Transcriptional and Translational Regulation of Photosystem I and II Genes in Light-Dark- and Continuous-Light-Grown Cultures of the Unicellular Cyanobacterium *Cyanothec* sp. Strain ATCC 51142

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Received 17 July 1997/Accepted 18 November 1997

*Cyanothec* sp. strain ATCC 51142, a unicellular, diazotrophic cyanobacterium, demonstrated extensive metabolic periodicities of photosynthesis, respiration, and nitrogen fixation when grown under N₂-fixing conditions. This report describes the relationship of the biosynthesis of photosynthesis genes to changes in the oligomerization state of the photosystems. Transcripts of the *psbA* gene family, encoding the photosystem II (PSII) reaction center protein D1, accumulated primarily during the light period, and net transcription reached a peak between 2 to 6 h in the light in light-dark (LD) growth and between 4 to 10 h in the subjective light when grown under continuous light (LL). The relative amount of the D1 protein (form 1 versus form 2) appeared to change during this diurnal cycle, along with changes in the PSII monomer/dimer ratio. D1 form 1 accumulated at approximately equal levels throughout the 24-h cycle, whereas D1 form 2 accumulated at significantly higher levels at approximately 8 to 10 h in the light or subjective light. The *psbB* gene, encoding the reaction center protein D2, also demonstrated differences between the two copies of this gene, with one copy transcribed more heavily between 6 to 8 h in the light. Accumulation of the PSI reaction center proteins PsA and PsB was maximal in the dark or subjective-dark periods, a period during which PSI was primarily in the trimeric form. We conclude that photosystem organization changes during the diurnal cycle to favor either noncyclic electron flow, which leads to O₂ evolution and CO₂ fixation, or cyclic electron flow, which favors ATP synthesis.

Cyanobacteria are capable of performing oxygenic photosynthesis very similarly to plants. In addition, the ability to fix atmospheric N₂ has been shown in several strains within all cyanobacterial morphological groups (10–12, 16, 56). Thus, they are unique microorganisms in that they perform two of the most important, though incompatible, biological processes—O₂-sensitive N₂ fixation and photosynthetic O₂ evolution. Cyanobacteria use primarily spatial and temporal separation of N₂ fixation and photosynthesis, along with high rates of respiration and the enzymatic removal of O₂-generated reactive species, as mechanisms to protect nitrogenase from O₂ inactivation (10, 11, 53). The most studied example of spatial separation is heterocyst development in filamentous strains of *Anabaena* spp. (16, 55). Heterocysts become the exclusive site for N₂ fixation by developing a thick envelope which interferes with O₂ diffusion, by having high rates of respiration, and by losing photosystem II (PSII) O₂ evolution. Therefore, in *Anabaena* spp., N₂ fixation and photosynthesis involving noncyclic electron transport are restricted to the heterocyst and vegetative cells, respectively.

Temporal separation of N₂ fixation and photosynthesis have been described for filamentous, nonheterocystous cyanobacteria such as *Plectonema* sp. (37) and *Oscillatoria* sp. (49, 50), as well as unicellular N₂-fixing cyanobacteria such as *Gloeothec* sp. (13, 35), *Synecococcus* strain RF1 (21, 41), and *Synecococcus* strains Miami BG 43511 and Miami BG 43522 (33, 34). We have begun a detailed analysis of regulation in the unicellular diazotroph *Cyanothec* sp. strain ATCC 51142 (formerly BH68), and rhythms of nitrogenase activity have been demonstrated under light-dark (LD) or continuous-light (LL) conditions (9, 38, 45). We have shown that photosynthesis, respiration, and N₂ fixation are temporally regulated under both growth conditions and that nitrogenase is regulated both at the transcriptional level and by proteolytic degradation in LD- and LL-grown cultures (9). Net transcription of the *nifHDK* operon, encoding the nitrogenase Fe protein and MoFe proteins, occurred only during a portion of the dark or subjective-dark period, and the proteins were degraded within a few hours (9). Thus, fresh nitrogenase proteins need to be synthesized each day.

