An increase in the level of 2-oxoglutarate promotes heterocyst development in the cyanobacterium *Anabaena* sp. strain PCC 7120

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Received 6 May 2003
Revised 30 July 2003
Accepted 5 August 2003

In the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120, a starvation of combined nitrogen induces differentiation of heterocysts, cells specialized in nitrogen fixation. How do filaments perceive the limitation of the source of combined nitrogen, and what determines the proportion of heterocysts? In cyanobacteria, 2-oxoglutarate provides a carbon skeleton for the incorporation of inorganic nitrogen. Recently, it has been proposed that the concentration of 2-oxoglutarate reflects the nitrogen status in cyanobacteria. To investigate the effect of 2-oxoglutarate on heterocyst development, a heterologous gene encoding a 2-oxoglutarate permease under the control of a regulated promoter was expressed in *Anabaena* sp. PCC 7120. The increase of 2-oxoglutarate within cells can trigger heterocyst differentiation in a subpopulation of filaments even in the presence of nitrate. In the absence of a source of combined nitrogen, it can increase heterocyst frequency, advance the timing of commitment to heterocyst development and further increase the proportion of heterocysts in a *patS* mutant. Here, it is proposed that the intracellular concentration of 2-oxoglutarate is involved in the determination of the proportion of the two cell types according to the carbon/nitrogen status of the filament.

INTRODUCTION

Organisms have evolved different mechanisms that allow them to find suitable nutrients in the environment. One example of adaptation to nutritional changes is the differentiation of nitrogen-fixing heterocysts as a result of combined-nitrogen depletion in filamentous cyanobacteria such as *Anabaena* sp. strain PCC 7120 (Wolk *et al*., 1994; Wolk, 2000; Meeks & Elhai, 2002). *Anabaena* sp. PCC 7120 can use different sources of combined nitrogen available in the growth medium. When filaments of *Anabaena* sp. PCC 7120 are starved for combined nitrogen, 5–10% of the cells on each filament are induced to differentiate into heterocysts specialized in the fixation of molecular nitrogen. The structure of heterocysts and the metabolic changes that occur in them ensure a microaerobic intracellular environment that prevents the nitrogenase from being inactivated by oxygen (Wolk *et al*., 1994).

Heterocysts are regularly intercalated among vegetative cells, giving rise to a regular pattern along each filament (Wolk *et al*., 1994; Wolk, 2000). According to one model (Wilcox *et al*., 1973; Meeks & Elhai, 2002), groups of cells may all be competent to respond to a depletion of combined nitrogen. Competition between contiguous cells is postulated to determine which one in every 10–20 vegetative cells will become a heterocyst and which will regress to the original vegetative state. Heterocyst pattern would thus be set up by the interaction of different signals, some promoting heterocyst development and others inhibiting it. So far, one signal, corresponding to the product of the *patS* gene (Yoon & Golden, 1998), has been identified. In a *patS* mutant, heterocyst frequency is increased, whereas increasing the level of the diffusible PatS peptide inhibits heterocyst differentiation. However, even in a *patS* null mutant, vegetative cells still represent the majority of cells on the filament (Yoon & Golden, 1998, 2001). This implies that, in the absence of PatS, either there are other signals that inhibit heterocyst development (Yoon & Golden, 2001) or the signals that activate heterocyst differentiation are insufficient to turn all cells into heterocysts.

It is still not known how filaments of *Anabaena* sp. PCC 7120 interpret nitrogen availability in the growth medium or what is the nature of the signal(s) that reflect the
depletion of combined nitrogen. The critical step for inorganic nitrogen assimilation in cyanobacteria is the set of reactions catalysed by glutamine synthetase (GS) and glutamate synthase (GOGAT), known as the GS–GOGAT cycle (Wolk et al., 1994; Merrick & Edwards, 1995; Herrero et al., 2001). In these reactions, 2-oxoglutarate serves as the carbon skeleton for the incorporation of ammonium (NH$_4^+$). This is particularly true in cyanobacteria, as they lack 2-oxoglutarate dehydrogenase, leaving the Krebs ‘cycle’ incomplete (Stanier et al., 1977).

