

# *pkn22* (*alr2502*) encoding a putative Ser/Thr kinase in the cyanobacterium *Anabaena* sp. PCC 7120 is induced by both iron starvation and oxidative stress and regulates the expression of *isiA*

Wen-Liang Xu<sup>a</sup>, Robert Jeanjean<sup>b</sup>, Yong-Ding Liu<sup>a</sup>, Cheng-Cai Zhang<sup>b,c,\*</sup>

<sup>a</sup>Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei 430072, PR China

<sup>b</sup>Laboratoire de Chimie Bactérienne, IBSM-CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France

<sup>c</sup>Key Laboratory of Agromicrobiology, Huazhong Agriculture University, Wuhan, Hubei 430070, PR China

Received 4 August 2003; revised 21 August 2003; accepted 21 August 2003

First published online 17 September 2003

Edited by P. Brzezinski

**Abstract** In cyanobacteria, the *isiA* gene is required for cell adaptation to oxidative damage caused by the absence of iron. We show here that a putative Ser/Thr kinase gene, *pkn22* (*alr2502*), is activated by iron deficiency and oxidative damage in *Anabaena* sp. PCC 7120. A *pkn22* insertion mutant is unable to grow when iron is limiting. *pkn22* regulates the expression of *isiA* (encoding CP43'), but not of *isiB* (encoding flavodoxin) and *psbC* (CP43). Fluorescence measurement at 77 K reveals the absence of the typical signature of CP43' associated with photosystem I in the mutant under iron-limiting conditions. We propose that Pkn22 is required for the function of *isiA*/CP43' and constitutes a regulatory element necessary for stress response. © 2003 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

**Key words:** Signal transduction; Iron; Photosynthesis; Protein phosphorylation

## 1. Introduction

In both prokaryotes and eukaryotes, protein phosphorylation catalyzed by protein kinases is an important signaling mechanism that allows cells to adapt to the constantly changing environment. It plays critical roles in cell responses to environmental stimuli by regulating gene expression and enzyme activities. Protein Ser/Thr and Tyr kinases are widespread in prokaryotes, although they were identified much later than their eukaryotic counterparts [1,2]. The first example of protein Ser/Thr kinases in cyanobacteria was reported in *Anabaena* sp. PCC 7120 [3], a filamentous cyanobacterium able to differentiate specialized cells (heterocysts) for molecular nitrogen fixation in the absence of combined nitrogen [4]. The complete sequencing of the genome of *Anabaena* sp. PCC 7120 revealed the existence of a family of 53 putative Ser/Thr and Tyr kinases [5–7]. For most of them, the function remains unknown. One of them, Pkn22 (Alr2502), is a putative protein Ser/Thr kinase belonging to subfamily STK-III as it has a protein kinase catalytic domain on the N-terminal region, and a C-terminal region [5,7]. *alr2502* is located on the chro-

mosome immediately upstream of a gene (*alr2503*) encoding a polypeptide similar to peroxiredoxin, a bacterioferritin comigrating protein [5]. Such a chromosomal organization could suggest that this protein kinase might play a role in the regulation of iron metabolism or iron stress response.

Cyanobacteria are often challenged with iron depletion since iron deficiency is believed to be one of the most important limiting factors for cyanobacterial growth in aquatic ecosystems [8]. Therefore, cell responses have been evolved to deal with frequently occurring conditions of iron limitation [8–12]. In bacteria, transcription factors, such as Fur or the Fur-like protein PerR, regulate the expression of genes involved in intracellular iron metabolism [13–15]. Iron homeostasis is also directly implicated in oxidative response since in the Fenton reaction, the reduced iron form can be used to convert less reactive hydrogen peroxide to more reactive oxygen species, causing oxidative damage [13]. Several reports have demonstrated that iron availability and oxidative stress can induce the expression of the same set of genes in bacteria [13–17].