We have initiated a thorough analysis of the responses of the photosynthetic mechanism to N₂-fixing conditions (29, 46, 47). We find that there are both short-term and long-term adaptations that are independent of the light regimen. Short-term adaptations (on the order of seconds to minutes) include state transitions and oligomeric changes in the organization of the photosystems. State transitions relate to a phenomenon, first detected in cyanobacteria by Murata (36), in which preferential excitation of PSI (state 1) caused an increase in energy transfer to PSII and a small decrease in energy transfer to PSI, whereas PSI-specific excitation (state 2) had the reverse effect. A physical model for state transitions in cyanobacteria has been developed by Rögner and colleagues (2, 26, 40), who have also invoked the oligomeric state of PSI and PSII in the overall mechanism. In this model, state 1 (which favors linear electron flow from O₂ evolution to CO₂ fixation) had a dimeric PSII and monomeric PSI with phycobilisomes primarily attached to
PSI. State 2 (which favors cyclic electron flow) had trimeric PSI complexes and monomeric PSII, and phycobilisomes could more readily attach to PSI.

It is important to note that cyanobacteria have retained small families of the psbD and psbA genes, which encode the PSI reaction center proteins. This phenomenon was first demonstrated by Golden et al. (15), who showed that Synechococcus sp. strain PCC 7942 has three copies of the psbA gene and two copies of the psbD gene (3). Importantly, Golden's lab has determined that the three psbA genes give rise to two different forms of D1, and they have produced the specific antibodies against these two forms that we used in this study (14, 43, 44). Using these antibodies and mutant strains which lack any two of the psbA genes, Kulkarni and Golden have shown that high light during growth favors the expression of genes that give rise to D1 form 2, whereas low-light growth conditions favor D1 form 1 (27).

This work has been extended somewhat by Öquist's lab (4, 5). They have shown that the overproduction of D1 form 2 renders Synechococcus sp. more tolerant to photoinhibition when treated with high light (4). They demonstrated that this was partially due to a change in most of the PSII centers from D1 form 1 to D1 form 2. They also demonstrated that the tolerance to high light was further strengthened by overexpressing the psbAIII gene during the photoinhibitory treatment, which leads to further enhancement of form 2. More important, they performed studies with Synechococcus sp. strain PCC 7942 which indicated that mutant cells which contain only D1 form 1 have lower photochemical energy capture efficiency and decreased resistance to photoinhibition than cells containing form 2. They obtained lower PSI fluorescence at 696 nm in cells containing form 1 than in cells containing form 2. In addition, Campbell et al. (4) find that cells containing form 1 are generally shifted toward state 2 (with PSI downregulated), whereas cells with form 2 tend to be more in state 1.

We demonstrated that such short-term adaptations are bountiful and important for Cynotheco sp. strain ATCC 51142 during N2-fixing conditions (29), and this report will show the involvement of longer-term, biosynthetic alterations (minutes and longer) in this process. This report will detail Northern and Western blot analyses of the major photosynthesis genes and gene products: psbA (PSI reaction center protein D1), psbD (PSI reaction center protein D2), psbC (PSI antenna CP43), and psbAB (PSI reaction center proteins PsA and PsB). We will describe the long-term changes that affect the photosystems and integrate this information into a model for the downregulation of PSI in Cynotheco sp. strain ATCC 51142 under N2-fixing conditions.

MATERIALS AND METHODS

Growth conditions. Cynotheco sp. strain ATCC 51142 (Cynotheco strain BH68K) was cultured at 30°C in a modified minimal salt medium (ASP2) without 1.5 g of NaNO3 per liter (38). Cultures were grown in different volumes: (i) 20-liter cultures of Cyanothece sp. strain ATCC 51142 (Cyanothece sp. strain ATCC 51142 [48]), (ii) anti-D1 form 1 (from Synechococcus sp. strain PCC 7942 [43]); (iii) anti-D2 (from Synechococcus sp. strain PCC 6803 [plasmid pKW1344 [7, 54]) and (iv) 2.8-kb EcoRI/BglII fragment of ppsA from Synechococcus sp. strain PCC 7942 (plasmid pAQPR80 [6]). A homologous probe from Cynotheco sp. strain ATCC 51142 corresponding to a 1.2-kb Cif fragment of the psbA gene (plasmid p2A5 [1]) was also used. The results were repeated with different samples at least twice and usually three to five times.