Several studies suggest that 2-oxoglutarate participates in the signalling of carbon/nitrogen metabolism in both Escherichia coli and the unicellular cyanobacterium Synechococcus sp. strain PCC 7942 (Forchhammer & Hedler, 1997; Ninfa et al., 2000; Muro-Pastor et al., 2001). In E. coli, the intracellular concentration of glutamine constitutes the signal for nitrogen and 2-oxoglutarate the signal for carbon (Senior, 1975; for a review, see Ninfa et al., 2000). Measurement of metabolite pools in the unicellular cyanobacterium Synechocystis sp. strain PCC 6803 suggested that the level of 2-oxoglutarate, but not that of glutamine, responds to the nitrogen availability (Muro-Pastor et al., 2001). Thus, 2-oxoglutarate might act as a signal in the perception of the nitrogen status. Consistent with this idea, 2-oxoglutarate stimulates the expression of the activity of nitrate (NO$_3^-$) reductase and the transcription of two nitrogen-regulated genes, nir and amtI, in Synechococcus sp. PCC 7942 (Vazquez-Bermudez et al., 2003). Two cyanobacterial proteins are known to interact with 2-oxoglutarate, NtcA (for a review, see Herrero et al., 2001) and PII (Forchhammer & Hedler, 1997). NtcA is a global control protein for nitrogen metabolism and it is a transcription factor belonging to the cAMP receptor protein family (Luque et al., 1994; Herrero et al., 2001). When the nitrogen source is limiting, NtcA enhances transcription of the glnA gene encoding glutamine synthetase or genes involved in the metabolism of an alternative nitrogen source, such as those involved in NO$_3^-$ acquisition and reduction (Luque et al., 1994; Cai & Wolk, 1997; Herrero et al., 2001). Interestingly, recent studies demonstrate that the presence of 2-oxoglutarate increases the binding affinity of NtcA for at least some of its target promoter elements and this interaction allows the initiation of transcription (Vazquez-Bermudez et al., 2002; Tanigawa et al., 2002). In this case, NtcA may constitute a good candidate for sensing the depletion of combined nitrogen by interacting with 2-oxoglutarate. In heterocystous cyanobacteria, like Anabaena sp. PCC 7120, NtcA and HetR (a master control protein specific for heterocyst differentiation) form a regulatory loop to activate heterocyst development (Frias et al., 1994; Muro-Pastor et al., 2002). The second cyanobacterial protein known to bind 2-oxoglutarate is PII, a signalling protein well-characterized in unicellular cyanobacterial strains. PII can respond to nitrogen status through reversible phosphorylation on a serine residue in Synechococcus sp. PCC 7942 (Forchhammer & Tandeau de Marsac, 1995). The binding of 2-oxoglutarate to PII stimulates the phosphorylation reaction of PII (Forchhammer & Tandeau de Marsac, 1995) and inhibits its dephosphorylation reaction (Immler et al., 1997; Ruppert et al., 2002). NtcA enhances the expression of the glnB gene encoding PII under nitrogen-limiting conditions (Lee et al., 1999). Recently, it was shown that PII is required for NtcA-mediated nitorgen control under nitrogen-deprivation conditions in Synechococcus sp. PCC 7942 (Fadi Aldehni et al., 2003; Paz-Yepes et al., 2003). In heterocystous cyanobacteria, PII might have distinct functions, since the PII-encoding glnB gene is essential in Nostoc punctiforme ATCC 29133 (Hanson et al., 1998). However, PII has not been functionally characterized in any filamentous strain described so far.

Because 2-oxoglutarate could be an intracellular signal for the nitrogen status in cyanobacteria, and one of its binding proteins, NtcA, is required for the initiation of heterocyst development, this metabolite could possibly exert a positive effect on heterocyst differentiation in these organisms. To test this model, we have constructed a strain of Anabaena sp. PCC 7120 that can take up 2-oxoglutarate in a controlled manner. By modulating the concentration of 2-oxoglutarate in the growth medium, we investigated the possible role of this metabolite in heterocyst development in Anabaena sp. PCC 7120 under different nitrogen regimes.

