Substrate-binding Lipoprotein of the Cyanobacterium 
Synechococcus sp. Strain PCC 7942 Involved in the 
Transport of Nitrate and Nitrite* 

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Of the four genes (nrtABCD) required for active transport of nitrate in the cyanobacterium Synechococcus sp. strain PCC 7942, nrtBCD encode membrane components of an ATP-binding cassette transporter involved in the transport of nitrate as well as of nitrate, whereas nrtA encodes a 45-kDa cytoplasmic membrane protein, the biochemical function of which remains unclear. Characterization of the nrtA deletional mutants showed that the 45-kDa protein is essential for the functioning of the nitrate/nitrite transporter. A truncated NrtA protein lacking the N-terminal 81 amino acids, expressed in Escherichia coli cells as a histidine-tagged soluble protein, was shown to bind nitrate and nitrite with high affinity (K_d = 0.3 μM). Immunoblotting analysis using the antibody against the 45-kDa protein revealed a 48-kDa precursor of the protein, which accumulated in the cyanobacterial cells treated with globomycin, an antibiotic that specifically inhibits cleavage of the signal peptide of lipoprotein precursors. These findings indicated that the nrtA gene product is a nitrate- and nitrite-binding lipoprotein. The N-terminal sequences of putative cyanobacterial substrate-binding proteins suggested that lipoprotein modification of substrate-binding proteins of ATP-binding cassette transporters is common in cyanobacteria.

Nitrates, the major source of nitrogen for photosynthetic organisms, are actively transported into the cell prior to its reduction to ammonium by the sequential action of nitrate reductase (NR) and nitrite reductase (NiR) (1). The transport of nitrate has been the least understood step of nitrate assimilation. Recently, nitrate transporter genes have been identified in various organisms (2–7), offering the opportunity to investigate the structure-function relationship and the regulation of the nitrate transporters.

The nitrA gene product of Synechococcus sp. strain PCC 7942 (nrtABCD, Refs. 2–4) are clustered with the NR gene (narB) and the NiR gene (nirA) to form the nirA-nrtABCD-narB operon (8). nrtB encodes a hydrophobic protein with structural similarities to the integral membrane components of ABC (ATP-binding cassette) transporters, and nrtC and nrtD encode proteins that resemble the ATP-binding proteins of ABC transporters, indicating that the nitrate transporter belongs to the ABC superfamily of transporters (9). The product of nrtA is the 45-kDa cytoplasmic membrane protein that is abundant in nitrate-grown cells (2). The hydrophilicity of the deduced amino acid sequence, the occurrence of a putative signal peptide, and its abundance seem to suggest that the 45-kDa protein is the substrate-binding protein of the nitrate transporter (4). However, the membrane-bound nature of the protein, which is not typical of the substrate-binding proteins of Gram-negative bacteria, has made biochemical studies difficult, leaving its function unclear.

On the basis of competitive interaction between nitrate and nitrite utilization (10) and the competitive inhibition by nitrite of nitrate transport (11), the cyanobacterial nitrate transporter has been assumed to transport nitrite as well. Luque et al. (12) verified this assumption by showing the inability of nrtD mutants to utilize low concentrations of nitrite. It is deduced that nrtB and nrtC, encoding the other components of the membrane transporter complex, are also involved in nitrite transport. It is yet to be examined whether the nrtA-encoded 45-kDa protein is involved in the transport of nitrite.

