2: Pairwise alignment between LinC from *S. japonicum* UT26S and the protein codified by the gene MAE_33900 of *Microcystis aeruginosa* NIES-843, Figure S3: Pairwise alignment between LinR from *S. japonicum* UT26S and the protein codified by the gene MAE_62070 of *Microcystis aeruginosa* NIES-843, Figure S4: Phylogenetic tree of proteins codified by cyanobacterial putative *linC* genes. The Maximum Likelihood phylogenetic tree was built in Phylogeny [14] using PhyML with default parameters. To assure statistical significance, 100 bootstraps were used in the computation of the tree (bootstrap values are indicated in the tree branches). The annotation of each sequence is represented, and the name of the organism appears in brackets. The sequence corresponding to *M. aeruginosa* NIES-843 is highlighted in bold.

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