**METHODS**

**Bacterial strains and growth conditions.** Wild-type Anabaena sp. PCC 7120 and its derivatives were grown in BG11 medium with either NO$_3^-$ or NH$_4^+$, or in BG110 medium without combined nitrogen, as described previously (Zhang 1993). The patS mutant (ΔpatS) strain AMC451 (Yoon & Golden, 1998) was kindly provided by Dr J. Golden, Texas A & M University, USA. Strain KGT5 was cultured in the presence of 50 μg neomycin ml$^{-1}$; strain AMC451 was cultured in the presence of 2 μg spectinomycin ml$^{-1}$ and 2 μg streptomycin ml$^{-1}$. E. coli strains used for cloning and conjugation have been described previously (Elhai & Wolk, 1988). Precautions were taken to remove trace amounts of copper, as described by Buikema & Haselkorn (2001).

**Molecular cloning of kgtP and the petE promoter.** The petE promoter from Anabaena sp. PCC 7120 (Ghassemian et al., 1994; Buikema & Haselkorn, 2001) was amplified by PCR using primers PetE-up (5’-ccttcagaggctttcaacgctt-3’) and PetE-down (5’-ccttcgaggtttgatttttctt-3’). The resulting fragment was cloned between the Xbal and PstI sites of pBluescript SK$^-$. The kgtP gene of E. coli (Seol et al., 1991; Vazquez-Bermudez et al., 2000) was amplified by PCR using primers KgtP-up (5’-ccttcagagctttgaggcag gagac-3’) and KgtP-down (5’-ccttaaggctttcaacgctt-3’). The PCR product was cloned between the PstI and HinDIII sites downstream of the petE promoter in pBluescript SK$^-$. The BamHI–EcoRI fragment containing the ppatS–kgtP fusion was subcloned into the shuttle vector pRL25c. pRL25c, like pAM1691, is a derivative of pDU1; the copy number of pAM1691 is about 17 per chromosome (Lee et al., 2003). The construct was conjugated into Anabaena sp. PCC 7120, and exconjugants were selected with 50 μg neomycin ml$^{-1}$.

**Incubation of 2-oxoglutarate with Anabaena cultures and statistical analysis of heterocyst frequency.** Heterocyst differentiation was induced in filaments of Anabaena sp. PCC 7120, and 2-oxoglutarate was added either at the time of heterocyst induction
or 1, 2 or 3 days before induction. Pre-incubation was required to observe the effect of 2-oxoglutarate on heterocyst development. Therefore, all subsequent experiments were carried out by incubating first with 2-oxoglutarate for 24 h before the induction of heterocyst differentiation. Heterocyst frequency was measured by randomly counting the number of vegetative cells between two heterocysts (heterocyst interval), as described by Yoon & Golden (1998, 2001). The concentration of 2-oxoglutarate was determined and used as follows: 25 mM 2-oxoglutarate for the wild-type strain, 1 mM 2-oxoglutarate for strain KGTP. The stock solution of 2-oxoglutarate was adjusted to pH 7.5.

**RESULTS**

**Engineering of a recombinant strain with a controlled expression of a 2-oxoglutarate permease**

To determine whether an increase of the 2-oxoglutarate concentration during combined-nitrogen depletion could affect heterocyst differentiation, we studied the effect on heterocyst development of 2-oxoglutarate added to the growth medium. The frequency of heterocyst was increased (data not shown), but only at a high concentration of 2-oxoglutarate in the medium (25 mM). The lack of effect of 2-oxoglutarate at low concentrations could be due to the absence of an efficient uptake system for this metabolite in *Anabaena* sp. PCC 7120 (see below), which could prevent its rapid accumulation in the cells. A known permease for 2-oxoglutarate is KgtP from *E. coli* (Seol & Shatkin, 1991). Although two proteins deduced from the sequenced genomes of *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 showed some sequence similarity (23 % identity) to KgtP, they appear not to be permeases for 2-oxoglutarate; one is the glucose permease GlcP from *Synechocystis* sp. PCC 6803 (Zhang et al., 1989) and the other is Alr3381 from *Anabaena* sp. PCC 7120, which is similar to proteins involved in antibiotic resistance (Kaneko et al., 2001). Thus, it is unlikely that a protein functionally equivalent to KgtP exists in *Anabaena* sp. PCC 7120, an observation consistent with our data obtained with the measurement of 2-oxoglutarate uptake (see below). For this reason, we constructed a recombinant strain of *Anabaena* sp. PCC 7120 that expressed the *kgtP* gene under the control of the copper-regulated promoter of the *petE* gene (Ghassemian et al., 1994; Buikema & Haselkorn, 2001). A similar strategy has been used in the unicellular cyanobacterial strain *Synechococcus* sp. PCC 7942 for studies into 2-oxoglutarate metabolism and regulation (Vazquez-Bermudez et al., 2000, 2003).

