JOURNAL OF BACTERIOLOGY, July 1983, p. 180–185
0021-9193/83/070180-06$02.00/0

Vol. 155, No. 1

The Wild-Type Gene for Glutamine Synthetase Restores Ammonia Control of Nitrogen Fixation to Gln⁻ (*glnA*) Mutants of *Rhodopseudomonas capsulata*

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Received 7 February 1983/Accepted 11 April 1983

The wild-type *glnA* gene, coding for glutamine synthetase, was cloned from the photosynthetic bacterium *Rhodopseudomonas capsulata* by using a cosmid library to complement the Gln⁻ phenotype of an *Escherichia coli glnA* deletion strain. The original cosmid plasmid contained 37 kilobase pairs (kbp) of *R. capsulata* DNA, of which only 2 kbp was necessary for Gln complementation in *E. coli*. A plasmid containing this 2-kbp insert was mobilized into G29, a Gln⁻ mutant of *R. capsulata* which is also unable to repress nitrogenase in ammonia-containing media (Nif^c phenotype). The 2-kbp fragment restored glutamine-independent growth and ammonia repression of nitrogenase, indicating that in *R. capsulata*, production of the signal for nitrogen repression of *nif* depends on the activity of the *glnA* gene. Repression of nitrogenase was shown, by hybridization of RNA to cloned *nif* DNA, to occur at the level of transcription in the wild-type and the complemented G29 strains.

Rhodopseudomonas capsulata is a purple, nonsulphur, photosynthetic bacterium, capable of fixing both nitrogen and carbon dioxide. Diazotrophy can be either microaerobic or anaerobic (11). Ammonia, obtained by nitrogenase activity or provided exogenously, can enter the nitrogen metabolic pathways only through the action of glutamine synthetase (L-glutamate ammonia ligase [ADP], EC 6.3.1.2), since glutamate dehydrogenase is not present in *R. capsulata* (7). Eight glutamine auxotrophs have been isolated, all of which map within 1.2 kilobase pairs (kbp) of the genome (22; D. P. Taylor, S. N. Cohen, W. Clark, and B. L. Marrs, submitted for publication). These mutants are also unable to repress nitrogenase activity in ammonia-containing media (Nif^c phenotype) (22). In *R. capsulata*, as in other photosynthetic bacteria, nitrogenase activity is regulated by ammonia at two levels. In addition to repressing synthesis of nitrogenase proteins, ammonia also leads to covalent modification of nitrogenase reductase. This modification, which consists of an added adenosine phosphate moiety, inactivates the enzyme (10, 16, 25).

In this report, we present evidence that both the glutamine (Gln⁻) and Nif^c phenotypes of an *R. capsulata* mutant can be complemented by a 2-kbp fragment of *R. capsulata* DNA containing the structural gene for glutamine synthetase. Regulation of *nif* gene expression by ammonia was further shown to occur at the level of transcription.

MATERIALS AND METHODS

Strains, media, and plasmids. SB1003 is an *R. capsulata* wild-type strain (23). G29 is a Gln⁻ mutant obtained from Judy Wall, University of Missouri, Columbia (22). ET8051 is an *Escherichia coli* Gln⁻ mutant (17) deleted for at least 11 kbp, including the entire *glnA ntrB ntrC* operon. Minimal medium was M9 (15) for *E. coli* and RCVB (8, 23) or RCVB-ammonia free for *R. capsulata*. Complete medium for *E. coli* was LB (15). Glutamine was added at 20 mM. The binary plasmid system pRK290-pRK2013 has been described previously (4). pPH1 is a gentamicin-resistant (Gen^r) R factor (P. R. Hirsch, Ph.D. thesis, University of East Anglia, Norwich, England) that is incompatible with pRK290. pDPT5Cm is a cosmid vector coding for tetracycline resistance (Tet^r) and chloramphenicol resistance (Cm^r) (20) that was kindly provided by Dean Taylor, Smith, Kline Laboratories, Philadelphia.