Western blot analysis. Whole-cell extracts were prepared as described previously (9, 45). The amount of protein was determined by using the Bradford reagent (0.1 mg of Coomassie blue G-250 [Bio-Rad, Hercules, Calif.] per ml, 5% ethanol, 8.5% phosphoric acid [Mallinckrodt] and bovine serum albumin [Sigma, St. Louis, Mo.], and the standard (9). Protein gels, transfer, and Western blot development were performed as described previously (9).

Antibody probes. Antibodies were diluted with 1× TBS (50 mM Tris, 150 mM NaCl, pH 8.0) and 0.1% NaN3. The following antibodies were used: (i) anti-D1; (ii) anti-D1 form 1 (from Synechococcus sp. strain PCC 7942 [43]); (iii) anti-D1 form 2 (from Synechococcus sp. strain PCC 7942 [43]); (iv) anti-D2 from Synechococcus sp. strain PCC 7942, prepared by Susan S. Golden, Texas A&M University [48]; (v) anti-CP43; and (vi) anti-PsA (from Synechococcus sp. strain PCC 7942, prepared by James Guikema, Kansas State University [48]). The results were replicated at least six times with each antibody.

RESULTS

Temporal relationship between N2 fixation, photosynthesis, and respiration in LD- and LL-grown cultures. Several physiological experiments have been performed to determine the temporal relationship between the metabolic activities of N2 fixation, photosynthesis, and respiration (9). An analysis in which the three metabolic activities were measured every hour from LD-grown cultures is shown in Fig. 1. In agreement with previous results, N2 fixation is restricted to the dark period, and its peak occurred approximately 8 to 16 h out of phase with maximum O2 evolution. The chlorophyll (Chl) concentration, photosynthetic electron transport, and respiration activities were measured at D4 for 2 consecutive days. Although the levels of nitrogenase activity remained low, there is an increase of respiration during the light phase. The maximum capacity of whole cells to evolve O2 occurred during the light, at L8 and L6 for the first and second days, respectively.
The minimum photosynthetic capacity was measured at D4 during maximum nitrogenase and respiratory activity. O$_2$ evolution started increasing during the dark period, beginning after D4, and reached its maximum during the middle to late light phase. The results for LL-grown cultures also demonstrated temporal separation, although the nitrogenase activity had a wider half bandwidth (data not shown).

**Transcriptional analysis of PSI and PSII genes in LD- and LL-grown cultures.** Northern blot analysis was performed to determine changes in transcript levels of photosynthesis genes. Messages corresponding to the proteins D1 (psbA gene product), D2 (psbD gene product), CP43 (psbC gene product), and PsaAB (psaAB gene product) were studied in samples from LD- and LL-grown cultures. Initially, RNA samples were collected from LD cultures every 2 h throughout a 24-h period, starting 108 h after subculturing, and hybridized to a 0.6-kb fragment of the psbA gene from Synechococcus sp. strain PCC 7142. (B) RNA samples (5 µg of total RNA/lane) were collected every 2 h for 2 consecutive days (days 5 and 6), starting 108 h after subculturing, and hybridized to a 1.2-kb fragment of psbA from Cyanothecae sp. strain ATCC 51142. Arrows indicate the calculated size for the psbA transcript. A similar experiment (Fig. 2B) was performed, with the major difference that RNA samples were prepared every 2 h for 2 consecutive days and hybridized to a homologous psbA probe (1). Basically, the same kinetics were observed in which maximum levels of the psbA message occurred during the light phase, with basal amounts of transcript present throughout most of the dark period. Therefore, these results suggested that the periodic photosynthetic activity observed in LD-grown cultures could be due to transcriptional regulation.

To gain a better understanding of the changes in the levels of psbA message during the D-L and L-D transitions, a more detailed transcriptional analysis was performed. RNA samples were prepared every hour throughout most of the dark (D2 to D10) and light (L0 to L10) periods. In addition, RNA was extracted every half hour around the D-L (D10 to L2) and L-D (L10-D2) transitions (RNA samples corresponded to h 108 to 132 in Fig. 1). A 1.2-kb psbA transcript, which is highly abundant during the light phase, was identified by using the homologous psbA probe from *Cyanothecae* sp. (Fig. 3). The levels of the psbA transcript increased around D2 to D4 and again near
D10, although these levels were substantially less than in the light. In a matter of 0.5 h (D11.5 to L0), the net accumulation of psbA message increased dramatically. Net transcription increased until reaching a maximum at L4, although the level of psbA transcripts remained high throughout the light period (Fig. 3B).