The *petE* promoter was inserted in front of the coding region of the *kgtP* gene encoding a 2-oxoglutarate permease in *E. coli* (Seol et al., 1991). This fusion was carried on the replicative plasmid pRL25c (Elhai & Wolk, 1988) and conjugated into *Anabaena* sp. PCC 7120. The recombinant strain was named KGTP.

As shown in Fig. 1, when the wild-type strain of *Anabaena* sp. PCC 7120 was incubated with 2-[1-14C]2-oxoglutarate, in the presence of either a small amount of copper or 1 mM copper, only a basal level of labelled metabolites was measured in the cells. In contrast, strain KGTP containing the permease gene could take up 2-oxoglutarate, and this effect was inducible by copper. With no copper, or a low level of copper (0.075 μM), the amount of 2-oxoglutarate entering into cells was as low as in the wild-type. However, with 1 μM copper, the uptake of 2-oxoglutarate became significant (Fig. 1). The apparent *Km* value was determined to be 35–54 μM, comparable to that (13–46 μM) reported for *E. coli* (Seol & Shatkin, 1992). The 2-oxoglutarate permease encoded by *kgtP* under the control of copper was thus functional in *Anabaena* sp. PCC 7120.

**Influence of exogenous 2-oxoglutarate on heterocyst development in strain KGTP**

We further examined the influence of exogenous 2-oxoglutarate on heterocyst differentiation with the help of strain KGTP in the presence of copper. A concentration of 2-oxoglutarate as low as 1 mM in the growth medium had

![Fig. 1. Uptake of 2-[1-14C]2-oxoglutarate in the wild-type strain and strain KGTP. 2-[1-14C]2-oxoglutarate, with concentrations ranging from 1 to 10 μM, was incubated with *Anabaena* sp. PCC 7120, and its uptake was measured. Copper was added to induce expression of the *kgtP* gene encoding the 2-oxoglutarate permease. ◇, Strain KGTP + 1 μM Cu2+; □, strain KGTP + 0-075 μM Cu2+; ○, wild-type + 1 μM Cu2+; △, wild-type + 0-075 μM Cu2+.

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already a noticeable effect on heterocyst frequency (Fig. 2), in contrast to the wild-type, where a concentration as high as 25 mM in the medium was needed to influence the pattern of heterocysts. When strain KGTP was cultured with 1 mM 2-oxoglutarate in the presence of 0–5 mM copper, the intervals of heterocyst decreased as compared to the control (Fig. 2). The presence of 2-oxoglutarate increased heterocyst frequency from 7 to 7·9 % 24 h after the depletion of combined nitrogen, and from 5·5 to 8 % 48 h after the depletion of combined nitrogen. This effect was even more pronounced when the concentration of 2-oxoglutarate was increased to 5 mM in the growth medium (data not shown). The increase in the level of 2-oxoglutarate did not disrupt the regular pattern of heterocysts (Fig. 2).

We also examined whether 2-oxoglutarate could trigger heterocyst differentiation in the presence of a combined-nitrogen source such as NO₃⁻ or NH₄⁺ (Fig. 3). In the presence of both 1 mM 2-oxoglutarate and 18 mM NO₃⁻, about one-third of the filaments had heterocysts, mostly spaced regularly on the filament. When just NO₃⁻ was present, only about 11 % of the filaments developed heterocysts. These results indicate that 2-oxoglutarate promotes heterocyst differentiation even in the presence of a weak inhibitor of heterocyst differentiation, such as NO₃⁻. In the presence of NH₄⁺, a strong inhibitor of heterocyst differentiation, no differentiation could be observed even in the presence of 2-oxoglutarate.

**Heterocyst frequency in the presence of 2-oxoglutarate and absence of PatS, an inhibitor of differentiation**

The product of the patS gene constitutes an inhibitory signal to heterocyst development (Yoon & Golden, 1998). As heterocyst differentiation and pattern formation probably depend on the combined effect of different signals, we thought it interesting to determine whether the increase of 2-oxoglutarate within cells of a null mutant of patS would lead to a further increase in the number of heterocysts.