Nucleic acids. A cosmid library in pDPT5Cm was constructed according to Collins (1). Genomic DNA, partially cut with *Hin*dIII, was purified through sucrose gradients (12) to eliminate small fragments. Efficiency of transformation of ET8051 was 5×10^4 colonies per μg of genomic DNA. Lysates from this library, obtained by infecting pooled colonies with phage λ, were stored at 4°C over chloroform. Plasmids were prepared from small volumes of culture according to Ish-Horowitz and Burke (6). Restriction enzymes were obtained from commercial sources and used according to supplier specifications. Gel electrophoresis was carried out by the method of Davis et al. (2). The DNA fragments were blotted onto nitrocellulose and hybridized with ^{32}P-labeled RNA according to Southern (19). Total RNA was isolated (G. Clark, personal communication) and 5′ labeled in vitro with

^{32}P according to Maxam and Gilbert (13). RNA-DNA hybridization was carried out at 37°C in 50% formamide–4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 18 h. Tn*5* mutagenesis with λ 467 was as previously described (3).

Nitrogenase assay. One milliliter of stationary-phase cells was placed in 5-ml rubber-stoppered glass vials and extensively flushed with O_2-free argon. Acetylene reduction was performed at 35°C with light provided by incandescent bulbs. One unit is defined as 1 μmol of ethylene produced per h per mg of protein. Protein was determined as described by Hallenbeck et al. (5). Precultures were grown at 35°C aerobically in the dark in RCVB medium, supplemented with 20 mM glutamine when necessary. Photosynthetic cultures were grown in 10-ml screw-cap tubes in a water bath at 35°C and illuminated with 40-W incandescent bulbs at a distance of 20 cm.

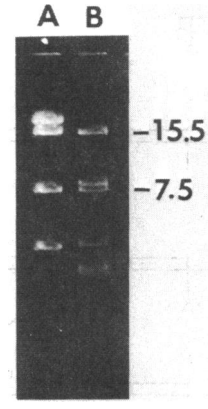

FIG. 1. Agarose gel electrophoresis of Gln^+ cosmid derivatives pCGCS210 (A) and pCGCF212 (B) cut with *Hin*dIII. Mobility of the common 15.5-kbp *R. capsulata* fragment and 7.5-kbp vector fragment in a 0.7% agarose gel are indicated.

RESULTS

Complementation of the Gln^- phenotype of *E. coli* ET8051. A λ lysate containing the cosmid library was used to infect *E. coli* ET8051, in which the entire *glnA ntrB ntrC* operon is deleted (17, 21). The transfectants were plated on LB-Cm-Gln medium, and after overnight growth, they were transferred by replica plating onto M9-Cm plates. After a week at 37°C, eight colonies were observed. When transferred to fresh plates, these colonies gave rise, at a low frequency, to clones which developed in 3 to 4 days. In turn, restreaking of these clones produced some colonies which grew overnight. We analyzed the *Hin*dIII restriction pattern of plasmid DNA isolated from slow-, intermediate-, and fast-growing clones. Two different cosmid plasmids were identified, pCGCS210 and pCGCF212 (Fig. 1), but no differences in the restriction patterns were found when plasmids isolated from the original clones and the more efficient derivatives were compared. Strain ET8051 was transformed with plasmid DNA extracted from slow- and fast-growing clones; the transformants retained the growth properties of the donor cells, indicating that plasmid rather than host mutations are responsible for the improved expression of *glnA*. Also, similar amounts of plasmid DNA were obtained in each case, indicating that no dramatic change in the copy number of the plasmids had occurred (data not shown). Examination of the restriction pattern of the two cosmids (Fig. 1) indicates that the only genomic band they have in common is a 15.5-kbp *Hin*dIII fragment, which therefore must contain the *glnA* gene, since both complement ET8051.