In cyanobacteria, the gene products of *isiA* and *isiB* are expressed when iron is limited [18–20]. It has been shown that *isiA* is absolutely required for the survival of *Synechococcus* PCC 7942 under light excess in iron-depleted medium [18]. The *isiA* gene, which encodes the chlorophyll binding protein CP43', has originally been assumed to function with photosystem (PS) II based on its sequence similarity with CP43 encoded by *psbC* [10]. Recent studies have clearly demonstrated that the product of *isiA* works as an antenna to PSI [19,20], and may play a role in thermal dissipation of excess light energy [18]. The *isiB* gene encodes flavodoxin that can replace ferredoxin for most of its functions under iron-limiting conditions [8]. A Fur protein homologue from *Synechococcus* PCC 7942 can repress the expression of a reporter gene driven by the *isiA* promoter in *Escherichia coli* [22]. In general, the regulation of *isiA* expression is still not well understood. In this report, we show that *pkn22* regulates *isiA* and is required for cell growth under iron-limiting conditions.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*Anabaena* sp. PCC 7120 was cultured in BG11 medium, at 30°C in air under continuous illumination. Moderate iron-limited conditions were achieved by removing iron compound (ferric ammonium) from BG11 medium, while for severe iron deficiency, different concentra-

\*Corresponding author. Fax: (33)-4-91718914.

E-mail address: cczhang@ibsm.cnrs-mrs.fr (C.-C. Zhang).

Abbreviations: Sp, spectinomycin; Sm, streptomycin; MV, methyl viologen; PS, photosystem

tions of the iron chelator 2,2'-dipyridyl were added. 2,2'-Dipyridyl is frequently used to deplete iron more severely and rapidly (for an example, see [23]).

## 2.2. PCR amplification and inactivation of *pkn22*

Two polymerase chain reaction (PCR) primers were designed to clone *pkn22* (*aln2502*) as shown in CyanoBase [5]: CTTCTGCAGATGAGCCTCTGCATAAA, and CTCTCGAGCTACTCTACATTGCCGC. Both primers have three extra bases and one *Pst*I or *Xho*I restriction site at the 5' end respectively. PCR was performed according to standard procedures using Taq DNA polymerase (Biostar, Canada). The amplified *pkn22* coding region was disrupted by insertion of a 1.5-kb spectinomycin/streptomycin (Sp/Sm) resistance cassette into the unique *Hpa*I site, cloned into the conjugative plasmid pRL271, and conjugated into *Anabaena* PCC 7120 as described [24]. The initial conjugants were selected by screening for resistance to 2.5 µg/ml of Sp and 2.5 µg/ml of Sm, and were then cultured on BG11 plates containing 5% sucrose to select for double recombinants [24]. Genomic DNA of the recombinant cells was analyzed by PCR.

## 2.3. RNA extraction and RT-PCR

Filaments of *Anabaena* sp. PCC 7120 were cultured in BG11 to mid-log phase and 50 ml of cells was collected and frozen in liquid nitrogen and was used as a sample grown in the presence of iron. The remainder of the culture was transferred to BG11 depleted of iron with 50 µmol/l of 2,2'-dipyridyl. Samples were then collected at different time intervals after the transfer and used as iron-starved cells. Total RNA was prepared using a TRIzol reagent kit (Invitrogen) according to the manufacturer's instruction. Residual DNA in RNA preparations was eliminated by digestion with RNase-free DNase (Promega). The absence of DNA was checked by PCR. Reverse transcription (RT)-PCR was performed using the Access RT-PCR kit (Promega). The log phase of RT-PCR was determined by measuring the amount of PCR products at different time intervals. For the final results, 15–20 cycles at the early log phase were used. A similar amount of total RNA (0.1 µg) was used for comparative studies.