Northern blot analysis was also performed on RNA samples from LL-grown cultures. Analysis of the psbA transcript was performed on samples collected every 2 h starting 108 h after subculturing for 2 consecutive days. A 1.2-kb transcript was detected when a DNA probe corresponding to the psbA gene from Cyanothece sp. was used (Fig. 4). This message was more abundant during the subjective light (LL0 to LL10), with basal levels of transcript detected during the subjective dark (LL12 to LL22). These results were comparable to those ones reported above (Fig. 2 and 3) for the kinetics of the psbA message on LL-grown cultures. Therefore, the cyclic photosynthetic activity observed on LL-grown cultures may also be partially regulated at the level of the transcript.

Similar Northern blot analysis was performed to determine the levels of transcripts corresponding to the psbC and psbD genes in LD-grown cultures (Fig. 5). Preliminary studies using a chloroplast psbD probe from spinach resulted in the identification of two transcripts (2.7 and 1.1 kb) that were differentially regulated throughout the light period (data not shown). We propose that the 2.7- and 1.1-kb messages correspond to transcripts from the psbDI/psbC operon and the psbDII loci, respectively. To confirm these results, similar experiments were done with probes from Synechocystis sp. strain PCC 6803 corresponding to the psbC (Fig. 5A) and psbD (Fig. 5B) genes. Both probes detected transcripts of the expected sizes and with comparable kinetics. A 2.4-kb transcript (psbDI/psbC) was identified with psbC (Fig. 5A); this transcript was more abundant during the light period and peaked around L2 to L4. The psbDII message (around 1.4 kb) was recognized with the psbC probe since the probe contains approximately 100 nucleotides of the 3' end of the psbDII gene. Similar results were obtained by using the psbD probe (Fig. 5B). The sizes of the two messages are 2.5 kb (psbDI/psbC) and 1.2 kb (psbDII). As with the psbC probe, the larger mRNA, corresponding to psbDII, was maximally detected early in the light period at L2, whereas the smaller message peaked around L8 (late light phase). Similar results were obtained for these probes on LL-grown cultures (data not shown).

Quantitative analysis of the Northern blot in Fig. 5B indicated that the net accumulation of the two psbD transcripts was somewhat different (Fig. 5C). Both transcripts were at high levels around L2, but psbDII transcripts increased to an even higher peak at L8. In addition, transcripts from both psbD genes accumulated similarly in the dark and reached peaks around D8. Therefore, genes for both PSII reaction center proteins were transcribed in the dark.

Analyses of the psaAB transcripts were performed for both LD- and LL-grown cultures (Fig. 6). In both cases, the level of transcription differed significantly throughout the diurnal cycle. In LD-grown cultures, psaAB transcripts were maximal around L8. In LL-grown cultures, psaAB mRNA began increasing toward the end of the subjective night (LL22) and peaked early in the subjective light (LL2 to LL4). The relationship of the transcriptional patterns of psbDII and psaAB was striking under both growth conditions but especially in LD cultures. Net transcriptional accumulation became evident near the end of the dark period, and there were two peaks in the light (with the highest peak at L8). As shown in Fig. 5C, the net accumulation of psaAB transcripts resembles that of psbDII, especially in the rise to a maximum at L8.

In summary, we have demonstrated that the messages corre-
responding to the genes psbA, psbC, psbD of PSII, and psaAB of PSI are differentially regulated in LD- and LL-grown cultures. The peak of mRNA accumulation differed among the genes; psbA reached a maximum at around L6 in LD and in LL cultures, psbDII peaked at around L2 to L4 in LD and LL cultures, and psbDII and psaAB peaked at L8 in LD cultures and at LL2 in LL cultures.

Translational analysis of PSI and PSII proteins in LD- and LL-grown cultures. Western blot analysis was performed to determine changes in the levels of the PSII proteins D1, D2, and CP43 and the PSI proteins PsAa and PsBb for cultures grown under either LD or LL conditions. Whole-cell extracts were prepared from samples harvested every 2 h throughout a 24-h period. In most instances, the fidelity of the results was confirmed by including a lane containing a Synechococcus photosynthetic membrane sample. The gels for Fig. 7 to 9 were loaded with equal amounts of protein/lane, and the Coomassie blue-stained gels indicated essentially identical protein loading and patterns in all lanes (data not shown). Therefore, any protein changes were not due to loading anomalies.

