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Variation in the synthesis of microcystin in response to saline and osmotic stress in *Microcystis aeruginosa* PCC7806

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ABSTRACT

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The growth and synthesis of microcystin in *Microcystis aeruginosa* PCC7806 was studied in cells grown in the presence of NaCl or with sucrose. The saline stress caused a decrease in the transcript levels of *mcyD*, one of the genes involved in microcystin synthesis, which was correlated with a decrease in the content of microcystin-LR in the cells. The cells treated with sucrose also had reduced levels of *mcyD* transcripts and contents of microcystin-LR.

Key words: Microcystin, *Microcystis aeruginosa* PCC7806, *mcyD*, *mcy* operon, salinity, osmotic stress.

RESUMEN

***Variación en la síntesis de microcistina como respuesta a salinidad y al estrés osmótico en* Microcystis aeruginosa**

Se ha estudiado el efecto de la salinidad y del estrés osmótico producido por sacarosa en la síntesis de microcistina por Microcystis aeruginosa *PCC7806. El estrés salino causa un decrecimiento de* mcyD, *uno de los genes del operón* mcy, *(el cluster génico implicado en la síntesis de microcistina), así como un correlativo decrecimiento de la microcistina producida. El estrés osmótico, producido por sacarosa, provoca también descenso de los niveles de* mcyD *y de microcistina-LR.*

Palabras clave: *Microcistina,* Microcystis aeruginosa *PCC7806,* mcyD, *operón* mcy, *salinidad, estrés osmótico.*

INTRODUCTION

Microcystins are hepatotoxins produced by some cyanobacteria that are a serious environmental and health risk. The synthesis of microcystin is inducible and is controlled by many environmental and nutritional factors, but laboratory and field studies have not yet clearly identified the factors that trigger microcystin synthesis (Kaebernick & Neilan, 2001; Schatz *et al.,* 2007). *Microcystis aeruginosa* is a freshwater cyanobacterium that naturally occasionally is also found in the tidal freshwater estuaries and the low salinity areas of coastal bays (Black *et al.,* 2011). Although blooms of *M. aeruginosa* are commonly associated with freshwater environments, an alarming increase in blooms has occurred in mesohaline estuaries (Black *et al.,* 2011). The increase in the salinity of freshwater ecosystems caused by agricultural practices, droughts, or rise in sea level is likely to affect phytoplankton populations, and thus, it is essential to determine the response of *Microcystis* to salt stress and examining changes in production of microcystin. Research was conducted on the effects of salinity on other species of cyanobacteria, but the data avail-
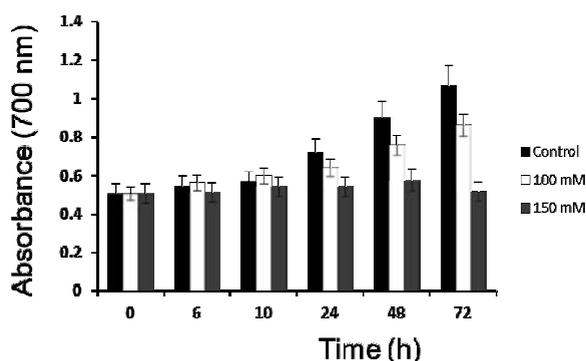
Figure 1. Growth of salt-stressed cells of *M. aeruginosa* PCC7806. Cells were grown in 100 and 150 mM NaCl, and the absorbance of aliquots was determined at 700 nm. *Crecimiento de células de* M. aeruginosa *PCC7806 en presencia de sal. Las células se sometieron a 100 y 150 mM de NaCl y la absorbancia de las alícuotas a distintos tiempos se utilizaron para medir su absorbancia a 700 nm.*