The following primers used for RT-PCR were designed based on the 3' coding region of each gene [5]: *pkn22* (*aln2502*), forward primer: ATGCCGAATCCACGATAACCAA, reverse primer CTACTCTACATTGCCGCTACGC; *aln2503*, forward primer: CACAGGTTTACATCAGGAATT; reverse primer: CTTATTAACTGCTTGAGC; *psbC*: forward primer, CCTCTACGTGGCCCTAACGGTCTT, reverse primer CTAGTCAAGGTCACCATAGCCATT; *isiA*, forward primer: GCGGTCAACACCTTAGCTTATCCT, reverse primer: TTACTCAGCACTACTAATGGCATT; *isiB*, forward primer: GGTAATTTGGTTGCCTACTT, reverse primer: TTACAAACCAATTCAGACTT.

## 2.4. Whole-cell fluorescence measurement at 77 K

Whole-cell and steady-state fluorescence spectra were measured using a Perkin Elmer LS50 at 77 K as described [19,21]. Filaments of

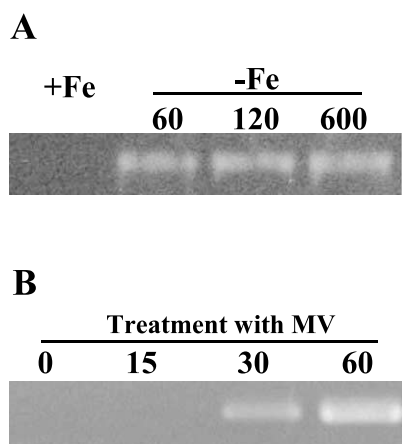


Fig. 1. RT-PCR analysis of *pkn22* expression under different conditions. A: Activation of *pkn22* by iron depletion. Total RNA was extracted from cells incubated in an iron-replete (+Fe) or -depleted medium (–Fe, with 50 µM of iron chelator) for 60, 120, and 600 min. B: Induction of *pkn22* expression in cells treated with 0.1 mM MV for 0, 15, 30 and 60 min.

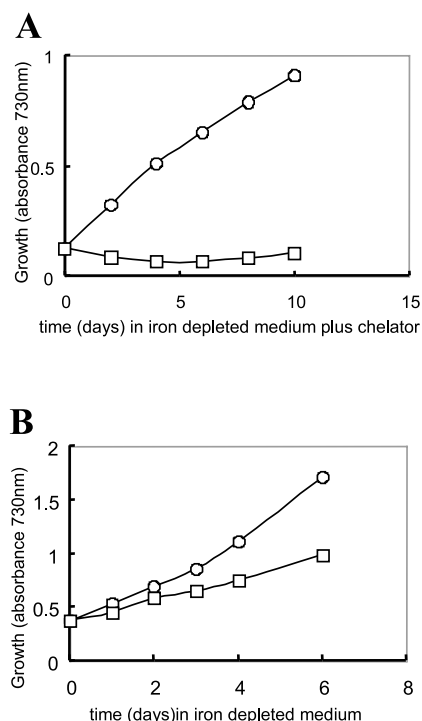


Fig. 2. Growth capacity of the wild-type (circles) and the *pkn22* mutant Mp22 (squares). A: Growth curves in medium lacking iron with 50 µM of the iron chelator 2,2'-dipyridyl. B: Growth curves in medium lacking iron without iron chelator.

*Anabaena* sp. PCC 7120 were grown in BG11 medium in the presence or absence of the iron compound ferric ammonium. Samples were excited at 570 nm and emission was detected at 600–800 nm.

## 3. Results and discussion

### 3.1. Regulation of the expression of the *pkn22* gene

Since the *pkn22* gene is located on the chromosome close to *aln2503* encoding a putative peroxiredoxin, a bacterioferritin comigration protein [5], the expression of these genes was studied under iron-depleted conditions. RT-PCR experiments were performed on total RNA extracted from samples cultured in the presence of iron or under iron-deficient conditions (see Section 2). The transcription of *pkn22* is strongly induced 1 h after iron deficiency, and remains at an equally high level 2 or even 10 h after (Fig. 1).