Results of the posttranslational analysis for the D1 protein are shown in Fig. 7A and B for LD- and LL-grown cultures, respectively. Three unique antisera were used to identify the different forms of the protein: anti-D1 immunoreacts with both forms (upper panels), and two form-specific antisera distinguish between form 1 (middle panels) and form 2 (lower panels). Under both light regimens, the levels of the D1 protein remained relatively constant throughout the 24-h period (upper panels). After dissecting these results by using the form-specific antisera, we have determined that the levels of form 1 remained constant (middle panels), but higher amounts of form 2 were detected during the light/subjective light phases (bottom panels).

Analyses of the D2 and CP43 proteins are shown in Fig. 8A and B for LD- and LL-grown cultures, respectively. Overall, similar results were obtained for both proteins under the two different light regimens. In LD, two immunoreactive bands (31 and 29 kDa) were detected with anti-D2 (Fig. 8A, upper panel). Although there appears to be a correlation between the amounts of both bands, the level of the faster-migrating protein (29 kDa) correlates better with the results from the Northern blot analysis in which the psbD message is maximally expressed during the light phase. Nevertheless, the levels of D2 protein (based on the lower band) started increasing at D10 until reaching a peak at L6 and decreased thereafter. Similar results were observed for the LL samples, although there was not a noticeable correlation between the two major reactive bands (Fig. 8B, upper panel). Again, low levels of D2 were found early in the subjective dark phase (LL12 to LL18) and increased toward the end of the period (LL20 to LL22). The maximum amount of D2 was detected during the subjective light period, specifically at LL6. Levels of the CP43 protein remained unchanged throughout the 24-h period under both light conditions (Fig. 8A and B, lower panels).

Western blot analysis of the PSI apoproteins PsAa and PsBb.
demonstrated that these proteins accumulated somewhat differently. Immunoreactive bands corresponding to the PsA and PsB proteins appeared higher early in the dark (D0 to D6) or subjective-dark (LL12 to LL20) phases (Fig. 9). In both growth conditions, PsAB was highest at 2 to 6 h into the dark phase. Similar results were obtained from two separate growth experiments for each condition and a total of six Western blots.

In summary, levels of the proteins D1 (form 1 and form 2), D2, CP43, and PsAB are similarly regulated in LD- and LL-grown cultures. Levels of total D1 protein (form 1 and form 2) remained relatively constant throughout the 24-h cycle, although a higher amount of form 2 was detected during the light/subjective-light phases. Also, the D2 protein is maximally detected during the light/subjective-light phases. On the contrary, the highest amount of PsAB was immunodetected early during the dark/subjective-dark period. The amount of CP43 protein remained constant throughout the 24-h cycle in LD and LL conditions.

**DISCUSSION**

The PSI and PSII genes in *Cyanothece* sp. strain ATCC 51142 demonstrated significant diurnal changes under N₂-fixing conditions. In particular, the genes coding for the PSII reaction center proteins D1 and D2 manifested interesting changes in net transcriptional accumulation, especially during the second half of the light or subjective-light periods. Non-reaction center protein genes, such as *psbC*, had less intricate changes but seemed to be transcribed at a higher level during the first half of the light period. Interestingly, very similar patterns for all of the PSI genes went through the 24-h cycle in LD-grown cultures. These are among many lines of evidence that support the concept of temporal regulation between N₂ fixation and photosynthesis in this cyanobacterium. In addition, this evidence suggests that the temporal regulation is controlled by an underlying circadian mechanism. For example, the results in Fig. 2 and 4 strongly suggest that transcription is ultimately controlled by a circadian clock (9).

There is now extensive evidence for the presence of circadian rhythms in cyanobacteria, a phenomenon first described for another unicellular diazotroph, *Synechococcus* sp. strain RF-1 (8, 17–20, 22), and studied in more detail in *Synechococcus* sp. strain PCC 7942 (23–25).