Digestion of the cosmid pCGCF212 with *Hin*dIII, followed by religation, transformation of ET8051, and selection on M9-Cm plates, yielded two plasmids (pJV21 and pJV22) containing the 15.5-kbp band in the two possible orientations (Fig. 2). Restriction of pJV22 with *Bam*HI, followed by religation, transformation of ET8051, and selection on M9-Cm plates, resulted in the isolation of plasmid pJV23, which retains a 9.2-kbp *Bam*HI-*Hin*dIII insert of *R. capsulata* DNA (Fig. 2). Note that by these steps the vector fragment is reduced to 7.2 kbp due to removal of a small *Bam*HI-*Hin*dIII fragment.

To localize the *glnA* gene within the 9.2-kbp fragment, we mutagenized pJV23 with Tn*5*. Mapping of the insertions was carried out by restricting plasmid DNA with *Hin*dIII, *Bam*HI, or both enzymes. Tn*5* has two *Hin*dIII sites symmetrically located within the IS*50* sequences and one *Bam*HI site to the right of the kanamycin resistance (Km^r) gene (18). Several hundred Tn*5* insertions in pJV23 were cut with both *Hin*dIII and *Bam*HI. Insertions in the vector part of pJV23 were identified by the disappearance of the 7.2-kbp *Hin*dIII-*Bam*HI band and were discarded. All remaining plasmids were digested with *Hin*dIII alone. Only one band is common to both the *Hin*dIII and *Hin*dIII-*Bam*HI cuts. This band corresponds to the fragment extending from the *Hin*dIII site of the 9.2-kbp *R. capsulata* insert to the proximal *Hin*dIII site of Tn*5*. Measurement of the size of this fragment allowed accurate mapping of each Tn*5* insertion. Each of 30 insertions which mapped to the 2.0-kbp region, indicated as *glnA* in Fig. 2, resulted in loss of the ability to complement ET8051. None of the more than 100 insertions which mapped to the remaining 7.2 kbp resulted in loss of complementing ability. Since the coding region for glutamine synthetase requires

