RESEARCH LETTER

Quorum quenching activity in *Anabaena* sp. PCC 7120: identification of AiiC, a novel AHL-acylase

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Abstract

Many bacteria use quorum sensing (QS) to coordinate responses to environmental changes. In Gram-negative bacteria, the most extensively studied QS systems rely on the use of *N*-acylhomoserine lactones (AHLs) signal molecules. Some bacteria produce enzymes that are able to inactivate AHL signals produced by other bacteria and hence interfere with QS-mediated processes via a phenomenon known as quorum quenching. Acylase-type AHL degradation activity has been found in the biomass of the filamentous nitrogen-fixing cyanobacterium *Anabaena* (*Nostoc*) sp. PCC 7120, being absent from the culture media. The gene *all3924* has been identified and cloned whose product exhibits homology to the acylase QuiP of *Pseudomonas aeruginosa* PAO1, demonstrating that it is at least partially responsible for the AHL-acylase activity. The recombinant enzyme, which was named auto-inducer inhibitor from *Cyanobacteria* (AiiC), shows broad acyl-chain length specificity. Because the presence of AHLs in the biomass of nitrogen-fixing cultures of *Anabaena* sp. PCC 7120 has been described recently, AiiC could represent a self-modulatory system to control the response to its own QS signals but could also be involved in the interference of signalling within complex microbial communities in which *Cyanobacteria* are present.

Introduction

Many bacterial species coordinate responses to environmental changes using complex cell–cell communication mechanisms in a population density-dependent manner. This phenomenon has become known as quorum sensing (QS) and is based in the accumulation of signal molecules in the culture media that control the expression of multiple genes (Fuqua *et al.*, 1994). Using QS bacterial populations can coordinate important biological functions including motility, swarming, aggregation, plasmid conjugal transfer, luminescence, antibiotics' biosynthesis, virulence, symbiosis or biofilm maintenance and differentiation (Swift *et al.*, 2001; Waters & Bassler, 2005; Williams *et al.*, 2007). In addition, many plant and animal pathogenic bacteria control virulence through QS (De Kievit & Iglewski, 2000; Swift *et al.*, 2001; Von Bodman *et al.*, 2003). While several chemically distinct families of QS signal molecules have now been described, the most-studied QS signalling system involves *N*-acylhomoserine lactones (AHLs), used by diverse Gram-negative bacteria (Whitehead *et al.*, 2001; Fuqua & Greenberg, 2002; Williams *et al.*, 2007). AHLs differ in the acyl side chain, which is usually between four and 18 carbons in length, with or without saturation or C3 hydroxy- or oxo-substitutions (Whitehead *et al.*, 2001).

Because many bacterial pathogens use AHLs to control the production of virulence factors, the interference with this cell density-dependent communication mechanism constitutes a novel and promising strategy to control bacterial infectious diseases (Dong & Zhang, 2005; Dong *et al.*, 2007). Evidence is beginning to accumulate indicating that inhibition of QS, a mechanism known as quorum quenching (QQ), may be a strategy already adopted by many organisms to disarm potentially pathogenic or competitor bacteria. An example of QQ is the production of AHL agonists by the red alga *Delisea pulchra* to inhibit their surface colonization by AHL-based QS microorganisms (Givskov *et al.*, 1996). Another strategy to block the

AHL-based QS is the enzymatic inactivation of signals found in mammalian cells (Xu *et al.*, 2003; Chun *et al.*, 2004) and in different bacteria (reviewed by Dong & Zhang, 2005). Plants can also interfere with AHL signals, probably through an enzymatic mechanism, although no enzyme responsible for this activity has been identified (Delalande *et al.*, 2005). Two main types of enzymes have been described so far with AHL-degrading activity in both cases, making the signal molecule unable to activate its cognate sensor/response regulator: the lactonases, which hydrolyse the HSL ring of the AHL molecule to produce corresponding acyl homoserines and are highly specific against AHLs, and the acylases, which cleave the AHL amide bond, generating the corresponding fatty acid and homoserine lactone (HSL) (Dong *et al.*, 2007). Lactonases have been identified in several strains of the genus *Bacillus* and *Arthrobacter* sp. but are also present in some Gram-negative bacteria such as *Klebsiella* or *Agrobacterium* (Dong & Zhang, 2005). AHL-acylase activity has been identified in *Pseudomonas aeruginosa* PAO1, *Ralstonia* sp., *Streptomyces* sp. and the Gram-positive *Rhodococcus erythropolis* (Lin *et al.*, 2003; Uroz *et al.*, 2005; Park *et al.*, 2005; Huang *et al.*, 2006; Sio *et al.*, 2006). AHL-acylase activity was also detected in *Variovorax paradoxus* but the enzyme responsible remains undetermined (Leadbetter & Greenberg, 2000).