In this work, we clarified the function of the nrtA gene product by molecular biological and biochemical analyses. Characterization of the mutants of Synechococcus sp. strain PCC 7942 with defined deletions in the nirA-nrtABCD-narB operon showed that nrtA is essential for the activity of the nitrate/nitrite transporter. Recombinant NrtA bound both nitrate and nitrite with high affinity. Studies using an inhibitor of lipoprotein processing indicated that the nrtA-encoded 45-kDa protein is a lipoprotein. We concluded that the nrtA gene product is a nitrate/nitrite-binding lipoprotein. On the basis of reported amino acid sequences of the putative substrate-binding proteins of ABC transporters from cyanobacteria, we propose that lipoprotein modification of substrate-binding proteins, which is unusual in other Gram-negative bacteria, is common in cyanobacteria.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—A derivative of Synechococcus sp. strain PCC 7942 which is cured of the resident small plasmid pUH24 (R2-SpC, Ref. 13; hereafter designated simply as strain PCC 7942) and the mutant strains derived therefrom were grown photoautotrophically at 30 °C under CO_2-sufficient conditions as described previously (14). The basal medium used was a nitrogen-free medium obtained by modification of BG11 medium (15) as described previously (14). Ammonium-containing medium and nitrate-containing medium were prepared by addition of 3.75 mM (NH_4)_2SO_4 and 60 mM KNO_3, respectively, to the basal medium unless otherwise stated. All media were buffered with 20 mM HEPES-KOH (pH 8.2). Expression of the nirA-nrtABCD-narB operon was induced by transfer of ammonium-grown cells to nitrate-
containing medium as described previously (8).

Deletional Mutagenesis—Two defined mutants of Synechococcus, NA2 and NA3, were constructed by deleting nrtA and nrtABCD from the nirA-nrtABCD-narB operon, respectively, by the marker exchange-eviction mutagenesis method (16), using a 5.8-kbp nptI-sacB cartridge excised from pBluescript II SK(−) (17) as the selection marker (Fig. 1A). In NA2, the nrtA and nrtB coding regions were separated by the 66-base-long nirA-nrtA intercistronic sequence. The nirA and narB coding regions in NA3 were separated by a segment of 228 nucleotides, consisting of the nirA-nrtA intercistronic sequence, ATG from the nirA start codon, and the 159 bases of the 5′ flanking sequence of narB.

DNA Isolation and Analysis—Chromosomal DNAs were extracted and purified from the Synechococcus cells as described by Williams (18). Manipulations and analyses of DNA were performed according to standard protocols (19). For Southern hybridization analysis of the genomic DNA digests, the following gene-specific probes were used (Fig. 1; 1) a 1.5-kbp EcoRI-BspHII fragment of nirA (probe 1), a 1.4-kbp BspHI fragment carrying nrtA (probe 2), and a 3.7-kbp BspHII-BglII fragment carrying nrtBCD (probe 3).

Measurements of Nitrate and Nitrite Uptake—Cells grown in nitrate-containing medium were washed with the basal medium supplemented with 5 mM NaHCO₃ and 20 mM HEPES-KOH (pH 9.6), and suspended in the same medium at a chlorophyll (Chl) concentration of 5 μg/ml. The reaction was started by the addition of 100 μM of KNO₃ or NaNO₂ to the cells at 30°C in the light (70 microeinsteins m⁻² s⁻¹). Aliquots were withdrawn from the cells suspensions at 6-min intervals, and after immediate centrifugation for 60 s at 15,000 × g to sediment the cells, nitrate and nitrite in the supernatant were determined.

Expression of Plasmid-encoded NrtA in Synechococcus—A shuttle vector (pSE1) for expression of cloned genes in strain PCC 7942 was constructed as follows. The AccI site in the polylinker of the expression vector pTrc99A (20) was eliminated from the polylinker by digestion of the plasmid with Sall and HindIII, followed by blunting of the termini and recircularization. The bla gene was then eliminated from the plasmid by digestion with BspHI, and after blunting of the termini, the linearized plasmid was ligated with a 1.3-kbp HindIII fragment of pUC4K (21) carrying the nptI gene. Finally, a 3.9-kbp Sall-Xhol fragment of pUC303 (13), carrying the origin of replication in strain PCC 7942 (22), was ligated into the AccI site located between lac I and the pMB1 replicon. A 1.4-kbp BspHI fragment of strain PCC 7942 DNA carrying nrtA was cloned into the Neos site of pSE1, and the resulting plasmid (pNRTA1) was used for expression of nrtA in the nrtA-deletional mutants.