To see if oxidative stress also leads to the expression of *pkn22*, as in the case of many iron-responsive genes in bacteria [13], 0.1 mM of methyl viologen (MV) was added in the growth medium and the expression of *pkn22* was examined by RT-PCR. The presence of MV, a strong electron donor, establishes an artificial electron transfer route, leading to the oxidation of inter-chain carriers (plastoquinone pool and *b6f* complex) and oxidative stress due to the production of reactive oxygen species. As shown in Fig. 1, no transcript of *pkn22* could be detected in cells in BG11 untreated with MV. Thirty minutes after the addition of MV, a strong induction of *pkn22* could be observed, and this transcriptional activity was further amplified 60 min after the treatment with MV.

### 3.2. Selection of *pkn22* insertion mutant and analysis of its mutant phenotypes

To further understand the role of *pkn22*, this gene was

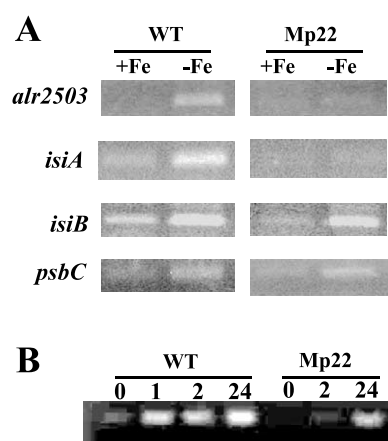


Fig. 3. RT-PCR analysis of the expression of *alr2503*, *isiA*, *isiB* and *psbC* in the wild-type and the *pkn22* mutant Mp22. A: Total RNA was extracted from cells incubated for 1 h in iron-replete or -depleted conditions (see Section 2). B: Kinetic studies with RT-PCR on *isiA* expression in the wild-type and the Mp22 mutant. Cells were cultured in BG11 in the presence of iron (time 0), or transferred into iron-free medium and incubated for different time intervals (in hours).

inactivated by homologous recombination. A 2-kb DNA fragment containing the coding region of *pkn22* was cloned from the genomic DNA after PCR amplification, and was interrupted by the insertion of a cassette bearing Sp and Sm resistance. After conjugation, several recombinants were analyzed by PCR to check whether double recombination events have occurred. One mutant, called Mp22, in which *pkn22* was completely inactivated and segregated, was successfully obtained since only a DNA fragment corresponding to the inactivated form of *pkn22* was amplified (data not shown).

In BG11 liquid medium, Mp22 grows only slightly more slowly than the wild-type strain. Since the expression of *pkn22* is induced by iron deficiency, the growth of Mp22 was compared to that of the wild-type strain under iron-replete and -depleted conditions (Fig. 2). When iron was moderately depleted by removing the ferric ammonium in the growth medium, the growth rate of Mp22 was half that of the wild-type strain. However, when iron was severely depleted by the presence 50  $\mu\text{mol/l}$  of the iron chelator 2,2'-dipyridyl, growth of the mutant was arrested, while the

wild-type strain could still sustain its growth albeit less efficiently (Fig. 2). *pkn22* inactivation did not produce a noticeable effect on heterocyst induction and development when the combined nitrogen source was limited in the growth medium (data not shown).