The presence of AHLs in the biomass of the nitrogen-fixing filamentous cyanobacterium *Anabaena* sp. PCC 7120 has been demonstrated recently (M. Romero *et al.*, unpublished results). The maximal concentration of AHLs was detected at the end of the exponential growth phase (48 h), disappearing abruptly 24 h later, which might indicate the possible presence of an AHL-degradation activity. Moreover, a sequence homologous to the QuiP acylase from *P. aeruginosa* PAO1 could be identified in the genome of *Anabaena* sp. PCC 7120 (Kaneko *et al.*, 2001). Both facts led to the investigation of the presence of enzymatic QQ activity in pure cultures of *Anabaena* sp. PCC 7120.

Materials and methods

Strains and culture conditions

Axenic cultures of *Anabaena* (*Nostoc*) sp. PCC 7120 were completely acclimatized to nitrogen-fixing conditions (diazotrophic cultures, $BG11_0$ medium, Rippka *et al.*, 1979) or to the presence of nitrate (nondiazotrophic cultures, BG11 medium). The axenicity of the cultures was checked daily (Rippka *et al.*, 1979). Cultures were grown in 300-mL tubes (diameter 4.5 cm) aerated with filter-sterilized air supplemented with CO_2 in order to maintain the pH at 7.0, and maintained at 30 °C with a circadian light cycle of 12 h light : 12 h dark at a light intensity of 118 μmol photon m^{-2} s^{-1} provided by OSRAM Daylight L36W/10 lamps. Cultures were inoculated with 50 mL of late logarithmic-phase cultures and allowed to reach the stationary phase (4 days).

Three biosensors of *Escherichia coli* JM109 transformed with the *lux*-based reporter plasmids pSB536, pSB401 or pSB1075 that were used to quantify the different AHLs with short, middle and long acyl chains, respectively, were grown on Luria–Bertani (LB) medium supplemented with the suitable antibiotic at 37 °C (Throup *et al.*, 1995; Winson *et al.*, 1998).

Detection of AHL-degradation activity

Samples (4 mL) were obtained from 48–hour-old completely acclimatized diazotrophic and nondiazotrophic cultures of *Anabaena* sp. PCC 7120. The samples were centrifuged for 10 min at 2000 ***g*** in order to separate the biomass from the culture media. The pellets were washed with 4 mL of PBS pH 6.5, resuspended in another 2 mL of the same buffer, sonicated in ice for 5 min and centrifuged at 16 000 ***g*** for 90 min. The crude cell extracts (CCEs) obtained in this way were filtered through a 0.22 μm and stored at 4 °C (Uroz *et al.*, 2005). For the detection of AHL degradation activity, 500 μL of culture media or CCEs were mixed with 500 μL of Tris-HCl buffer 100 mM pH 7.0 and 6 μL of a 1 mg mL^{-1} solution of *N*-dodecanoyl-L-homoserine lactone (C12-HSL). The mixture was incubated at 30 °C for 1 h and the reaction was stopped by heating at 95 °C for 5 min (Park *et al.*, 2005). Finally, 10 μL of the reaction mix was pipetted into a microtitre plate and the amount of remaining intact AHL was measured on addition of a 1/100 dilution of an overnight culture at 37 °C of the *lux*-based biosensor strain *E. coli* JM109 pSB1075 (Winson *et al.*, 1998) using a luminometer (Ultra Evolution Xfluor4beta E 4.51e). A positive control was set with 10 μL of Tris-HCl 100 mM pH 7.0/40 μM C12-HSL heated at 95 °C for 5 min. Negative controls were established with 10 μL of CCE and 10 μL Tris-HCl 100 mM pH 7.0. To monitor the evolution of AHL degradation activity along the growth curve 4 mL samples were taken at 12, 24, 36, 48, 72 and 96 h after inoculation of diazotrophic cultures ($n = 4$), centrifuged and processed to obtain the corresponding CCEs.