able on *Microcystis* response to varied levels of salinity are limited (Liu, 2006; Srivastava *et al.*, 2009; Black *et al.*, 2011). The strain *M. aeruginosa* PCC7806 was described in some works as tolerant to high salinity, with tolerances of up to 10 g/L (171 mM) (Tonk *et al.*, 2007), whereas salt concentrations of 14 g/L (239 mM) caused mortality of *Microcystis*. These concentrations suggest that an input of salt could be a management option to suppress *Microcystis* blooms (Verspagen *et al.*, 2006). Salt stress decreased the production of microcystin in *M. aeruginosa* in laboratory cultures and in field samples (Salomon *et al.*, 2001; Black *et al.*, 2011). However, in other research, no relationship was found between salinity and toxin concentration (Liu, 2006). Orr *et al.* (2004) related toxin production with salt tolerance; a lower tolerance of salinity increased the toxicity of the bloom, but elevated salt concentrations resulted in higher tolerance of salinity of the population, which resulted in decreased toxicity. The data are not available to determine whether this effect of salinity was caused by the presence of ions or the changes in external osmolarity and loss of turgor pressure.

In this study, microcystin-LR was quantified in *M. aeruginosa* PCC7806 cell aliquots grown in saline solution and under osmotic stress with sucrose. Additionally, quantitative real-time PCR was used to monitor changes in the level of tran-

scripts encoding *mcyD*, a gene from the microcystin synthesis gene cluster. The expression of *mcyD* is essential in microcystin synthesis; the protein is involved in the synthesis of the amino acids responsible for the toxicity, and without the protein, microcystin synthesis does not occur (Kaebernick & Neilan, 2001). Furthermore, previous work on identification of environmental conditions that trigger microcystin production used *mcyD* to evaluate *mcy* operon expression (Sevilla *et al.*, 2008, 2010, 2012; Kuniyoshi *et al.*, 2013). For these reasons, we chose this gene as a suitable candidate to be a marker for induction of microcystin production.

EXPERIMENTAL CONDITIONS

Growth conditions

The axenic strain *Microcystis aeruginosa* PCC7806 was provided by the Pasteur Culture Collection (Paris, France) and was grown in BG11 media (Rippka, 1979) with 2 mM of $NaNO_3$ and 10 mM of $NaHCO_3$ as recommended by the Pasteur Institute. The cells were grown in batch cultures with continuous aeration at 25 °C. The cyanobacteria were grown under a light intensity of 28 μmol of photons m^{-2} s^{-1}. Light was measured with a Quantum Sensor photometer (SKP 200; Skye Instruments). All cultures, control and stressed cells, if not otherwise indicated, were started with equal aliquots of 0.5 OD from a culture in the exponential phase of growth. The treatment amounts of salt or sucrose were added at time zero; control flasks were maintained during all the experiments with the same conditions. Aliquots of cells were harvested at the indicated times, unless otherwise noted, with centrifugation at 9000 rpm for 10 min. The experiments were performed in 1 L Roux flasks to avoid strong volume changes during sampling.

Analytical methods and quantification of microcystin-LR

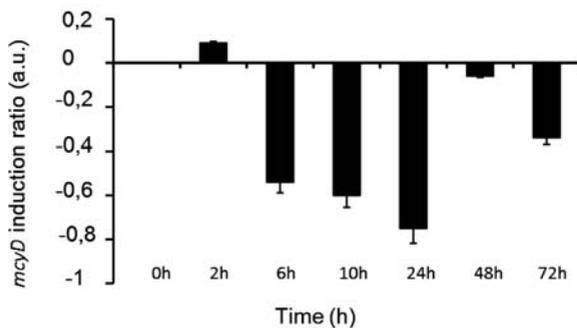Samples of 1 ml and 5 ml were collected for chlorophyll *a* and protein determinations, re-

Figure 2. Level of expression of *mcyD* mRNA in *M. aeruginosa* PCC7806 cells in response to salt stress. Total RNA was extracted and reverse-transcribed, and real-time PCR was performed using the Light Cycler system with specific primers for *mcyD* and 16S rRNA genes. The induction of expression of *mcyD* mRNA in stressed cells was calculated relative to the expression in control cells (as fold increase) using the threshold cycle (Ct) value of the log-linear portion of the amplification curve after normalisation with the endogenous reference gene 16S rRNA. The value of the fold increase calculated for each time was normalised to the fold increase at the beginning of the experiment, when the ratio was 1 (Pfaffl 2001). Results are expressed as ratio −1. *Niveles de RNA de mcyD en* M. aeruginosa *PCC7806 como respuesta a estrés salino. El RNA obtenido se retrotranscribió, y se llevó a cabo PCR a tiempo real utilizando un "Light Cycler System". Se utilizaron cebadores específicos para mcyD y 16S. Los cambios de expresión de mcyD se calculan en base a la eficiencia de su amplificación, y a la diferencia entre los ciclos umbral de amplificación (Ct) de la condición control y las células estresadas, tras normalizar con la referencia endogena de la expresión del gen 16S. El cambio calculado para cada tiempo se normaliza con respecto a los valores determinados en el principio del experimento, cuando la razón de ambos es 1. Los resultados obtenidos se expresan en la gráfica como ratio −1.*