### 3.3. Inactivation of *pkn22* affects the transcription of *isiA* under conditions of iron limitation

Both *isiA* and *isiB* genes are expressed when cyanobacteria are faced with iron limitation [8,10,25,26]. To understand the phenotypes of Mp22, the influence of *pkn22* on the expression of these genes was studied. In filamentous cyanobacterial strains *Anabaena* sp. PCC 7120 and *Fischerella muscicola* sp. PCC 73103, *isiA* and *isiB* are localized at different positions on the chromosome [25]. In the unicellular strain *Synechocystis* sp. PCC 6803, these two genes are organized into an operon [26]. As shown in Fig. 3, both *isiA* and *isiB* are induced by iron limitation, consistent with results previously obtained in *Anabaena* PCC 7120 and other cyanobacterial strains [10,25]. The expression of *isiA* under iron starvation was practically abolished in the *pkn22*-null mutant 1 h after iron removal. By contrast, the transcription of the *isiB* gene does not appear to be affected by the inactivation of *pkn22* (Fig. 3). These results suggest that *pkn22* differentially affects the expression of *isiA* and *isiB*. Similarly, the inactivation of *pkn22* did not produce a strong effect on the regulation of *psbC* encoding the CP43 protein (Fig. 3). As previously shown [10], the *psbC* transcripts were more abundant under iron-depleted conditions. The effect of *pkn22* inactivation was further examined in prolonged iron limitation. The results demonstrate that *isiA* expression remained low 2 h after iron depletion, and recovered slightly 24 h after iron depletion. Another factor may be present to account for the weak expression of *isiA* under long-term iron-deficient conditions in the absence of *pkn22*.

Although *alr2503* encoding a protein similar to peroxiredoxin was closely linked to *pkn22* [5], no co-transcript could be identified between these two genes by RT-PCR (data not shown), suggesting that they may be transcribed separately. However, the inactivation of *pkn22* also has a negative effect of the expression of *alr2503* that is also induced by iron limitation in the wild-type strain (Fig. 3). *pkn22* therefore regulates *isiA* and *alr2503* induced by iron limitation.

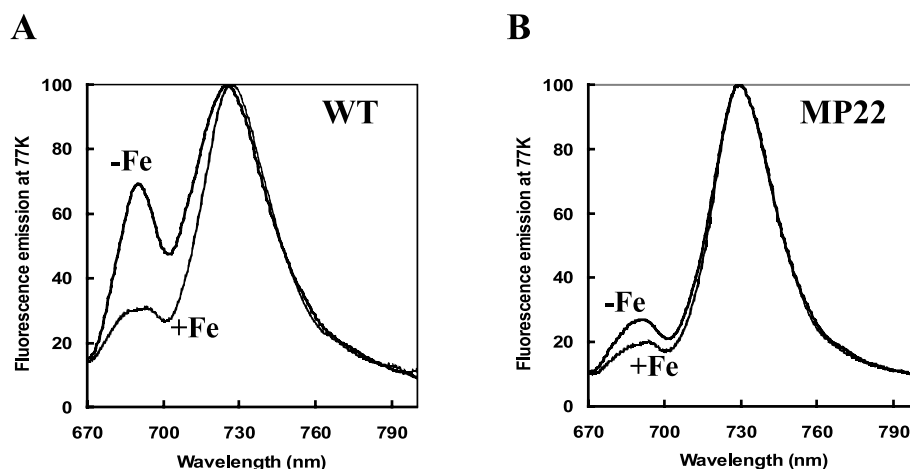


Fig. 4. 77 K fluorescence emission spectra for whole cells of *Anabaena* sp. PCC 7120. Wild-type (A) and Mp22 mutant (B) were cultured with iron (bold line) or without iron (light line), excited at 570 nm, and their emission was then recorded. Both spectra were normalized to the maximal fluorescence emission of P700.

### 3.4. Fluorescence spectra at 77 K in the wild-type and the Mp22 mutant

The phenotypes of the mutant Mp22 under iron-limited conditions could be mostly attributed to the weak expression of *isiA* since this gene plays a major role in the adaptation of PSI to iron deficiency [19–21]. We thus sought to determine whether the coding product CP43' of *isiA* can be found in the PSI complex in vivo.