Specificity of the degradation activity

Samples (300 mL) were taken from diazotrophic cultures of *Anabaena* sp. PCC 7120 72 and 96 h after inoculation and the CCEs were prepared in 30 mL of PBS (pH 6.5) as explained before, and were used to determine the specificity of the degradation. Acetonitrile stocks of AHLs were pipetted in triplicate in a 96-well microtitre plate in order to obtain a final concentrations of 100, 10, 1 and 0.1 μg mL^{-1} in the reaction mixture. The solvent was allowed to evaporate and AHLs were reconstituted in 100 μL CCE and

incubated at 30 °C. Controls were set for each AHL concentration in which 100 μL of PBS, pH 6.5, were added. After 4 h, 100 μL of a 1 : 100 dilution of a suitable *lux*-based sensor (*E. coli* pSB401, pSB536 or pSB1075) was added to each well and incubated for another 2 h at 37 °C. Plates were photographed by a light camera and the differences in bioluminescence were evaluated to determine the degree of degradation of each AHL in comparison with controls. The AHLs tested varied in their *N*-acyl side chains, which were as follows: *N*-butyryl-L-homoserine lactone (C4-HSL), *N*-(3-oxobutyryl)-L-homoserine lactone (OC4-HSL), *N*-(3-hydroxybutyryl)-L-homoserine lactone (OHC4-HSL), *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-(3-oxohexanoyl)-L-homoserine lactone (OC6-HSL), *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (OHC6-HSL), *N*-octanoyl-L-homoserine lactone (C8-HSL), *N*-(3-oxooctanoyl)-L-homoserine lactone (OC8-HSL), *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (OHC8-HSL), *N*-decanoyl-L-homoserine lactone (C10-HSL), *N*-(3-oxodecanoyl)-L-homoserine lactone (OC10-HSL), *N*-(3-hydroxydecanoyl)-L-homoserine lactone (OHC10-HSL), *N*-dodecanoyl-L-homoserine lactone (C12-HSL), *N*-(3-oxododecanoyl)-L-homoserine lactone (OC12-HSL), *N*-(3-hydroxydodecanoyl)-L-homoserine lactone (OHC12-HSL), *N*-tetradecanoyl-L-homoserine lactone (C14-HSL), *N*-(3-oxotetradecanoyl)-L-homoserine lactone (OC14-HSL) and *N*-(3-hydroxytetradecanoyl)-L-homoserine lactone (OHC14-HSL).

Identification of the type of degradation activity

CCEs obtained from 4-day-old diazotrophic cultures using the procedure mentioned before were used for the identification of the type of AHL degradation activity. The assays were carried out using OC12-HSL as described previously (Uroz *et al.*, 2005). A sample of 50 μL of OC12-HSL (100 mM in acetonitrile) was evaporated to dryness under nitrogen flux and incubated with 5 mL of the CCE at 30 °C for 4 h. Every hour, 1 mL samples of the reaction mixtures were obtained and processed for the detection of acylase and lactonase activity. For the detection of acylase activity, the presence of free HSL was determined by 'chemically trapping' the free amine released by dansylation with dansyl chloride (Uroz *et al.*, 2005). The reaction was performed by adding 125 μL of 10 mM NaOH to a 250 μL CCE sample, followed by the addition of 125 μL of 5 mM dansyl chloride and incubation overnight at 37 °C. The presence of dansylated HSL was analysed by reverse-phase HPLC separation of 20 μL of the mixture. As a control, synthetic HSL (final concentration 1 mM) was dansylated in parallel with the same reagents.

For the detection of a possible lactonase activity, which enzymatically opens the lactone ring, 250 μL of the sample was extracted twice with ethyl acetate, evaporated to dryness and resuspended in acetonitrile in order to quantify the remaining concentration of OC12-HSL present in the sample by reverse-phase HPLC as described before (Uroz *et al.*, 2005). In parallel, another 250 μL was treated with 10 mM HCl in order to adjust the pH of the sample to 2.0 and incubated overnight at room temperature to induce the recyclization of the lactone ring. Samples were extracted and analysed by reverse-phase HPLC in order to detect the increase in AHL concentration that would be produced in comparison with the nontreated sample, in case the degradation was caused by a lactonase (Uroz *et al.*, 2005).