spectively. The chlorophyll *a* was quantified according to Mackinney (1941), and the protein contents were estimated with the bicinchoninic acid method (BCA™ Protein Assay Reagent Kit from Pierce). For the intracellular microcystin-LR analysis (Sevilla *et al.,* 2008), aliquots of 10 ml of cell culture were collected, centrifuged for 5 min at 4000 rpm, and then frozen at −20 °C until analysed. The samples were extracted for 1 h with methanol and 0.1% trifluoroacetic acid (TFA), with stirring. After centrifugation at 12 000 g for 5 min, the extraction of the pellet was repeated for another hour, and the extracts were then pooled. The toxin content was determined with high-performance liquid chromatography (HPLC) using a Waters Simmetry 300 analytical reverse phase column C_{18}

(5 m, 4.6 mm × 250 mm). A linear gradient (A: water and 0.05% TFA and B: acetonitrile and 0.05% TFA) was performed in 30 min. The flow rate was 1 ml/min, and the detector was set at 238 nm. The toxin content was quantified by using commercial microcystin-LR standards (Alexis Biochemicals).

Sampling and RNA isolation

The sampling was performed very carefully to avoid changes in RNA during the manipulation. An aliquot of 25 ml of each of the cultures was centrifuged at 4000 g for 2 min at 4 °C. After removing the supernatant, each pellet of cells was resuspended in 600 µl of 50 mM Tris-HCl (pH 8), 100 mM EDTA and 130 µl of chloroform and was incubated on 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, cell pellets were frozen in liquid nitrogen and stored at −70 °C until RNA isolation.The total RNA was extracted using the "FastRNA Pro Blue kit" (Qbiogene, Inc.), following the recommendations of the manufacturer. The cells were disrupted in "FastPrep" (Qbiogene, Inc.) using four cycles of 20 s at 6.0 m/s. The samples were kept on ice between each cycle. The total RNA was resuspended in 20–50 µl of DEPC-H_2O.

Reverse transcription (cDNA synthesis)

Before RT-PCR, the total RNA was treated with 40 units of DNase (Pharmacia) in a volume of 100 µl using a buffer that contained 4 µl of 1 M Tris-HCl (pH 7.5) and 0.6 µl of 1 M $MgCl_2$ in DEPC-H_2O. The sample was incubated at 37 °C for 45 min, and heating for 10 min at 65 °C stopped the digestion.The RNA integrity was checked on a 1% agarose gel. The concentration of RNA was determined by measuring the absorbance at 260 nm, and the purity was assessed by the ratio $A_{260\,nm}/A_{280\,nm}$.For reverse transcription, 2 µg of total RNA was mixed with 300 ng of random hexamer primers (Invitrogen Corp.) and then diluted with the annealing buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA, and 150 mM

KCl) to a final volume of 20 µl. The mixture was heated at 85 °C for 10 min and then incubated at 50 °C for 1 h. The sample was then divided into two aliquots of 10 µl each. One aliquot was incubated with 1 µl of 10 mg/ml DNase free-RNase A for 30 min at room temperature; this aliquot was used as a control to verify the absence of DNA by PCR. Both aliquots (control and stressed) were reverse transcribed with 200 U of SuperScript™ (GibcoBRL) and 2 µl of deoxyribonucleoside triphosphate mixture (2.5 mM of each one), 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 with DEPC-H_2O. The mixture was incubated at 47 °C for 1 h and then heated at 75 °C for 15 minutes.