In a patS-inactivated strain, AMC451 (Yoon & Golden, 1998), the peak of heterocyst intervals was about eight vegetative cells 48 h after the transfer from NO₃⁻ to combined-nitrogen-free medium (Fig. 4A). When heterocyst differentiation was induced with the mutant incubated in the presence of 25 mM 2-oxoglutarate, the peak of heterocyst intervals was decreased to four vegetative cells (Fig. 4B).

Moreover, the addition of 2-oxoglutarate also significantly increased the percentage of double heterocysts in the patS mutant. When heterocysts were observed 48 h after induction by removal of combined nitrogen, double heterocysts represented 71 % of all heterocysts in the presence of...
oxoglutarate, as compared to 59% without 2-oxoglutarate (Fig. 4). The percentage of double heterocysts remained higher in the presence of 2-oxoglutarate than in its absence in the following days (data not shown). Double heterocysts were rare in the wild-type and KGTP strains, even in the presence of 2-oxoglutarate. It is possible that 2-oxoglutarate affects heterocyst differentiation synergistically with the effect of the absence of PatS.

**2-Oxoglutarate advances the timing of commitment to heterocyst differentiation**

If 2-oxoglutarate promotes heterocyst differentiation, it possibly also affects the timing of commitment in the process of heterocyst development. Within a few hours after induction of heterocyst differentiation, the addition of a combined-nitrogen source causes reversion of differentiating cells to their vegetative states (Yoon & Golden, 1998; also see Fig. 5). After a certain point, however, the inhibition of heterocyst differentiation becomes less and less effective. This moment was defined as the point of commitment to heterocyst differentiation, the time at which differentiation becomes irreversible (Yoon & Golden, 2001).

To examine the effect of 2-oxoglutarate on commitment to heterocyst development, heterocyst differentiation was induced in strain KGTP at time zero, in the presence or absence of 2-oxoglutarate, and samples were taken at different time intervals, to which NH$_4$Cl was added; heterocyst frequency was counted 24 h after induction. As shown in Fig. 5, in the absence of 2-oxoglutarate, 6 h after heterocyst induction, NH$_4$Cl still inhibited heterocyst development in the recombinant strain KGTP. However, in the presence of 5 mM 2-oxoglutarate, 6 h after induction, heterocyst differentiation had reached a stage at which the presence of NH$_4$Cl no longer completely repressed heterocyst formation. Furthermore, 8 h after induction, the percentage of heterocysts was significantly higher in the presence of 2-oxoglutarate than in its absence. Similar results were also obtained with the wild-type strain treated with 25 mM 2-oxoglutarate (data not shown). These results indicate that 2-oxoglutarate advanced the timing of commitment in the process of heterocyst differentiation.

**DISCUSSION**

2-Oxoglutarate added to the growth medium led to a higher heterocyst frequency and earlier commitment to heterocyst development in the wild-type strain. In view of our results, one of the physiological functions of 2-oxoglutarate could be the regulation of the proportion of heterocysts relative to vegetative cells in accordance with the need of the filament. Usually, the number of heterocysts is at its highest when they are first induced by the deprivation of combined nitrogen, then it decreases over time (Fig. 2). During the period of time from the beginning of starvation of combined nitrogen to heterocyst maturation (about 24 h), an important imbalance of carbon/nitrogen could be created within filaments with the accumulation of 2-oxoglutarate.
Once mature heterocysts become functional, glutamine can be synthesized through the actions of nitrogenase and glutamine synthetase. Since glutamate synthase is absent in heterocysts (Martin-Figueroa et al., 2000), glutamine can then be used in vegetative cells for the synthesis of glutamate using 2-oxoglutarate as the carbon skeleton. In this case, the imbalance of carbon/nitrogen along the filaments becomes less dramatic, so that fewer heterocysts are induced. This is consistent with the inhibitory effect of the product of nitrogen fixation on heterocyst differentiation proposed by Yoon & Golden (2001). If the intracellular concentration of 2-oxoglutarate is artificially maintained at a high level with exogenous 2-oxoglutarate, it may also create a carbon/nitrogen imbalance within the filaments, thus creating a situation where more heterocysts are required to consume the excessive amount of 2-oxoglutarate (Fig. 2).