Preparation of Recombinant NrtA and Binding Assay—A 1.2-kbp DNA fragment, carrying a truncated nrtA coding region lacking the first 241 bases, was cloned between the BamHI and HindIII sites in the polylinker of the expression vector pQE-30 (Qiagen). The resulting plasmid (pNRTA2) carried a chimeric gene, which encodes a fusion protein consisting of an N-terminal amino acid segment carrying six consecutive histidine residues (MRGS)SHGS) and truncated NrtA lacking the N-terminal 81 amino acids. The plasmid was transformed into E. coli M15/pREP4 (Qiagen), expression of the chimeric gene was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and the histidine-tagged protein was purified on Ni²⁺-nitrilotriacetic acid resin (23).

Binding of nitrate and nitrite to the recombinant NrtA protein was measured at 30°C by equilibrium dialysis in a buffer containing 20 mM sodium phosphate, pH 8.0, and 100 mM NaCl; aliquots of protein solution (1.0 mg/ml) were dialyzed for 1.5 h against the same volume of the buffer containing various concentrations of nitrate or nitrite, using paired Teflon cells separated by dialysis membrane (Spectrum). For determination of the total substrate concentration in the protein solution ($S_{total} = [S]_{protein}$), the protein was denatured by heat treatment at 100°C for 10 min to dissociate the substrate, and the denatured protein was removed by centrifugation at 15,000 × g for 5 min, followed by passage through a 0.22-μm (pore size) cellulose acetate filter (Toyo Roshi).

Immunoblotting Analysis—Cells were collected by centrifugation, resuspended in the sample buffer for SDS-polyacrylamide gel electrophoresis (24), and lysed by heat treatment at 100°C for 5 min. After gel electrophoresis in the buffer system of Laemmli (24), polypeptides were electrotransferred to a polyvinylidene difluoride membrane and allowed to react with the affinity-purified antibody against the 45-kDa protein (2). A goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Bio-Rad) was used as the second antibody.

Other Methods—NR and NiR activities were determined at 30°C, using toluene-permeabilized cells with dithionite-reduced methylviologen as the electron donor (25, 26). Nitrate and nitrite were determined with a flow-injection analyzer (NOX-1000, Tokyo Chemical Industry Co., Ltd.). Chl and protein were determined according to Mackinney (27) and Lowry et al. (28), respectively.

RESULTS

Characterization of nrtA Deletion Mutants—Fig. 1B shows the results of Southern hybridization analysis of the EcoRI-HindIII digests of genomic DNAs from the wild-type strain and the nrt deletional mutants. As expected from the restriction map (Fig. 1A), the nrtA-, nrtA-, and nrtBCD-specific probes

![Figure 1](image)

**TABLE I**

NR and NiR activities in ammonium- and nitrate-grown cells of the wild-type and mutant strains

Ammonium-grown cells of wild-type Synechococcus sp. strain PCC 7942 (WT) and the mutants NA2 and NA3 were transferred to nitrate (60 mM)-containing medium, and the enzyme activities were assayed before and 12 h after the transfer.

| Strain | NH₄⁺ | NR activity (μmol mg⁻¹ Chl h⁻¹) | NO₃⁻ | NiR activity (μmol mg⁻¹ Chl h⁻¹) | NO₃⁻ |
|--------|------|--------------------------------|------|--------------------------------|------|
| WT     | 7    | 150                            | <5   | 80                             |
| NA2    | 7    | 280                            | <5   | 270                           |
| NA3    | 5    | 290                            | <5   | 200                           |