DNA manipulation and cloning of the *all3924* gene

All DNA handling was performed following standard protocols (Sambrook *et al.*, 1989). Chromosomal DNA was isolated from cultures of *Anabaena* sp. PCC 7120 following the protocol of Wu *et al.* (2000). The ORF encoding the putative acylase gene (bases 4733973 to 4731430, obtained from www.cyanobase.com; Kaneko *et al.*, 2001) was amplified from chromosomal DNA by PCR using the forward primer 5′-TATGAATTC*ATG*GCACAGCACCCAAATATG-3′ and the reverse primer 5′-TATATCGAT*TCA*GTGATGGT GATGGTGATGTTTGGGCATTAACTGCAATTTT-3′ which contain EcoRI and ClaI restriction sites, respectively (underlined; start and stop codons are indicated in italics). The PCR product was digested with both restriction enzymes and inserted into the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible shuttle expression vector pME6032 cut with the same enzymes (Heeb *et al.*, 2002). The insert was sequenced with a CEQ8000 Genetic Analysis System using the primers P6032: 5′-CCCTCACTGATCCGCTAGTC-3′ and PTAC: 5′-CGGCTCGTATAATGTGTGGA-3′. The sequence of the cloned gene product was compared with other bacterial acylase sequences available in the databases (QuiP and PvdQ of *P. aeruginosa* PAO1, AhlM of *Streptomyces* sp., AiiD of *Ralstonia* sp. and QsdA of *R. erythropolis* from the GenBank protein database) and with sequences of putative acylases from other *Cyanobacteria* (GLR1988 of *Gloeobacter violaceus* PCC 7421, NPR0954 *Nostoc punctiforme* PCC 73102 and SLR0378 of *Synechocystis* sp. PCC 6803) that were identified using the Cyanobase BLAST tool (Kaneko *et al.*, 2001). The phylogenetic analysis of sequences was carried out using the MULTALIN (multiple sequence alignment with hierarchical clustering) software (Corpet, 1988; http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html).

Expression of *all3924* in *E. coli* and detection of AHL-acylase activity

Escherichia coli DH5α transformed with plasmids pME6032 or pME6032::*all3924* was grown for 24 h at 37 °C or 48 h at

28 °C in 100 mL LB medium supplemented with 25 μg mL^{-1} tetracycline and 1 mM IPTG. Cells were harvested by centrifugation, washed twice with 10 mL of PBS, pH 7.0, sonicated 5 min in ice and centrifuged 15 min at 16 000 ***g*** to obtain a CCE.

The acylase activity of the obtained CCEs towards C12-HSL was determined using a bioluminescence assay. Aliquots consisting of 100 μL of CCE containing 100 μg mL^{-1} of synthetic AHLs were prepared. After 4 h of incubation at 30 °C, half of the reaction mixtures were acidified to pH 2.0 with HCl and incubated overnight at room temperature. Finally, all the reaction mixtures were extracted twice with ethyl acetate and the extracts obtained were evaporated to dryness. For the bioassay, the extracts were resuspended with a 1/100 dilution of an overnight culture of the *lux*-based sensor strain *E. coli* pSB1075, triplicate samples were deposited in 96-well microtitre plates at 37 °C and light emission was measured over time.

Results

AHL degradation activity

Acylase activity was found in the biomass of both diazotrophic and nondiazotrophic cultures of *Anabaena* sp. PCC 7120, but no degradation activity could be detected in the cell-free supernatants of any of them (data not shown). Because the concentration of AHL seemed to be strictly modulated in diazotrophic cultures (M. Romero *et al.*, unpublished results), while no AHLs could be found in any of the nondiazotrophic cultures analysed, further characterization of the AHL degradation activity was performed using only diazotrophic cultures. The degradation activity varied along the growth curve. The activity was low in early cultures (12 h after inoculation), increasing up to 36 h and declining abruptly at the early stationary phase, between 36 and 48 h, and recovering thereafter (Fig. 1).

To investigate the specificity of the AHL-degrading activity for AHLs with different acyl chain lengths and substitutions, a selection of AHLs with chains ranging from C4 to C14 were tested. Although the CCEs of diazotrophic cultures of *Anabaena* sp. PCC 7120 were able to degrade a wide range of AHLs, they showed a preference for long-chain AHLs (Table 1).

