JOURNAL OF BACTERIOLOGY, July 1992, p. 4356–4360
0021-9193/92/134356-05$02.00/0

Vol. 174, No. 13

Tyrosine Phosphorylation of a Membrane Protein from *Pseudomonas solanacearum*

MERELEE ATKINSON,* CAITILYN ALLEN, AND LUIS SEQUEIRA

Department of Plant Pathology, University of Wisconsin, 1630 Linden Drive, Madison, Wisconsin 53706

Received 4 November 1991/Accepted 22 April 1992

We have investigated a tyrosine kinase activity from *Pseudomonas solanacearum*, an economically important plant pathogen. In vitro incubation of membrane fractions with [γ-^{32}P]ATP and subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed an 85-kDa phosphoprotein. Phosphorylation of this protein on tyrosine residues was demonstrated by phosphoamino acid analysis of base hydrolysis products and by immunoanalysis of Western blots (immunoblots) with antiphosphotyrosine monoclonal antibody. In vitro incubation of membranes with ATP was not required for recognition by the antibody, indicating that the 85-kDa protein is phosphorylated in vivo. These results demonstrate that membranes from *P. solanacearum* exhibit a tyrosine kinase activity toward an endogenous membrane protein. This bacterium provides an opportunity to study the structure and function of a prokaryotic tyrosine kinase.

Reversible protein phosphorylation plays a central role in the regulation of prokaryotic cellular activities. As in eukaryotes, the majority of phosphoproteins in prokaryotes are modified at serine and threonine residues (5, 23). In addition, a family of environmentally responsive regulatory systems that use phosphorylated histidine and aspartate residues may be unique to prokaryotes (23, 25). Despite the importance of tyrosine phosphorylation in the regulation of eukaryotic cell growth, development, and malignant transformation, its significance in prokaryotes is uncertain. Many attempts to demonstrate phosphotyrosine or tyrosine kinase activity in prokaryotes have been unsuccessful (10, 17, 24), leading to the general view that this modification is absent or rare. Although phosphotyrosine has been detected in protein hydrolysates from *Escherichia*, *Acinetobacter*, *Rhodospirillum*, *Pseudomonas*, *Bacillus*, and *Clostridium* species (4, 6, 7, 14, 16, 17, 21, 29), some of these reports may reflect the generation of phosphotyrosine by acid hydrolysis of proteins containing adenylated or uridylated tyrosine residues (10). This phenomenon cannot, however, account for apparent in vitro tyrosine kinase activities in bacterial extracts incubated with γ- but not α-labelled [^{32}P]ATP (7, 16). In any case, the extent of tyrosine phosphorylation in prokaryotes is uncertain, and, where present, its function is unknown (5).

Pseudomonas solanacearum E. F. Sm. causes wilt diseases of a wide range of crop plants in tropical and warm-temperate zones worldwide (11). Its pathogenicity depends on many factors, including the synthesis and secretion of endoglucanases, polygalacturonases, and extracellular polysaccharide (1–3, 22). The mechanisms by which this bacterium senses the host environment and responds by expressing these and other genes required for pathogenicity are not well understood. As part of a broad effort to identify these regulatory mechanisms, we are studying protein phosphorylation in *P. solanacearum*.