FIG. 1. Southern hybridization analysis of genomic DNA from the wild-type strain and the nrt deletional mutants. A, restriction map of the nirA-narB region of the genome of the wild-type strain (WT) and the NA2 and NA3 mutants. The thick bars above the map represent the probes used for Southern hybridization analysis (probes 1–3). Restriction endonuclease sites are abbreviated as follows: Ba, BamHI; Bg, BglII; Bs, BspHI; E, EcoRI; H, HindIII. B, Southern hybridization analysis of genomic DNA from wild-type (lanes 1, 4, and 7), NA2 (lanes 2, 5, and 8), and NA3 (lanes 3, 6, and 9). DNA samples (5 μg/lane) were digested with EcoRI plus HindIII, fractionated on a 0.7% agarose gel, transferred to positively charged nylon membrane (Hybond N⁺; Amer sham), and hybridized with the 32P-labeled gene-specific probes as indicated.
hybridized with an 8.5-kbp fragment of DNA from the wild-type strain (Fig. 1B, lanes 1 and 4). The nrtA-specific probe hybridized with 7.1- and 3.4-kbp fragments in the digests of DNAs from NA2 and NA3, respectively (Fig. 1B, lanes 2 and 3). The nrtA-specific probe hybridized with neither of the NA2 and NA3 DNA (Fig. 1B, lane 5 and 6). The nrtBCD-specific probe hybridized with the 7.1-kbp fragment of NA2 DNA but not with NA3 DNA (Fig. 1B, lanes 8 and 9). These results indicated that nrtA and nrtBACD had been deleted from the genome of NA2 and NA3, respectively.

In the mutants as well as the wild-type strain, NR and NiR activities were null in ammonium-grown cells and induced by transfer of the cells to nitrate-containing medium (Table I). Since NR and NiR are encoded by narB and nirA, respectively, these results indicated that the deletion of nrtA or nrtBACD from the nirA operon had not essentially affected the expression of the other genes in the operon. Similar to the nrtA insertional mutant previously described (2), NA2 and NA3 expressed higher NR and NiR activities than the wild-type strain (Table I) and yet failed to grow in a medium containing 2 mM nitrate (data not shown). Medium containing 60 mM nitrate supported the growth of the mutants, showing that they are defective in active transport of nitrate (data not shown).

Uptake of nitrate and nitrite by the Synechococcus cells was measured at pH 9.6, under which condition the passive diffusion of nitrous acid (HNO₂) into the cells is negligible (29). As reported previously (30), suspensions of the wild-type and mutant cells used nitrate or nitrite until its exhaustion, with a calculated uptake rate of 44 μmol/ml Chl/h for either substrate (Fig. 2A). NA2 and NA3, on the other hand, could not take up nitrate at the low concentrations at all, and the rate of nitrite uptake was about 30% of that in the wild-type strain (Fig. 2, B and C).

Complementation of the Mutant Phenotype with Plasmid-encoded NrtA—Cells of NA2 and NA3 were transformed with plasmid pNRTA1 to kanamycin resistance, and the resulting transformants were designated NA21 and NA31, respectively. As described previously (2), the wild-type cells accumulated the nrtA-encoded 45-kDa protein when grown with nitrate but not when grown with ammonium (Fig. 3A, lanes 1 and 2). The protein was absent in NA2 and NA3 cells irrespective of the source of nitrogen (Fig. 3A, lanes 3, 4, 9, and 10). Cells of NA21 and NA31 accumulated the 45-kDa protein without IPTG treatment and irrespective of the nitrogen source (Fig. 3A, lanes 5, 7, and 11), indicating IPTG-independent expression of nrtA from pNRTA1. The non-induced NA21 cells grew as rapidly as the wild-type strain in a medium containing 2 mM nitrate (data not shown) and used up nitrate and nitrite in medium (Fig. 2D), showing that the amount of the 45-kDa protein (Fig. 3A, lane 7) was sufficient to support nitrate and nitrite transport. The restoration by the plasmid-encoded 45-kDa protein of the wild-type rate of nitrate and nitrite uptake (Fig. 2D) indicated that the protein itself participates in the transport of nitrate and nitrite.