Identification of the type of degradation activity

To investigate whether the AHL-degrading activity was caused by lactonolysis, 1 mM OC12-HSL treated with CCE of diazotrophic *Anabaena* sp. PCC 7120 during 4 h was acidified to enable the recyclization of any opened HSL rings. HPLC analysis of the acidified samples showed the inability to recover the original levels of intact AHLs (Fig. 2a), suggesting that the degrading activity observed is

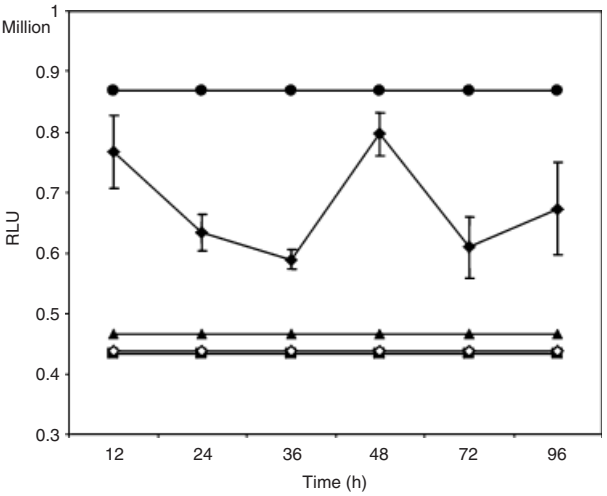

Fig. 1. Changes in the AHL degradation activity of CCEs obtained at different times of the growth curve of diazotrophic cultures ($n = 4$, scale bars represent SD) of *Anabaena* sp. PCC 7120. Activity of CCE with 40 μM solution of C12-HSL (♦) as measured with the bioluminescent sensor strain *Escherichia coli* JM109 pSB1075. Controls: (■) no addition, (▲) TRIS, (●) TRIS + AHL, (◇) CCE without AHL. RLU: relative light units.

Table 1. Degradation of different AHLs (100 μg mL^{-1}) by CCEs of 4 days old diazotrophic cultures of *Anabaena* sp. PCC 7120 and 2 days old culture of *Escherichia coli* DH5α transformed with pME6032 expressing the *aiiC* gene

	CCE	
AHL	*Anabaena* sp.	*E. coli*
Short length AHLs		
C4 HSL	+	+
OC4 HSL	+	ND
OHC4 HSL	+	ND
C6 HSL	+	+
OC6 HSL	+	ND
OHC6 HSL	+	ND
Middle length AHLs		
C8 HSL	+	++
OC8 HSL	+	ND
HC8 HSL	+	ND
C10 HSL	+++	++
OC10 HSL	++	+++
OHC10 HSL	+++	+++
Long length AHLs		
C12 HSL	+++	+++
OC12 HSL	++	+++
OHC12 HSL	+++	+++
C14 HSL	+++	++
OC14 HSL	+++	ND
OHC14 HSL	+++	ND

The degradation was measured as a decrease of the AHL activity in a liquid media bioassay, with three biosensors for short, middle and long side chain AHLs. *Escherichia coli* cultures were carried out at 28 °C and degradation assays at 30 °C.
ND, not determined.