Real-time PCR analysis of gene expression

The RT-PCR was conducted with the ABI Prism 7000 HT Sequence Detection System, as previously described (Sevilla *et al.*, 2008). The reaction mixture was 5 µl of cDNA, 10 µl of TaqMan Universal PCR Master Mix, 1 µl of PCR primers (Table 1) and TaqMan MGB probes (FAM dye-labelled) from Assay-on-Demand Service (all reagents from Applied Biosystems, Foster City, CA, USA), and 4 µl of sterile milliQ-H_2O. All samples were run in triplicate. The PCR program consisted of one cycle of denaturation at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and at 60 °C for 60 s. Sequence Detector Software (SDS) (Applied Biosystems) was used for data analysis. A threshold cycle (Ct) value was determined from each amplification plot.

The triplicate samples were measured and plotted as the fold increase between stressed and control samples. The slope of the calibration curve was used to assess each gene before it was analysed, with Ct values on the y-axis and logarithm of the equivalent amount of total RNA on the *x*-axis ($E = 10^{[-1/\text{slope}]}$), according to Pfaffl (2001). The value of the fold increase calculated for each sample time was normalised to the fold increase at the beginning of the experiment, which was a ratio of 1.

The relative expression ratio or the fold change of the target gene was calculated based on its real-time efficiency (E) and the difference between the mean Ct of the sample in stressed conditions and the mean Ct of the control sample; the value was normalised for the expression of the endogenous reference gene 16S rRNA (AF139299) (Pfaffl, 2001). The equation used was as follows:

$$ratio = [E_{\text{target gene}} \cdot \exp{(\Delta Ct_{\text{target gene}} \cdot}$$
$$\cdot\ (\text{control-problem}))]/[E_{\text{ref gene}} \cdot$$
$$\cdot\ \exp{(\Delta Ct_{\text{ref gene}} \cdot (\text{control-problem}))]}$$
$$(\text{Pfaffl, 2001}).$$

RESULTS

Effect of salt on growth of *M. aeruginosa* PCC7806

Microcytis aeruginosa PCC7806 cells were grown under 28 µmol of photons m^{-2} s^{-1} until they reached an early exponential phase of growth ($OD_{700\,nm} = 0.5$). Then, aliquots were

Table 1. Oligonucleotides used as primers for PCR, and primers and TaqMan MGB probes used for the real time RT-PCR analysis (Sevilla *et al.*, 2008). *Oligonucleotidos utilizados como cebadores para PCR y sondas TaqMan MGB para el estudio de PCR a tiempo real (Sevilla* et al.*, 2008).*

|	Primer sequences (5′-3′)	Description
mcyD-N	ACCCGGAACGGTCATAAATTGG	Forward primer for mcyD real-time PCR
mcyD-C	CGGCTAATCTCTCCAAAACATTGC	Reverse primer for mcyD real-time PCR
mcyD MGB probe	CTGCTGCACCTATTTCA	Probe for mcyD real-time PCR
16S-N	TGCGTAGAGATTGGGAAGAACATC	Forward primer for 16S rRNA real-time PCR
16S-C	GCTTTCGTCCCTGAGTGTCA	Reverse primer for 16S rRNA real-time PCR
16S MGB probe	CCAGTAGCACGCTTTC	Probe for 16S rRNA real-time PCR
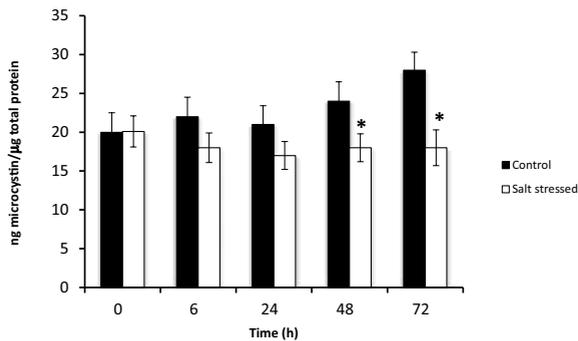
Figure 3. Content of microcystin-LR in *M. aeruginosa* PCC7806 cells in response to salt stress. Intracellular microcystin-LR content in cells growing in 150 mM NaCl was determined by HPLC and is presented as total soluble protein. Data were analysed with a one-way ANOVA statistical test with a significance level of 0.05 (*). *Cuantificación de la microcistina-LR intracelular en* M. aeruginosa *creciendo en estrés salino. La microcistina-LR en células creciendo en 150 mM de NaCl se determinó por HPLC, y se expresa en referencia a la proteína soluble total. * Datos significativos analizados con ANOVA (0.05).*