Although non-induced NA31 cells synthesized the 45-kDa protein in an amount similar to that in NA21 (Fig. 3A, lane 11), it failed to utilize low concentrations of nitrate, and the rate of
purified on Ni\textsuperscript{2+} fraction from the IPTG-induced expression strain; the expression strain after 1-h treatment with IPTG; gel and stained with Coomassie Blue. The amount of the loaded protein (masses are indicated in kilodaltons). The amount of the loaded protein.

In addition to the hydrophobic core of the putative signal peptide (amino acids 9–23), the deduced NrtA polypeptide has a hydrophobic amino acid segment extending from amino acid 58 to 81 (3). Truncated NrtA lacking both hydrophobic segments was expressed as a histidine-tagged protein in \textit{E. coli} and purified in a soluble form to near homogeneity (Fig. 4A). Equilibrium dialysis experiments showed that the protein binds both nitrate and nitrite (Fig. 4B). From the Scatchard plot (31) of the data (Fig. 4B, insets), the dissociation constants were calculated to be 0.32 and 0.34 \mu M for nitrate and nitrite, respectively. The concentration of the bound substrate under saturation was calculated to be 24.3 and 23.8 \mu M for nitrate and nitrite, respectively. These values were similar to the protein concentration used, 23.9 \mu M, as calculated from the protein concentration of 1 mg/ml and the calculated molecular mass of 41,823 Da, suggesting that one molecule of protein carries one substrate-binding site. Nitrate and nitrite inhibited the binding of each other to the protein (Table II), but other anions added at a concentration 10-fold higher than nitrate and nitrite did not affect the binding of nitrate or nitrite (Table II). These findings indicated that the truncated NrtA specifically binds nitrate and nitrite.

**Detection of the Precursors of the 45-kDa Protein**—NA21 and NA31 did not grow in the presence of IPTG, showing that overexpression of \textit{nrtA} was inhibitory to the growth (data not shown). IPTG-treated NA21 and NA31 cells accumulated large amounts of the 45-kDa protein and also of 48- and 46-kDa proteins reacting with the antibody against the 45-kDa protein (Fig. 3A, lanes 6, 8, and 12), which suggested that the latter proteins are precursors of the 45-kDa protein.

When transcription of the \textit{nirA-nrtABCD-narB} operon was induced by transfer of the wild-type PCC 7942 cells from ammonium-containing medium to nitrate-containing medium, the progressive increase of the 45-kDa protein was accompanied by increased accumulation of the 48-kDa protein at 30 °C for 1.5 h. The concentration of the bound substrate ([S]\textsubscript{bound}) was plotted against that of the free substrate ([S]\textsubscript{free}). Inset, Scatchard plot of the data.

**Table II**

| Competitive substrate | Concentration | Nitrate bound | Nitrite bound |
|-----------------------|---------------|---------------|---------------|
| Nitrate               | 50 \mu M      | ND            | ND            |
| Nitrate               | 100 \mu M     | ND            | 22            |
| Nitrate               | 500 \mu M     | ND            | 8             |
| Nitrite               | 50 \mu M      | 59            | ND            |
| Nitrite               | 100 \mu M     | 36            | ND            |
| Sulfate               | 500 \mu M     | 101           | 96            |
| Sulfite               | 500 \mu M     | 100           | 98            |
| Chlorate              | 500 \mu M     | 100           | 101           |
| Chlorite              | 500 \mu M     | 101           | 99            |
| Bicarbonate           | 500 \mu M     | 103           | 97            |
| Borate                | 500 \mu M     | 97            | 99            |
| Molybdate             | 500 \mu M     | 99            | 98            |

**DISCUSSION**

The \textit{nrtABCD} deletion mutant (NA3) of \textit{Synechococcus} sp. strain PCC 7942 was totally defective in nitrate uptake but showed significant activity of nitrate uptake (Fig. 2C). The results are in conflict with the previous report that \textit{nrtD} insertional mutants are totally defective in uptake of low concentra-
tions of nitrite (12) and indicate the occurrence of a nitrite-specific transporter. The nitrite uptake activity of the \textit{nrtA} deletion mutant (NA2) was similar to that of NA3 and hence was ascribed to the nitrite-specific transporter. The recovery of the wild-type rates of nitrate and nitrite uptake by expression of \textit{nrtA} from a plasmid in NA2 (Fig. 2D) indicates that the \textit{nrtA} gene product is an essential constituent of the nitrate/nitrite transporter.