Fluorescence measurement at 77 K in unicellular cyanobacteria showed that CP43' encoded by *isiA* is associated with PSI [19–21]. The same experiment was carried out with the wild-type and the Mp22 mutant in *Anabaena* PCC 7120 grown in the presence or absence of the iron source (ferric ammonium) in the culture medium (Fig. 4). In the wild-type, a characteristic spectral shift that was attributed to the association of CP43' with PSI [19,21] was clearly detected in cells cultured without iron as compared to cells cultured in the presence of iron. This typical signature of CP43' associated with PSI was completely lost in the Mp22 mutant studied under similar conditions (Fig. 4). These results suggest that no CP43' was associated with PSI in the Mp22 mutant under iron-limited conditions.

In conclusion, our results indicate that *pkn22* was required for cell adaptation to iron limitation, and maybe also oxidative stress. Pkn22 could exercise its function by regulating the expression of a set of genes induced under iron-depleted conditions, including *isiA* and *alr2503* (Fig. 3). Iron homeostasis and oxidative response are highly coordinated in bacteria [13]. It is well documented that when freely available iron is in excess, it may be used to generate highly reactive oxygen radicals, thus creating oxidative damage (for a review, see [13]). The *pkn22* gene is induced by both iron starvation and oxidative stress. It is likely that iron limitation may also cause oxidative stress in photosynthetic organisms since it can strongly limit the electron transfer capability due to the requirement of iron for many components in photosynthetic complexes and electron transfer chains [8]. In this case, light-generated electrons, not sufficiently consumed, may cause oxidative stress. This situation is reminiscent of that obtained with the treatment of cells with MV, a strong artificial donor of photosynthetic complexes and electron transfer chains (for a review, see [8]). It is also consistent with the proposed function of *isiA* in thermal dissipation and the requirement of *isiA* under high light stress [18] that may generate too many electrons for cells to handle in the absence of *isiA*. Since *pkn22* is necessary for the activation of *isiA* which is in turn involved in stress response under iron-limited conditions [18], it is likely that the *pkn22*-null mutant undergoes oxidative stress under similar conditions. The overoxidation of P700 in the mutant (unpublished) suggests indeed that under iron-deficient conditions it has lost the ability to adapt to iron limitation.

Both Fur [22] and Pkn22 regulate the expression of *isiA*. The regulatory effect of Fur on *isiA* expression was only demonstrated in *E. coli* [22]. Therefore, *pkn22* can be considered at the moment the only regulatory element of *isiA* with in vivo studies. While Fur is a negative transcriptional repressor, Pkn22 exerts a positive effect, either directly or indirectly, on the expression of *isiA*. The *pkn22* gene itself may also be under the control of Fur since a putative Fur box can be found upstream of the *pkn22* coding region (unpublished results). It would be interesting to study the regulatory mecha-

nism of *isiA* by Fur and *pkn22*, since it is one of the major elements induced by iron stress in cyanobacteria.

**Acknowledgements:** Work at the Institute of Hydrobiology, Chinese Academy of Sciences, was supported by grants to Y.-D.L. from the National Natural Sciences Foundation of China (30070154), the Frontier Science Program of the Institute of Hydrobiology (220316) and State Key Project (K99-05-35-01). We are also grateful for the support from the Cheung Kong Scholars Program, the National Natural Science Foundation and the National 863 Program for work done by C.C.Z. at the Huazhong Agricultural University. Work in France was supported by the ATIPE-Microbiologie program of the CNRS, and the program Environnement et Santé from AFSSE.