The mechanism of the early perception of nitrogen deple-
tion is possibly conserved in cyanobacteria (Luque et al., 1994; Cai & Wolk, 1997; Herrero et al., 2001). A signalling role for 2-oxoglutarate in carbon and nitrogen metabolism has long been suggested in *Synechococcus* sp. PCC 7942 (Forschhammer & Hedler, 1997). It was also found that changes in the intracellular pool of 2-oxoglutarate correlated to the expression of genes dependent on NtcA, a global nitrogen control protein highly conserved among cyanobacteria (Muro-Pastor et al., 2001; Vazquez-Bermudez et al., 2003). 2-Oxoglutarate was therefore proposed as a signal reflecting the nitrogen status within cells (Muro-Pastor et al., 2001). Furthermore, the binding affinity of NtcA towards its target DNA fragment, such as the promoter region of *glnA*, was enhanced by 2-oxoglutarate (Vazquez-Bermudez et al., 2002; Tanigawa et al., 2002). In *Anabaena* sp. PCC 7120, NtcA also constitutes the global regulator for nitrogen metabolism, as in unicellular strains (Luque et al., 1994; Frias et al., 1994; Cai & Wolk, 1997; Herrero et al., 2001), but it is also the first protein known so far in the regulatory cascade required for heterocyst differentiation (Wolk, 2000; Herrero et al., 2001). These results suggest the following model for the early signalling pathway leading to heterocyst development in heterocystous cyanobacteria. On depletion of combined nitrogen, the intracellular level of 2-oxoglutarate increases rapidly to reach a threshold concentration, and binds to and activates the transcription factor NtcA. The signalling effect of 2-oxoglutarate can be further amplified through the positive autoregulation of NtcA (for a review, see Herrero et al., 2001). As a result, the transcription of ntcA reaches the maximal level, enabling it subsequently to activate genes involved in nitrogen metabolism and heterocyst formation. This hypothesis agrees with the observation that the presence of 2-oxoglutarate leads to more heterocyst differentiation even in the presence of NO$_3^-$ (Fig. 3). By increasing the level of 2-oxoglutarate, as we did in strain KGTP in this study, this metabolite may also help cells to reach the critical threshold concentration faster, thus leading to earlier commitment to heterocyst development.

However, more experiments need to be done to confirm the hypothesis.

The signalling protein PII is also involved in the perception of the 2-oxoglutarate signal in unicellular strains and is even required for NtcA-mediated gene expression under nitrogen-deprivation conditions (Fadi Aldehni et al., 2003; Paz-Yepes et al., 2003). However, little is known about the function of PII in filamentous cyanobacteria. In light of the results in unicellular strains, it is conceivable that PII may interact with 2-oxoglutarate and NtcA for heterocyst differentiation in heterocystous cyanobacteria.

PatS and 2-oxoglutarate may act in concert to determine heterocyst frequency. Although the increase of 2-oxoglutarate by itself led to more heterocysts, it did not produce double heterocysts. When the level of 2-oxoglutarate was increased in a PatS$^-$ mutant (Yoon & Golden, 1998; 2001), not only was heterocyst frequency further increased, but also there was an increase in the number of double heterocysts. It seems that various signals, including 2-oxoglutarate, the products of nitrogen assimilation or fixation (Yoon & Golden, 2001) and the inhibitor of heterocyst differentiation PatS (Yoon & Golden, 1998), act together to determine heterocyst frequency, possibly as a means to manage the efficiency of exchanges of different metabolites between vegetative cells and heterocysts.

**ACKNOWLEDGEMENTS**

This study was supported by the ATIPE-Microbiology programme of the CNRS, and the programme of Environnement-santé from AFFSSE. V.K. was supported by a fellowship from the Ministry of Research of France. J.-H.L. was supported by fellowships from the FRM foundation and the K.-C. Wong foundation. S.L. is a recipient of a fellowship for PhD thesis from the Ministry of Education. C.-C.Z. would like to thank the Cheung-Kong scholarship programme and the Natural Science Foundation of China for support. We would like to thank A. Janicki for her technical assistance, Drs T. Arcondéguy, K. Jäger and R. Jeanjean for their critical reading of the manuscript, and Professor J. Golden for the patS mutant. We are grateful to Drs A. Cornish-Bowden and M. I. Cardenas for help with the English version of this manuscript and for insightful discussion.

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