Bacterial ABC importers require a substrate-binding protein that has a high affinity for its specific substrate (9, 33). A recombinant NrtA protein was shown to bind nitrate and nitrite with a \(K_d\) value of approximately 0.3 \(\mu\)M for either substrate (Fig. 4B), which is low enough to account for the apparent \(K_m\) (NO\(_3^-\)) of the nitrate transport of strain 7942, 1 \(\mu\)M (2). These findings strongly suggest that the \textit{nrtA} gene product is the substrate-binding protein of the nitrate/nitrite transporter. Since one molecule of the protein binds one molecule of nitrate or nitrite (Fig. 4B), and since nitrate and nitrite inhibit the binding of each other to the protein (Table II), we speculate that the same binding site recognizes nitrate and nitrite.

The predicted amino acid sequence around the presumed signal cleavage site of NrtA (Table III) conforms to the consensus sequence recognized by signal peptidase II, (LV)(x)ASTG(GA)C, in which one mismatch is acceptable in the first two amino acids (34). The inhibition by globomycin of the accumulation of the \textit{nrtA}-encoded 45-kDa protein (Fig. 3B) confirms the involvement of signal peptidase II in the maturation of the protein. Since the enzyme cleaves the signal peptide from an S-glycylde derivative of prolipoproteins with the modified cysteine at the signal cleavage site (32), the 45-kDa protein is deduced to be a lipoprotein with a lipoyl acid at the N terminus, and the 48-kDa precursor of the protein (Fig. 3B) is deduced to be the S-glycyl derivative of NrtA. The 46-kDa form of NrtA (Fig. 3A) is tentatively identified as the nascent NrtA polypeptide. Thus, the 45-kDa cytoplasmic membrane protein is a substrate-binding lipoprotein, which is rarely found in Gram-negative bacteria (35). The NrtA sequences from other strains of cyanobacteria, Synechocystis sp. strain PCC 6803 (36) and \textit{Plectonema boryanum} (37), also have the consensus sequence for signal peptidase II (Table III), and are hence probably lipoproteins. Although the sequence from \textit{Phormidium laminosum} (38) does not exactly match the consensus, this may represent divergence in specificity of signal peptidase II in cyanobacteria.

As shown in Table III, most of the genetically and/or biochemically characterized substrate-binding proteins of cyanobacterial ABC transporters have the lipoprotein consensus sequence. The \textit{cmpA} gene of strain PCC 7942 encodes a 42-kDa cytoplasmic membrane protein similar to the 45-kDa nitrate/nitrite-binding protein (3, 39). Since \textit{cmpA} is clustered with other genes encoding membrane components of an ABC transporter (40), its product is assumed to be a substrate-binding protein, although the substrate is yet to be identified. It is blocked at the N terminus and is tightly bound to the cytoplasmic membrane in spite of the hydrophilicity of the predicted sequence (39). These observations and the presence of the signal peptidase II recognition sequence suggest that the \textit{cmpA} gene product is a lipoprotein. The \textit{sbpA} gene of strain PCC 6803 was cloned by immunoscreening of an expression library using antisera raised against total cytoplasmic membrane proteins (41), which implies association of the sulfate-binding protein to the membrane and hence its lipophilic modification. While the phosphate-binding protein \textit{PstS} from \textit{Synechococcus} sp. strain WH 7803 is a soluble periplasmic protein with a signal peptide typical of periplasmic proteins (42), one of the two \textit{PstS} homologues from \textit{Synechocystis} sp. strain PCC 6803 has the lipoprotein consensus sequence (36, 43). The genome sequencing project of strain PCC 6803 has identified 21 putative substrate-binding proteins of ABC transporters including those in Table III (36, 12 of which have the lipoprotein consensus sequence (data not shown). Thus, many of the substrate-binding proteins seem to be lipoproteins in cyanobacteria.