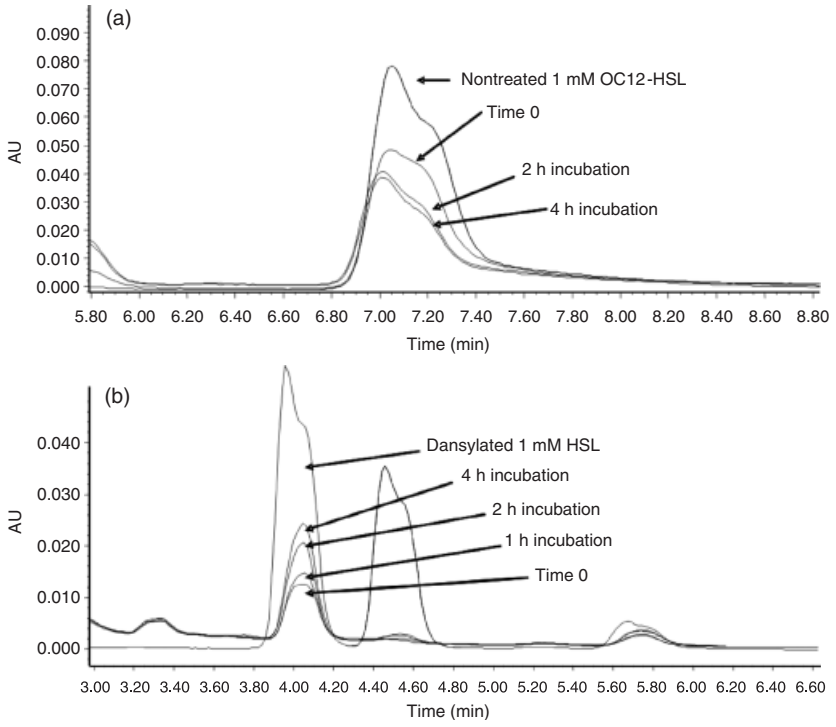

Fig. 2. Identification of the type of AHL degradation activity in CCEs of diazotrophic *Anabaena* sp. PCC 7120 by HPLC. (a) Degradation of OC12-HSL by CCE at different times of incubation as measured by HPLC after acidification to pH 2.0. No recovery of the AHL activity following acid treatment is observed, which dismisses the presence of lactonase activity. (b) Dansylation of OC12-HSL treated with CCE to reveal the presence of free HSL. The concentration of dansylated-HSL increases with the time of exposure of OC12-HSL to the CCE of *Anabaena* sp. PCC 7120, confirming the presence of an acylase that cleaves the amide bond linking the HSL ring and the acyl chain. AU, absorbance units.

not due to lactonolysis. This suggested that this degradation may be due to acylase activity instead. To test this possibility, dansylation experiments were performed and the dansylated products were analysed by HPLC. Figure 2b shows the presence of dansylated HSL, resulting from the cleavage of the amide bond between the HSL and the acyl chain. This dansylated product increased in concentration with time in samples obtained by exposing OC12-HSL for 4 h to the CCE (Fig. 2b). These results clearly indicate the presence of an AHL acylase in CCEs of *Anabaena* sp. PCC 7120 that specifically released the HSL from OC12-HSL. The difference between control (nontreated 1 mM OC12-HSL) and Time 0 sample in the acidification assay (Fig. 2a) is due to the partial recovery of AHL from the organic matrix during the extraction procedure because at least two sequential extractions are recommended for efficient recovery (Ortori *et al.*, 2007). In addition to this, a few minutes could be enough to obtain some degradation of the AHL, as shown by the presence of free HSL at Time 0 in the dansylation assay (Fig. 2b).

Cloning of the *aiiC* acylase gene

Using the *P. aeruginosa* PAO1 quorum signal utilization and inactivation acylase QuiP (PA1032) as a query, the product of *all3924* (GenBank protein accession No. BAB75623) was identified in a BLAST search at the Cyanobase web site as a gene encoding a potential acylase in *Anabaena* sp. PCC 7120. QuiP and the product of *all3924* share 29% identity and 47% similarity between them. The *all3924* gene was cloned by PCR into the expression vector pM6032 in *E. coli* DH5α. The sequence of the cloned insert was identical to the sequence available in the genome database. Incubation for 4 h of CCEs from the recombinant *E. coli* expressing *all3924* with a C12-HSL solution showed a decrease of 50% of AHL activity as monitored with the AHL biosensor pSB1075 (Fig. 3). Very little activity was recovered after acidification of the mixture to pH 2.0, which confirms that the reduction of AHL concentration is not due to a lactonase but instead due to the presence of acylase activity in the CCE of the transformed strain. The slight recovery of activity observed after acidification of the treated sample is due to the recovery of the portion of the AHL that is spontaneously degraded at a neutral pH after exposure to CCE, as indicated by the same trend being present in samples treated with the CCE of *E. coli* DH5α transformed with the empty vector (Fig. 3). Interestingly, the acylase activity in the clone was dependent on the growth temperature, because no activity was found when grown at 37 °C (data not shown). The *all3924* gene has been named as *aiiC* for being the first *a*uto*i*nducer *i*nhibitor identified in a cyanobacterium.

Discussion

AHLs accumulate in the biomass of *Anabaena* sp. PCC 7120 during exponential growth of completely acclimatized