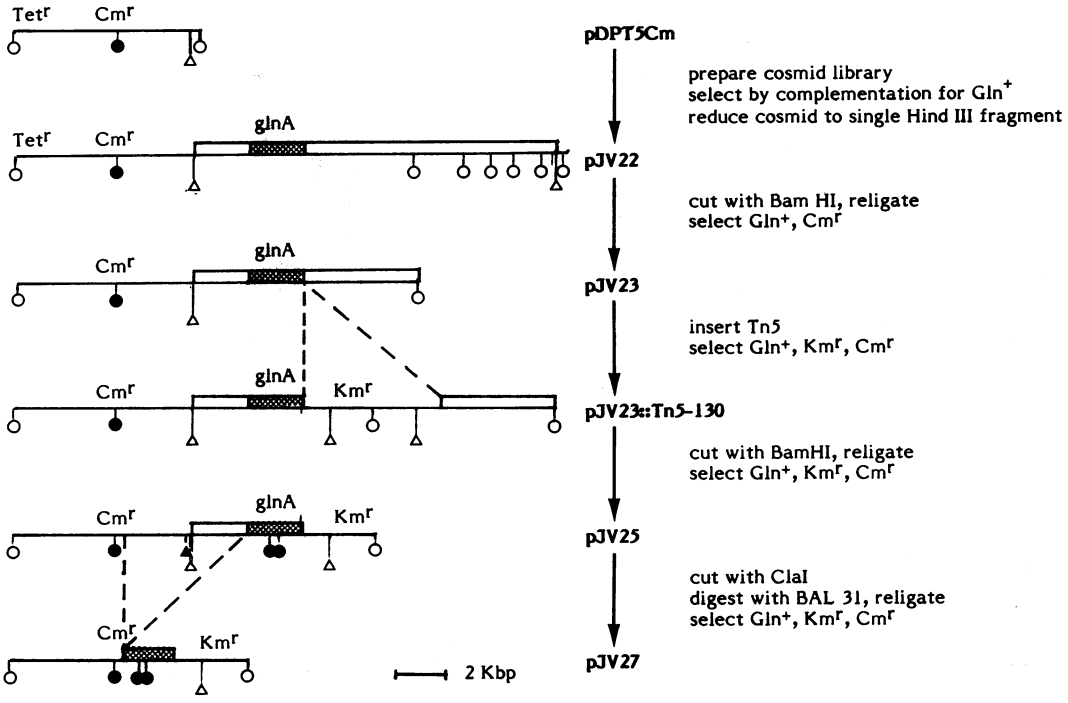

FIG. 2. Restriction map and construction plan leading to pJV27, which contains only the *R. capsulata glnA* gene. Vector DNA is shown as a thin horizontal line; *R. capsulata* DNA is shown as an open box. The *glnA* gene, defined by Tn*5* insertional inactivation, is indicated by the gray area. Symbols: ○, *Bam*HI; ●, *Eco*RI; △, *Hin*dIII; ▲, *Cla*I.

~1.6 kbp, it is unlikely that the 2.0-kbp region required to complement ET8051 codes for more than one protein.

Tn*5* insertion 130 is approximately 50 bp from the right end of the gene, because the next insertion to the left is that distance from 130, but it inactivates *glnA*. The orientation of insertion 130 is shown in Fig. 2. By restricting pJV23::Tn*5* 130 with *Bam*HI and religating, 4.5 kbp of genomic DNA to the right of *glnA* was deleted, yielding pJV25. Further reduction was achieved by cutting pJV25 with *Cla*I and treating with the exonuclease *Bal* 31. After religation, this DNA was used to transform ET8051, selecting for Cm^r and Km^r. Colonies were screened for their ability to grow in the absence of glutamine, and the smallest plasmid containing an active *glnA* gene was selected (pJV27). ET8051(pJV27) forms colonies overnight on M9-Cm plates. Restriction of pJV27 and of several Gln$^-$ plasmids (produced by *Bal* 31 digestion) with *Hin*dIII showed no difference in band sizes in 0.7% agarose gels, indicating that less than 100 bp remains to the left of *glnA* in pJV27.

Having reduced the cloned *R. capsulata* DNA fragment to the minimum size needed to complement an *E. coli glnA* deletion, we wished next to transfer the fragment to *R. capsulata* Gln$^-$ mutants. Such experiments would determine the nature of the *R. capsulata* mutation (e.g., the fragment should be able to complement only *glnA* mutants) and the relationship between the Gln$^-$ and Nif^c phenotypes. For that purpose, it was necessary to construct a more complex vector, because pJV27 could not be mobilized into *R. capsulata*. This was done by opening pJV27 at its unique *Hin*dIII site and inserting the entire fragment into the *Hin*dIII site of pRK292, a derivative of pRK290 with similar replication and mobilization properties (4). The resulting plasmid, pPS27, could be mobilized into *R. capsulata* G29 by conjugation with pRK2013 to provide the necessary *trans* mobilizing function (4). All of the exconjugants obtained were Tet^r, Km^r, and Gln$^+$. This result indicated that the mutation in G29 was either in the *glnA* gene itself or in a regulatory gene needed to turn on the chromosomal *glnA*. (Note that the plasmid-borne *glnA* gene had acquired a mutation to enhanced expression during its selection in *E. coli*.) To distinguish between these two possibilities, the plasmid pPS27 was chased out of G29(pPS27) by conjugation with *E. coli* HB101 (pPH1), selecting for the Gen^r marker on the pPH1 plasmid which is incompatible with pPS27. All of the colonies selected on gentami-

cin plates were Tet^s and Km^s, indicating that pPS27 was lost. Of these colonies, 2% remained Gln^+. Since G29 reverts to Gln^+ at a frequency of 10^{-8}, this 2% represents rescue of the marker in G29 by the fragment cloned in pPS27. Since pPS27 contains only the *glnA* gene, the original G29 mutation must have been in the *glnA* gene.