treated with two different salt concentrations (100 or 150 mM NaCl) or maintained as controls. For accurate interpretation of the effect of each of the factors studied, growth curves were determined for cells used in the transcriptional analysis of *mcyD* with RT-PCR and used in the quantification of microcystin, and the results are displayed in Figure 1. The cells growing in different stress conditions were monitored versus nonstressed cells. Figure 1 shows the changes in absorbance at 700 nm caused by the growth of *M. aeruginosa* during the experiments. The cells stressed with 100 mM NaCl grew more slowly than control cells, whereas 150 mM NaCl affected the viability of *M. aeruginosa*, and after 72 h, the cells died. Tonk *el al.* (2007) described this strain as salt tolerant until a concentration of 170 mM NaCl (10 g/L); however, in our case, the cells were affected at a lower concentration of salt.

Effect of salinity on levels of *mcyD* transcripts determined by RT-PCR

The RT-PCR is increasingly used because of high sensitivity, good reproducibility, and a wide, dynamic range of quantification. Quantification is

performed with two approaches, absolute and relative. In our study, relative quantification was used, which determines the expression level of a gene in comparison with a reference gene, as an internal control. In our study, two housekeeping genes were used, 16S rRNA and *rpo*. Similar results were obtained, indicating that both housekeeping genes were adequate. The results presented were calculated using 16S rRNA as the reference. The normalisation of all samples was performed with the relative expression as described previously (Pfaffl, 2001).

The relative levels of *mcyD* transcription that were measured with real-time efficiency (E) and cycle threshold (Ct) values under saline stress were compared with the levels in the control culture.

The changes in expression of *mcyD* mRNA in stressed cells were measured at different times, and results for the 150 mM NaCl cultures are shown in figure 2. A similar trend was obtained for the 100 mM NaCl cultures (data not shown). A clear decrease in *mcyD* transcripts was detected, which indicated that microcystin synthesis is not induced under this type of stress. Using RPAs, Kaebernick *et al.* (2000) found a decrease in *mcyB* transcripts in cells stressed with 250 mM NaCl after 1 h, but these data should be interpreted with caution because it is a lethal salt concentration for *M. aeruginosa*. At 48 and 72 h, the decrease in transcripts was lower than at previous times, most likely because of acclimation to the stress.

Cell contents of microcystin-LR in the presence of salt

The contents of microcystin-LR in the stressed cells were consistent with the changes found during the transcriptional analysis of the *mcyD* gene (Fig. 3). The microcystin-LR content was determined by HPLC, and the strain also had a minor amount of D-Aps[3] MC-LR (Wiedner *et al.*, 2003). The results are expressed as total protein, with the values shown in Figure 3, which shows a decrease in proteins at a salt concentration of 150 mM. This result indicated that microcystin production was not induced by the stress of in-
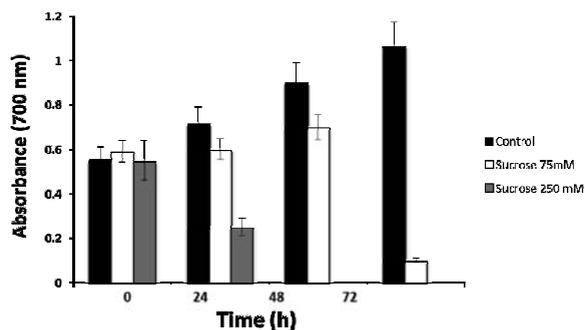
Figure 4. Effect of sucrose on the growth of *M. aeruginosa* cells. The absorbance of aliquots of cells growing in 75 and 250 mM sucrose was measured at 700 nm. *Efecto de la sacarosa en el crecimiento de* M. aeruginosa*. Se determinó la absorbancia a 700 nm de alícuotas de células creciendo en 75 y 250 mM de sacarosa.*

creased salinity, and there was a decline in microcystin. The decline was most likely caused by the lower growth rate and the general effects of salinity on photosynthesis (Sudhir *et al.,* 2005) because the activity of the photosynthetic electron chain affects the production of microcystin (Sevilla *et al.,* 2008).