### References

- [1] Zhang, C.-C. (1996) *Mol. Microbiol.* 20, 9–15.
- [2] Bakal, C.J. and Davies, J.E. (2000) *Trends Cell Biol.* 10, 32–38.
- [3] Zhang, C.-C. (1993) *Proc. Natl. Acad. Sci. USA* 90, 11840–11844.
- [4] Wolk, C.P. (2000) in: *Prokaryotic Development* (Brun, Y.V. and Shimkets, L.J., Eds.), pp. 83–104, ASM Press, Washington, DC.
- [5] Kaneko, T., Nakamura, Y., Wolk, C.P., Kuritz, T., Sasamoto, S., Watanabe, A., Iriguchi, M., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kohara, M., Matsumoto, M., Matsuno, A., Muraki, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takazawa, M., Yamada, M., Yasuda, M. and Tabata, S. (2001) *DNA Res.* 8, 205–213.
- [6] Ohmori, M., Ikeuchi, M., Sato, N., Wolk, C.P., Kaneko, T., Ogawa, T., Kanehisa, M., Goto, S., Kawashima, S., Okamoto, S., Yoshimura, H., Katoh, H., Fujisawa, T., Ehira, S., Kamei, A., Yoshihara, S., Narikawa, R. and Tabata, S. (2002) *DNA Res.* 8, 271–284.
- [7] Wang, L., Sun, Y.-P., Chen, W.-L., Li, J.-H. and Zhang, C.-C. (2003) *FEMS Microbiol. Lett.* 217, 155–165.
- [8] Straus, N.A. (1994) in: *Molecular Biology of Cyanobacteria* (Bryant, D.A., Ed.), pp. 731–750, Kluwer Academic, Dordrecht.
- [9] Burnap, R.L., Troyan, T. and Sherman, L.A. (1993) *Plant Physiol.* 103, 893–902.
- [10] Leonhardt, K. and Straus, N.A. (1994) *Plant Mol. Biol.* 24, 63–73.
- [11] Poncelet, M., Cassier-Chauvat, C. and Chauvat, F. (1994) *Gene* 145, 153–154.
- [12] Falk, S., Samson, G., Bruce, D., Hunter, N.P.A. and Laudenbach, D.E. (1995) *Photosynth. Res.* 45, 51–60.
- [13] Touati, D. (2000) *Arch. Biochem. Biophys.* 373, 1–6.
- [14] Zheng, M., Doan, B., Schneider, T.D. and Storz, G. (1999) *J. Bacteriol.* 181, 4639–4643.
- [15] Horsburgh, M.J., Clements, M.O., Crossley, H., Ingham, E. and Foster, S.J. (2001) *Infect. Immun.* 69, 3744–3754.
- [16] Thompson, D.K., Beliaev, A.S., Giometti, C.S., Tollaksen, S.L., Khare, T., Lies, D.P., Nealson, K.H., Lim, H., Yates, J3rd., Brandt, C.C., Tiedje, J.M. and Zhou, J. (2002) *Appl. Environ. Microbiol.* 68, 881–892.
- [17] Rodriguez, G.M. and Smith, I. (2003) *Mol. Microbiol.* 47, 1485–1494.
- [18] Park, Y., Sandstrom, S., Gustafsson, P. and Oquist, G. (1999) *Mol. Microbiol.* 32, 123–129.
- [19] Bibby, T.S., Nield, J. and Barber, J. (2001) *Nature* 412, 743–745.
- [20] Boekema, E.J., Hifney, A., Yakushevskaya, A.E., Piotrowski, M., Keegstra, W., Berry, S., Michel, K.P., Pistorius, E.K. and Kruij, J. (2001) *Nature* 412, 745–748.
- [21] Bibby, T.S., Nield, J. and Barber, J. (2001) *J. Biol. Chem.* 276, 43246–43252.
- [22] Gasseman, M. and Straus, N.A. (1996) *Microbiology* 142, 1469–1476.
- [23] Baicho, N., Wang, T. and Hellmann, J.D. (2002) *Mol. Microbiol.* 45, 1613–1629.
- [24] Cai, Y.P. and Wolk, C.P. (1990) *J. Bacteriol.* 172, 3138–3145.
- [25] Geiss, U., Vinnemeier, J., Kunert, A., Lindner, B., Gemmer, B., Lorenz, M., Hagemann, M. and Schoor, A. (2001) *Appl. Environ. Microbiol.* 67, 5247–5253.
- [26] Vinnemeier, J., Kunert, A. and Hagemann, A. (1998) *FEMS Microbiol. Lett.* 169, 323–330.