When the mutant G29 is grown on limiting glutamine with excess ammonia, nitrogenase activity is derepressed (22). We wished to determine whether pPS27 restored ammonia regulation of nitrogenase, as well as glutamine independence, to G29. Controls for these experiments were provided by mobilizing the plasmid vector, pRK292, into both wild-type *R. capsulata* (SB1003) and G29. All three strains, SB1003(pRK292), G29(pRK292), and G29(pPS27), were grown for 16 h in RCVB medium containing 20 mM glutamine. No nitrogenase activity was detected. The cells were washed carefully, suspended in RCVB-ammonia-free medium, and each culture was divided into two. After 90 min of incubation under photosynthetic conditions, each had nitrogenase activity of 5 to 6 units. Ammonium sulfate was then added to one sample of each strain, and incubation was continued for 60 min. Nitrogenase activity in the cultures of SB1003(pRK292) and G29(pPS27) dropped to 0.2 to 0.5 units, whereas that of the G29(pRK292) culture was unchanged.

To determine whether *nif* gene expression was regulated at the level of transcription, total RNA was extracted from each of the cultures described above, labeled with ^{32}P by polynucleotide kinase following brief treatment with alkali, and then hybridized to a blot containing *nif* DNA. The DNA on the blot consisted of two fragments cut from plasmid pNRK76. This plasmid contains a 7.6-kbp *Eco*RI fragment of *R. capsulata* DNA, which includes the structural genes *nifH* and *nifD*, cloned in the *Eco*RI site of pRK290 (P. A. Scolnik et al., manuscript in preparation). Cutting pNRK76 with *Eco*RI provides a 20-kbp vector fragment and a 7.6-kbp *nif* fragment. Since each strain from which RNA was prepared contained pRK292 or the derivative pPS27, hybridization to the vector DNA band serves as a control on the RNA preparation. SB1003 and G29(pPS27) repress *nif* transcription when ammonia is added, but G29 does not (Fig. 3). Thus, the regulation of *nif* gene expression requiring the product of the *glnA* gene occurs at the level of transcription.

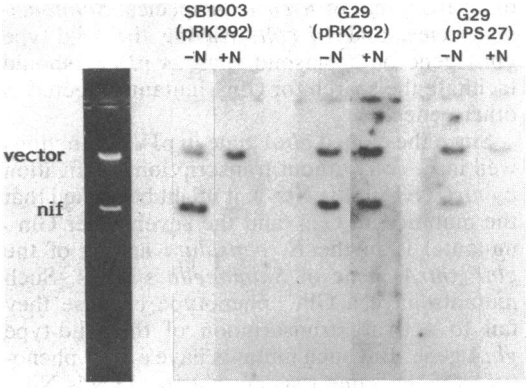

FIG. 3. Hybridization of RNA from cells induced (−N) or repressed (+N) for nitrogenase to cloned *R. capsulata nif* DNA. Cultures of the three strains shown were induced for nitrogenase (−N) and then ammonia was added (+N). Total RNA was extracted 60 min later, labeled in vitro, and hybridized to blots of *Eco*RI-cut pNRK76. The 20-kbp band corresponds to the vector pRK292. The 7.6-kbp band contains the *nifH* and *nifD* genes of *R. capsulata*. Each of six lanes contained 1 μg of cut pNRK76, whose stained pattern is shown on the left. The autoradiograms shown are the result of hybridization with RNA in the six labeled preparations. Vector transcripts are present in all six preparations, whereas *nif* transcripts are present only in induced cells and in G29 (+N), which fails to repress *nif* gene transcription.

DISCUSSION

We have shown that a plasmid containing little more *R. capsulata* DNA than is needed to code for the enzyme glutamine synthetase suffices to restore transcriptional control of *nif* genes in a Gln^- Nif^c *R. capsulata* mutant. The simplest interpretation of this result is that glutamine, or one of its metabolites, is an element of the repressing side of *nif* gene regulation. Although nitrogenase is not repressed by ammonia in strain G29, it is repressed by 20 mM glutamine, as described above relating to the experiment of Fig. 3.