Net accumulation of transcripts of photosynthesis genes from LD-grown cultures peaked at three to four periods throughout the 24-h cycle. During the dark phase, accumulation of transcripts was somewhat greater around 2 h and 8 to 10 h, with the most significant peak near the end of the dark phase. In the light phase, net accumulation of transcripts was high near L2 but especially strong at L8 to L10. The peak around L8 appeared to correlate with an increase in protein accumulation of the PSII proteins D1 form 2 and D2 (Fig. 7 and 8). Interestingly, the PSI reaction center proteins increased during the dark phase. A unifying hypothesis for these changes is that cellular bioenergetic needs are key. The cell emphasizes noncyclic electron flow (which leads to O₂ evolution and CO₂ fixation) in the light phase but shifts to cyclic electron flow (which favors ATP synthesis) in the dark. The cell requires substantial levels of energy for nitrogen fixation, and respiration becomes maximal near D4. It is possible that a change in the organizational structure of PSI is required for, or caused by, the need to enhance respiration.

The conclusions drawn from fluorescence kinetics and spectral experiments indicate that the photosystems of *Cyanothece* sp. strain ATCC 51142 are in dynamic flux (29). During LD growth, PSI change from trimers in the dark to primarily monomers in the early portion of the light period (L0 to L6) and then back to trimers by the beginning of the next dark phase. PSII mostly exists as monomers in the late dark and early light phases and then shows a strong switch to dimers around L6 to L12. The cells are mostly in state 1 (favoring linear electron flow) during the middle to late light period, at a time when photosynthesis activity reaches a peak. The synthesis of D1 form 2 at this time (Fig. 7) is consistent with the results of Campbell et al. (4) and indicates a relationship between state 1 and form 2. The L6 to L10 period represents afternoon, and this might be associated with high light intensities. Thus, the switch to D1 form 2 may be an adaptation to permit high rates of photosynthesis during a period of high light flux, whereas form 1 is best during the lower light intensities in the morning (Fig. 10).

The results for PsAB were both striking and puzzling, since they suggested that there is more PSI that accumulates in the early part of the dark period. Yet physiological and biophysical measurements have provided additional support for this finding. Misra and Desai (30) showed that in *Plectonema boryanum*, PSI activity increased during N₂ fixation under microaerobic and anaerobic conditions. We have now determined that the capacity for PSI electron transport is somewhat higher in the dark phase relative to the light phase in LD-grown cultures of *Cyanothece* sp. strain ATCC 51142 (52). In addition, N₂ fixation activity is more dependent on PSI than PSII in continuous light, implying a possible role for cyclic phosphorylation (29–32). We have also observed substantial changes in the organization of PSI throughout the diurnal cycle, and PSI is mostly trimeric during the late light and early dark periods. It is possible that the short-term switch to trimers and the biosynthetic increase in PSI protein in the L8 to L12 period alters the input of excitation energy from PSII in favor of PSI. This could be one cause of the PSII downregulation after L8.

The short-term and long-term changes appear to converge in...
the afternoon (L6 to L12) and are schematically outlined in Fig. 10. This is at a time of significant heterogeneity in PSI, as seen on both the reducing side (29) and the oxidizing side (28) of the photosystem. New PSI complexes (which include D1 form 2) are formed, and PSI is primarily dimeric and in state 1 (favoring noncyclic electron flow). At the same time, more PSI is synthesized and the organization of PSI becomes more trimeric. This favors coupling of the phycobilisomes to PSI (2, 40) and thus can shift excitation away from PSI. This then shifts the cells in the direction of state 2 (favoring cyclic electron flow around PSI), which is what we see early in the dark phase. In Fig. 10, we suggest that the successive synthesis of D1 form 2 for PSI and PsAB for PSI acts to facilitate the switch from state 1 to state 2. Operationally, this leads to the attachment of a higher proportion of the phycobilisomes to PSI, thus protecting PSI and favoring cyclic flow around PSI (and ATP synthesis). At D4, which is the peak of respiratory and nitrogenase activity, PSI is virtually all trimeric and cyclic electron flow is greatly favored. Thus, in LL growth, PSI can produce additional ATP for N\textsubscript{2} fixation. The results suggest that the longer-term, biosynthetic changes reported here act to amplify the short-term changes already under way.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical help of Wendy O. Adamowicz and Hsiao-Yuan (Vicky) Tang at various times throughout this project and the kindness of Sue Golden and others who provided DNA probes and antibodies. This work was supported by USDA grant 93-37306-9238 (to L.A.S.) and by fellowships from the American Society for Microbiology, from the National Hispanic Scholarship Foundation, and from NIH (F31 GM16400-01) (to M.S.C.-L.).

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