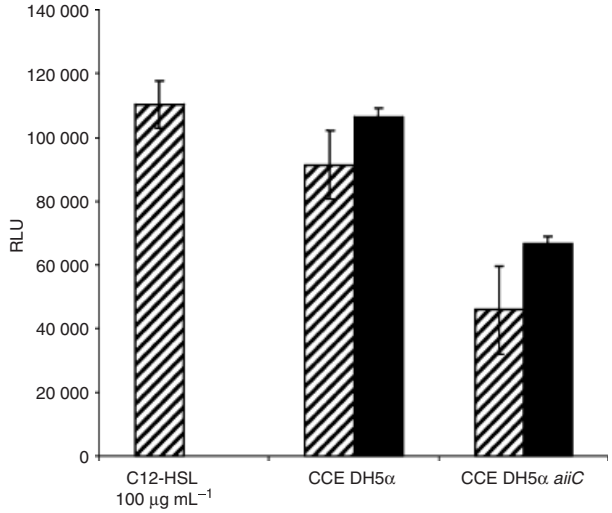

Fig. 3. C12-HSL degradation activity of CCE of *Escherichia coli* DH5α transformed with pME6032 carrying (CCE DH5α *aiiC*) or not (CCE DH5α) the *aiiC* gene before (hatched bars) and after acidification to pH 2.0 (solid bars). Luminescence values of negative controls (nontreated biosensor or biosensor plus solvent) were subtracted in order to normalize the results. *E. coli* cultures were carried out at 28 °C and degradation assays were carried out at 30 °C.

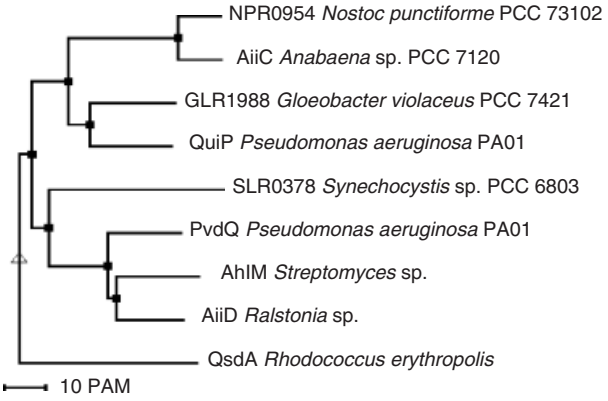

Fig. 4. Tree showing the phylogenetic relationship between the AiiC acylase of *Anabaena* sp. PCC 7120 and homologous sequences from known or putative acylases present in diverse bacteria and *Cyanobacteria*. PAM, percent accepted mutation.

diazotrophic cultures and disappear abruptly when the early stationary phase is achieved (M. Romero *et al.*, unpublished results), indicating that the level of the signals could be modulated through the presence of degradation mechanisms. This fact encouraged a search for enzymes able to degrade the AHLs in *Anabaena* sp. PCC 7120. The degradation activity found, demonstrated to be acylase-type by the dansylation assay, can be at least partially attributed to the *aiiC* acylase gene cloned in this paper. Even though the presence of additional acylases in *Anabaena* sp. PCC 7120 cannot be completely excluded, the similarity between the specificity of AHL degradation of the cyanobacterial CCE and the cloned enzyme (Table 1) seems to indicate that AiiC is the only enzyme responsible for the degradation of AHLs in *Anabaena* sp. PCC 7120.

The 2544 bp *all3924* gene, here named *aiiC*, encodes an 847 amino acid protein. As reported for the homologous quorum signal utilization and inactivation protein QuiP in *P. aeruginosa* PAO1, the presence of a signal sequence and a putative transmembrane domain located in the *N*-terminal region (residues 20–40) suggests that AiiC has a periplasmic localization. Acylase activity has been localized primarily in the cell debris after lysis in *Ralstonia* sp. (Lin *et al.*, 2003), while in *Streptomyces* sp. it has been found in the culture media (Park *et al.*, 2005). The predicted secondary structure of AiiC and the absence of acylase activity in the cell-free culture supernatants suggest that in the case of *Anabaena* sp. PCC 7120, the enzyme is cell-bound.