Mutant G29 is representative of all eight Gln^- mutants of *R. capsulata* isolated by Wall and Gest (22). All map between 0.13 and 0.23 map units with GTA-mediated gene transfer, which means that they lie within a 1.2-kbp region of the chromosome. All have the Nif^c phenotype with respect to ammonia. It is likely, therefore, that all eight are *glnA* mutants. This result is interesting for several reasons. In *Klebsiella* species, roughly one-half of the *glnA* mutants are Nif^- (9), presumably because the mutations are polar and affect the *ntrC* gene, which is needed to activate *nif* transcription, downstream. Secondly, in *Salmonella* species, a number of Gln^- mutants are mutated in *glnF* (*ntrA*), a gene whose product is required for activation of both *glnA* and the *nif* genes (14). Thus, although the number of Gln^- mutants of *R. capsulata* examined thus far is still small, the absence of such mutants with Nif^- phenotypes suggests that the nitrogen regulatory circuit of *R. capsulata* may

differ from that of *Klebsiella* species, *Salmonella* species, and *E. coli*. Having the wild-type *glnA* gene on a plasmid such as pJV27 should facilitate the search for Gln$^-$ mutants affected in other genes.

Since the cloned *glnA* gene in pJV27 functions well in *E. coli* without transcriptional activation by *ntrC* (ET8051 is Ntr$^-$), it might be argued that the mutation in G29 (and the seven other Gln$^-$ mutants) is in the *R. capsulata* analog of the *glnF* (*ntrA*) gene of *Salmonella* species. Such mutants have a Gln$^-$ phenotype because they fail to activate transcription of the wild-type *glnA* gene. But such mutants have a Nif$^-$ phenotype for the same reason, whereas G29 is Nif^c. Moreover, pJV27, which contains only *glnA*, rescues the marker in G29 by recombination. Therefore, the G29 mutation must be in *glnA*.

Plasmid pJV27 is useful for studies in which one wishes only to provide glutamine synthetase. For studying the regulation of the *R. capsulata glnA* gene itself, it will be necessary to use other constructions based on the original cosmid clones, because the clone selected in ET8051 for rapid growth in the absence of glutamine has probably accumulated mutations in the promoter region, the ribosome-binding site, or both.

In wild-type *R. capsulata*, addition of ammonia not only represses *nif* gene transcription (Fig. 3), but also leads to inactivation of nitrogenase reductase by covalent modification (10). The inactivation system is probably a fine-tuned, short-term, ATP-saving mechanism which switches off nitrogenase long before the enzyme is diluted by growth after repression. It is also possible that the inactivation system responds to a lower threshold of the combined nitrogen level than is needed for repression. Since G29 has the same nitrogenase activity with or without ammonia, it appears that the switch-off also requires glutamine or a metabolite of glutamine. Hallenbeck et al. have previously suggested that the signal for modification of nitrogenase reductase is not ammonia (5). It is possible, of course, that the signals for enzyme modification and for repression are the same.

Finally, it should be noted that, although the direction of transcription of the *glnA* gene of *R. capsulata* has not been determined in this work, the gene contains two *Eco*RI sites about 300 bp apart. Two *Eco*RI sites with the same spacing are found in the *glnA* genes of *Salmonella* species, *E. coli*, and *Klebsiella* species (3) near the amino terminal. If the *R. capsulata* gene is homologous, transcription will be from left to right in Fig. 2.

ACKNOWLEDGMENTS

We are grateful to Lillie Hsu for mapping all of the Tn*5* insertions in pJV23.

This research was supported by Public Health Service grant GM21823 from the National Institutes of Health, a contract from Chevron Research Corp., and, in part, by a postdoctoral fellowship to P.A.S. from the National Institute of General Medical Sciences.