In Gram-positive bacteria and mycoplasma, substrate-binding proteins of ABC transporters are generally lipoproteins anchored to the cytoplasmic membrane (44–49). Since the cells of these organisms are surrounded by a single membrane and have therefore no periplasmic space, the lipoprotein modification is supposed to have a role in maintaining the substrate-binding proteins at the cell surface. In Gram-negative bacteria, which have an outer membrane and periplasmic space, the substrate-binding proteins are usually soluble proteins located in the periplasm (33). Since cyanobacteria belong to Gram-negative bacteria, the common occurrence of the substrate-binding lipoproteins, as suggested by this study, may have a role other than to keep the proteins from escaping away from the cell surface.

REFERENCES

1. Guerrero, M. G., Vega, J. M., and Losada, M. (1981) \textit{Annu. Rev. Plant Physiol.} \textbf{32}, 169–204
2. Omata, T., Ohmori, M., Arai, N., and Ogawa, T. (1989) \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{86}, 6612–6616
3. Omata, T. (1991) \textit{Plant Cell Physiol.} \textbf{32}, 151–157
4. Omata, T., Andriesse, X., and Hirano, A. (1993) \textit{Mol. Gen. Genet.} \textbf{236}, 193–202
5. Unkles, S. E., Hawker, K. L., Grieve, C., Campbell, E. I., Montague, P., and Kinghorn, J. R. (1991) \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{88}, 204–208
6. Tsay, Y.-F., Schroeder, J. I., Feldmann, K. A., and Crawford, N. M. (1993) \textit{Cell} \textbf{72}, 705–713
7. Quesada, A., Galván, A., and Fernández, E. (1994) \textit{Plant J.} \textbf{5}, 407–419
8. Suzuki, I., Sugiyama, T., and Omata, T. (1993) *Plant Cell Physiol.* **34**, 1311–1320
9. Higgins, C. F., Hyde, S. C., Mimmack, M. M., Gileadi, U., Gill, D. R., and Gallagher, M. P. (1990) *Bioenerg. Biomembr.* **22**, 571–592
10. Madueno, F., Flores, E., and Guerrero, M. G. (1987) *Biochim. Biophys. Acta* **896**, 109–112
11. Rodriguez, R., Lara, C., and Guerrero, M. G. (1987) *Biochim. Biophys. Acta* **896**, 109–112
12. Luque, I., Flores, E., and Herrero, A. (1994) *Biochim. Biophys. Acta* **1184**, 296–298
13. Kubalemeier, C. J., Thomas, A. A. M., van der Ende, A., van Leen, R. W., Borrias, W. E., van den Hondel, C. A. M. J. J., and van Arkel, G. A. (1983) *Plasmid* **10**, 156–163
14. Suzuki, I., Sugiyama, T., and Omata, T. (1996) *J. Bacteriol.* **178**, 2688–2694
15. Stanier, R. Y., Kunisawa, R., Mandel, M., and Cohen-Bazire, G. (1971) *Bacteriol. Rev.* **35**, 171–205
16. Ried, J. L., and Collmer, A. (1987) *Gene* (**Amst.**) **57**, 239–246
17. Cai, Y., and Wolk, C. P. (1990) *J. Bacteriol.* **172**, 3138–3145
18. Williams, J. G. K. (1988) *Methods Enzymol.* **167**, 766–778
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Amann, E., Ochs, B., and Abel, K.-J. (1988) *Gene* (**Amst.**) **69**, 301–315
21. Vieira, J., and Messing, J. (1982) *Gene* (**Amst.**) **19**, 259–268
22. van der Plas, J., Oosterhoff-Teertstra, R., Borrias, M., and Weisbeek, P. (1992) *Mol. Microbiol.* **6**, 653–664
23. Hochuli, E., Dobeli, H., and Schacher, A. (1987) *J. Chromatogr.* **411**, 177–184