Effect of Sucrose on growth of *M. aeruginosa*

To establish the difference between stress caused by presence of ions in the cytosol and osmotic changes, sucrose was used as an osmolite to cause stress. There are no previous studies that examined the effects of hyperosmotic stress on *Microcystis*, although effects were described for other cyanobacteria. For example, *Synechocystis* reacted to salt and hyperosmotic stresses as different perturbations, with different effects on cytoplasmic volume and on gene expression (Kanesaki *et al., 2002*). We found that *M. aeruginosa* was more sensitive to hyperosmotic pressure in the media than other species because we used sucrose in concentrations in the range used in studies on other cyanobacteria. For the other species, a typical sucrose concentration that caused cell death within a few hours was 250 mM (Fig. 4). The 100 mM sucrose treatment caused also cell death in this study, and at 75 mM, growth continued until 48 h, but the cells were then severely affected (Fig. 4). Thus, osmotic stress affected the growth of *M. aeruginosa* PCC7806 in the laboratory.
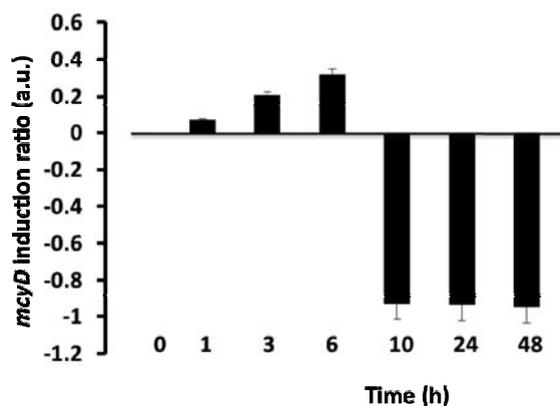

Figure 5. Effect of sucrose on the expression of *mcyD* in *M. aeruginosa* cells. Induction of expression of *mcyD* mRNA is shown as the fold increase in cells treated with 75 mM sucrose. The data are calculated relative to the expression in nonstressed cells and are normalised with the expression of the reference gene 16S rRNA. The value of the fold increase calculated for each time was normalised to the fold increase at the beginning of the experiment, when the ratio was 1 (Pfaffl 2001). Results are expressed as ratio −1. *Efecto de la presencia de sacarosa en la expresion de mcyD en* M. aeruginosa*. Inducción de* mycD *en células tratadas con 75 mM de sacarosa. Los datos se calculan relativos a la expresión de las células control, y se normalizan en referencia a la expresión del 16S rRNA. Los valores estan normalizados con respecto a la expresión en el inicio del experimento, con una razón igual a 1 (Pfaffl 2001). Los resultados obtenidos se expresan en la gráfica como ratio −1.*

Effect of sucrose on *mcyD* expression

Figure 5 shows the changes in levels of *mcyD* transcripts in cells that were exposed to 75 mM sucrose; similar results were obtained at the 100 mM concentration in the initial samples, before the cells died (data not shown). An initial small increase occurred in the levels of mRNA, which was followed by a decrease after 10 h of exposure to sucrose in the culture media.

Effect of sucrose on cellular levels of microcystin-LR

The level of microcystin-LR in cells was consistent with the transcriptional analysis data, and figure 6 shows the contents of microcystin-LR in cell aliquots, referred to as total protein. Protein synthesis was strongly affected by hyperosmotic stress (data not shown), and the microcystin produced by stressed cells decreased. The results
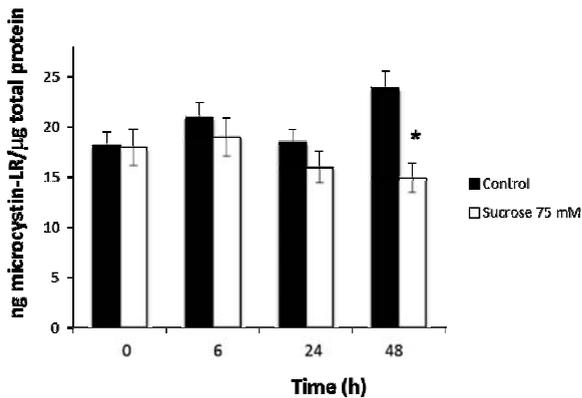
Figure 6. Content of intracellular microcystin-LR in sucrose-treated cells. Microcystin-LR content was determined by HPLC, and the value is presented as total soluble protein in *M. aeruginosa* cells. Data were analysed with a one-way ANOVA statistical test with a significance level of 0.05 (*).*Microcistina-LR intracelular en células tratadas con sacarosa. El contenido en microcistina-LR se cuantificó mediante HPLC y se refiere a la proteína soluble total presente en las células de* M. aeruginosa. *Datos significativos analizados con ANOVA (0.05).*