Genomic searches with acylase sequences revealed the presence of genes homologous to the *Pseudomonas* acylases in the genomes of the filamentous cyanobacterium *N. punctiforme* and in the unicellular *G. violaceus* and *Synechocystis* sp., indicating that this activity could be widespread among the *Cyanobacteria*. The phylogenetic analysis shows that AiiC and most of the other cyanobacterial putative acylases are closely related to *P. aeruginosa* PAO1 QuiP (Fig. 4). Only the potential homologous acylase of the unicellular nondiazotrophic cyanobacterium *Synechocystis* sp. PCC 6803 is located in a different branch, closer to the PvdQ acylase of *P. aeruginosa*. Out of these two clusters is the *R. erythropolis* acylase QsdA, which shows no homology with the others. The QuiP cluster proteins share in average 30% identities between them, while the PvdQ cluster shares a more variable 23–40% identity in their peptide sequences. Despite their relatively low overall sequence homologies, conserved homologous domains of 11–12 residues can be observed in both the QuiP cluster (GYXHAQDRLWQM) and in the PvdQ cluster (GLLLXNPHXPN). The identity between the two AHL-acylases clusters is only 18%, without any noticeable conserved motif in their sequences. Despite showing a higher similarity to the *P. aeruginosa* PAO1 acylases, those are only able to degrade long-chain specifically AHLs (Huang *et al.*, 2006; Sio *et al.*, 2006), while AiiC can also degrade short-chain AHLs, (Table 1), as it has been described for QsdA of AiiD *R. erythropolis* and *Ralstonia* sp. or the activity found in *Variovorax paradoxus* (Leadbetter & Greenberg, 2000; Lin *et al.*, 2003; Uroz *et al.*, 2005).

Because the lowest acylase activity in the biomass of diazotrophic cultures is observed at the early stationary phase, correlating with the highest AHL concentration in the biomass (M. Romero *et al.*, unpublished results), the possibility that this enzyme could be directly involved in the

intracellular inactivation of the signals should be investigated. The use of the QQ enzymes for the self-modulation of the level of the signals has already been found in *Agrobacterium tumefaciens*, for which the lactonase activity of AttM seems to be crucial for the inhibition of conjugation of the Ti plasmid (Zhang *et al*., 2002). The hypothesis of a possible role of AiiC in the regulation of the AHLs intracellular level is further supported by the fact that the AHL acylase activity could be found only in the biomass. The continuous periplasm of filamentous *Cyanobacteria* is thought to be a channel for the transfer of signals and nutrients, which is essential for the multicellular nature of these organisms (Mariscal *et al*., 2007). The transfer of the signals through the continuous periplasm would be consistent with the finding of AHLs in the biomass of *Anabaena* sp. PCC 7120 and a possible periplasmic location of the acylase.

Although the possible physiological role of AHLs in *Anabaena* sp. PCC 7120 is not yet completely defined, the presence of AHLs only in the biomass of diazotrophic cultures and a strong effect of the addition of exogenous AHLs on the nitrogenase activity indicate a possible role of AHLs in the control of heterocyst differentiation or any other process related to nitrogen fixation (M. Romero *et al*., unpublished results). Another fact supporting the hypothesis of a role of AHLs and AHL-acylase in the complex network of genes controlling nitrogen fixation in *Anabaena* sp. PCC 7120 is the presence of a putative binding site for NtcA at position -48 in the *aiiC* promoter. NtcA is the global transcription regulator belonging to the cyclic AMP receptor protein family, which controls a number of genes involved in nitrogen and carbon metabolism and is responsible for the initiation of heterocyst differentiation (Herrero *et al*., 2004).

On the other hand, and even though the hypothesis that AiiC could play a self-regulatory function cannot be completely excluded, the presence of acylase activity in diazotrophic cultures, in which no AHLs could be detected, suggests a more general physiological role of AiiC. The unicellular nondiazotrophic cyanobacterium *Synechocystis* sp. PCC 6803 also possesses a putative acylase and this may indicate an alternative physiological role for this enzyme. Because the presence of AHLs has been demonstrated in the natural microbial communities in which the *Cyanobacteria* are present or even dominate, such as microbial mats or cyanobacterial blooms (Bachofen & Schenk, 1998; Braun & Bachofen, 2004), AiiC could also be involved in the interference of signalling in these complex bacterial systems.

Despite their ecological significance, the importance of QS systems have been questioned due to the relatively low number of *Proteobacteria* species in which the real physiological functions controlled by the AHL-mediated QS system has been identified (Manefield & Turner 2002). The increasing number of bacteria presenting AHL-degrading mechanisms reinforces the view on the ecological significance of these signals. In the case of *Cyanobacteria*, the presence of AHL signals only in the biomass suggests a different usage of these molecules that may rather act as intracellular signals. Even though some results indicate that AiiC could be involved in the self-regulation of the AHL levels in the diazotrophic filaments, the cyanobacterium could also use the enzyme as a defence barrier in order to avoid the interference of external signals with its own signalling system. The presence of AHL-acylase sequences in diverse *Cyanobacteria* would support the latter hypothesis. Further studies are required in order to elucidate the possible physiological role of AiiC in *Anabaena* sp. PCC 7120.

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