24. Laemmli, U. K. (1970) *Nature* **227**, 680–685
25. Herrero, A., Flores, E., and Guerrero, M. G. (1981) *J. Bacteriol.* **145**, 175–180
26. Herrero, A., and Guerrero, M. G. (1986) *J. Gen. Microbiol.* **132**, 2463–2468
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
28. Stachel, G. A. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
29. Inukai, M., Takeuchi, M., Shimizu, K., and Arai, M. (1978) *J. Antibiot.* **31**, 1203–1205
30. Suzuki, I., Kikuchi, H., Nakanishi, S., Fujita, Y., Sugiyama, T., and Omata, T. (1995) *J. Bacteriol.* **177**, 6137–6143
31. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
32. Inukai, M., Takeuchi, M., Shimizu, K., and Arai, M. (1978) *J. Antibiot.* **31**, 1203–1205
33. Tam, R., and Saier Jr., M. H. (1993) *J. Bacteriol.* **177**, 2259–2264
34. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hiroswawa, M., Sugiyama, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimo, T., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. (1996) *DNA Res.* **3**, 109–136
35. Suzuki, I., Kikuchi, H., Nakanishi, S., Fujita, Y., Sugiyama, T., and Omata, T. (1995) *J. Bacteriol.* **177**, 6137–6143
36. Scanlan, D. J., Mann, N. H., and Carr, N. G. (1993) *Mol. Microbiol.* **10**, 181–191
37. Kaneko, T., Tanaka, A., Sato, S., Kotani, H., Suzuki, T., Miyajima, N., Sugiyama, M., and Tabata, S. (1995) *Plant Mol. Biol.* **28**, 759–766
38. Omata, T., Carlson, T. J., Ogawa, T., and Pierce, J. (1990) *Plant Physiol.* **93**, 305–311
39. Omata, T. (1992) in *Research in Photosynthesis* (Murata, N., ed) Vol. III, pp. 807–810, Kluver, Dordrecht, The Netherlands
40. Kohn, C., and Schumann, J. (1993) *Plant Mol. Biol.* **21**, 409–412
41. Alloing, G., de Philip, P., and Claverys, J. P. (1994) *J. Mol. Biol.* **241**, 44–58
42. Chang, Z., Choudhary, A., Lathigra, R., and Quiocho, F. A. (1994) *J. Biol. Chem.* **269**, 1956–1958
43. Jenkinson, H. F., Baker, R. A., and Tannock, G. W. (1996) *J. Bacteriol.* **178**, 68–77
44. Bartsevich, V. V., and Grossman, A. R. (1991) *J. Bacteriol.* **173**, 2739–2750
45. Park, S. F., and Richardson, P. T. (1995) *J. Bacteriol.* **177**, 2259–2264
46. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hiroswawa, M., Sugiyama, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimo, T., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. (1996) *DNA Res.* **3**, 109–136
47. Suzuki, I., Kikuchi, H., Nakanishi, S., Fujita, Y., Sugiyama, T., and Omata, T. (1995) *J. Bacteriol.* **177**, 6137–6143
48. Scanlan, D. J., Mann, N. H., and Carr, N. G. (1993) *Mol. Microbiol.* **10**, 181–191
49. Kaneko, T., Tanaka, A., Sato, S., Kotani, H., Suzuki, T., Miyajima, N., Sugiyama, M., and Tabata, S. (1995) *PLASMID* **10**, 156–163
50. Laudenbach, D. E., and Grossman, A. R. (1991) *J. Bacteriol.* **173**, 2739–2750
51. Bartsevich, V. V., and Grossman, A. R. (1991) *J. Bacteriol.* **173**, 2739–2750
52. Bartsevich, V. V., and Grossman, A. R. (1991) *J. Bacteriol.* **173**, 2739–2750
53. Bartsevich, V. V., and Grossman, A. R. (1991) *J. Bacteriol.* **173**, 2739–2750