indicated that microcystin synthesis is not a response to osmotic stress.

DISCUSSION

For many years, considerable effort was invested in the study of the relationship between environmental factors and microcystin synthesis. In addition to heterogeneity of strains and laboratory conditions, the difficulties inherent with interpretation of field data illustrate the necessity for a different approach. Currently, increased salinity is one of the most important changes occurring in the environment, and it is necessary to predict how this change will affect the synthesis of microcystin in the toxic strains. For the experimental conditions, we found that high levels of salinity decreased the microcystin content in *M. aeruginosa* PCC7806 cells (Figure 3), which was consistent with the results of Black *et al.* (2011) and Liu (2006). In support of this observation, a decrease in *mcyD* transcripts was found in salt-stressed cells, a result described for the first time in this work (Fig. 2). For salt tolerance, we found that *M. aeruginosa* PCC7806 cells were affected by

lower salt concentrations than other strains, with described limits of 170 mM (Tonk *et al.*, 2007) and above 215 mM (Black *et al.*, 2011). In our study, the cells were seriously affected at 8.7 g/l (150 mM). The average marine salinity is approximately 35 g/l (598 mM), whereas estuaries range between 0.5 and 17 g/l (8-290 mM). Assuming that the behaviour of this strain of *Microcystis* can be extrapolated to other strains and to natural conditions, according to our results, only oligohaline or low mesohaline estuaries could support a bloom of *M. aeruginosa*, and the production of microcystin would decrease as the concentration of salt increases.

The growth of cells was severely affected by salt and sucrose, particularly by the sugar. The effects observed on the expression of *mcyD* and on the levels of microcystin were caused by the effects on growth. The growth rate itself was positively correlated with the production of microcystin, as previously reported (Downing *et al.*, 2005). However, results from previous studies indicated that the microcystin synthesis of cells could be affected without any effects on growth, as occurred, for example, with a phosphate deficiency (Sevilla *et al.*, 2012; Kuniyoshi *et al.*, 2013). By contrast, some stresses affected growth but not levels of toxin, as occurred, for example, with dark conditions, excess phosphate or nitrate deficiency (Sevilla *et al.*, 2010, 2012; Kuniyoshi *et al.*, 2013).

With the decrease in *mcyD* expression, there was a decrease in levels of microcystin-LR, and the salt-stressed cells had lower levels of toxin than nonstressed cells (150 mM; Fig. 2 and Fig. 3). The same behaviour was found at salt concentrations of 75 and 100 mM (data not shown). The addition of sucrose resulted in a hypertonic solution of a nonpenetrating solute, and the growth of *M. aeruginosa* was severely affected at 75 mM (Fig. 4). At 150 mM sucrose, the turgor pressure in *Microcystis* declined four-fold (Comte *et al.*, 2007), a likely reason for the effects on growth (Fig. 4). The levels of expression of *mcyD* and production of microcystin decreased in the presence of sucrose, to a similar extent as the decrease with salt. Nonpenetrant osmolites are present in low

concentrations in natural waters. Moreover, literature reports on salt tolerance of *Microcystis* indicate variability, depending on the strain used, and thus, extrapolation to and prediction of effects in estuaries are difficult. Nevertheless, fortunately, the production of microcystin decreases in saline conditions, which is consistent with results of published works (Liu, 2006; Tonk *et al.*, 2007; Black *et al.*, 2011).

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