LITERATURE CITED

1. **Collins, J.** 1979. *Escherichia coli* plasmids packageable *in vitro* in λ bacteriophage particles. Methods Enzymol. **68**:309–325.
2. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics, p. 148–158. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **deBruijn, F. J., and F. M. Ausubel.** 1981. Cloning and transposon Tn5 mutagenesis of the *glnA* region of *Klebsiella pneumoniae*: identification of *glnR*, a gene involved in the regulation of the *nif* and *hut* operons. Mol. Gen. Genet. **183**:289–297.
4. **Ditta, G., S. Stanfield, D. Corbin, and D. Helinski.** 1980. Broad host range cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. **77**:7347–7354.
5. **Hallenbeck, P. C., C. M. Meyer, and P. M. Vignais.** 1982. Nitrogenase from the photosynthetic bacterium *Rhodopseudomonas capsulata*: purification and molecular properties. J. Bacteriol. **149**:708–717.
6. **Ish-Horowitz, D., and J. F. Burke.** 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989–2998.
7. **Johansson, B. C., and H. Gest.** 1976. Inorganic nitrogen assimilation by the photosynthetic bacterium *Rhodopseudomonas capsulata*. J. Bacteriol. **128**:683–688.
8. **Johansson, B. C., and H. Gest.** 1977. Adenylylation/deadenylylation control of the glutamine synthetase of *Rhodopseudomonas capsulata*. Eur. J. Biochem. **81**:365–371.
9. **Leonardo, J. M., and R. B. Goldberg.** 1980. Regulation of nitrogen metabolism in glutamine auxotrophs of *Klebsiella pneumoniae*. J. Bacteriol. **142**:99–110.
10. **Ludden, P. W., and R. H. Burris.** 1979. Removal of an adenine-like molecule during activation of dinitrogenase reductase from *Rhodospirillum rubrum*. Proc. Natl. Acad. Sci. U.S.A. **76**:6201–6205.
11. **Madigan, M. T., J. D. Wall, and H. Gest.** 1979. Dark anaerobic dinitrogen fixation by a photosynthetic microorganism. Science **204**:1429–1430.
12. **Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, and C. O'Connell.** 1978. The isolation of structural genes from libraries of eucaryotic DNA. Cell **15**:687–701.
13. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–559.
14. **Merrick, M. J.** 1982. An new model for nitrogen control. Nature (London) **297**:362–363.
15. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Nordlund, S., J. Erickson, and A. Baltscheffsky.** 1977. Necessity of a membrane component for nitrogenase activity in *Rhodospirillum rubrum*. Biochim. Biophys. Acta **462**:187–195.
17. **Pahel, G., F. R. Bloom, and B. Tyler.** 1979. Deletion mapping of the *polA-metB* region of the *Escherichia coli* chromosome. J. Bacteriol. **138**:653–656.
18. **Rothstein, S. J., R. A. Jorgensen, K. Postle, and W. S. Reznikoff.** 1980. The inverted repeats of Tn5 are functionally different. Cell **19**:795–805.
19. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
20. **Taylor, D. P.** 1982. Plasmid and cosmid vectors bearing chloramphenicol or spectinomycin resistance. Plasmid **8**:100.
21. **Tuli, R., R. Fisher, and R. Haselkorn.** 1982. The *ntr* genes of *Escherichia coli* activate the *hut* and *nif* operons of *Klebsiella pneumoniae*. Gene **19**:109–116.
22. **Wall, J. D., and H. Gest.** 1979. Derepression of nitrogenase activity in glutamine auxotrophs of *Rhodopseudo-*

monas capsulata. J. Bacteriol. **137**:1459–1463.
23. **Weaver, P. F., J. D. Wall, and H. Gest.** 1975. Characterization of *Rhodopseudomonas capsulata*. Arch. Microbiol. **105**:207–216.
24. **Yen, H.-C., and B. Marrs.** 1976. Map of genes for carotenoid and bacteriochlorophyll biosynthesis in *Rhodopseudomonas capsulata*. J. Bacteriol. **126**:619–629.
25. **Zumft, W. G., K. Alef, and S. Mummler.** 1981. Regulation of nitrogenase activity in *Rhodospirillaceae*, p. 190–193. *In* A. H. Gibson and W. E. Newton (ed.), Current perspectives in nitrogen fixation. Elsevier/North-Holland, New York.