MATERIALS AND METHODS

P. solanacearum K60 (wild-type race 1, biotype 1) was grown at 28°C in Casamino Acids-peptone-glucose broth (12) supplemented with yeast extract (1 g liter^{-1}). Bacteria were harvested at late log phase, washed once in 10 mM Tris-HCl (pH 7.4), and resuspended in the same buffer containing 0.75 M sucrose and 200 μg of lysozyme ml^{-1}. The bacterial suspension was incubated at 0 to 4°C for 30 min, after which 2 volumes of 1.5 mM EDTA containing the protease inhibitors leupeptin (4μM), pepstatin (1 μM), and phenylmethylsulfonyl fluoride (0.5 mM) was slowly added with stirring. The suspension was sonicated three times for 30 s each at 7 A and centrifuged at 4,000 × *g* for 15 min to remove unbroken cells. The supernatant was centrifuged at 83,000 × *g* for 1 h. The supernatant (cytosolic fraction) was saved, and the pellet (total membrane fraction) was resuspended in 10 mM Tris-HCl (pH 7.4). Membranes were further separated into low- and high-density fractions by sucrose gradient centrifugation (9). The low-density fraction was used for all experiments unless indicated otherwise. All cellular fractions were stored at −80°C. Protein was determined by the bicinchoninic acid method (26), and 2-keto-3-deoxyoctonic acid and NADH oxidase were assayed as described previously (19, 20).

Protein kinase assays were started by adding 1 μCi of [γ-^{32}P]ATP (final ATP concentration, 10 μM) to reaction mixtures containing 8 μg of protein in 20 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 50 mM KCl, and 0.025% Triton X-100 in a 20-μl volume. Where washing was indicated, membranes were incubated in the washing buffer at 4°C for 1 h, pelleted by centrifugation at 150,000 × *g* for 1 h, and resuspended in 10 mM Tris buffer prior to assay. After a 15-min (unless otherwise indicated) incubation at 10°C, kinase reactions were stopped by adding concentrated Laemmli (15) sample buffer and heating at 90 to 100°C for 10 min. Proteins were separated on sodium dodecyl sulfate (SDS)–8 or 9% polyacrylamide gels, and dried gels were exposed to X-ray film to detect phosphoproteins. For quantitative analysis of kinase activity, reactions were stopped by spotting reaction mixtures onto GF/F (Whatman) glass fiber disks and then immediately immersing the disks in 10% trichloroacetic acid (TCA). The radioactivities of the washed disks were determined by liquid scintillation counting (13).

Base hydrolysis products of the 85-kDa protein were used for phosphoamino acid analysis. To prepare the hydrolysate,

* Corresponding author.

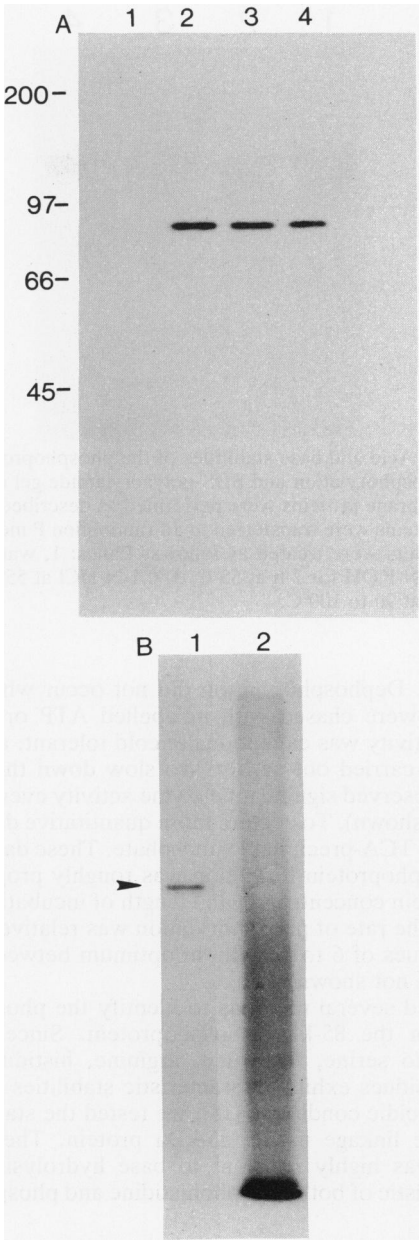

FIG. 1. In vitro phosphorylation in cellular fractions from *P. solanacearum*. (A) Lanes: 1, cytosol; 2, total membrane; 3, low-density membrane fraction; 4, low-density fraction washed with 10 mM Tris-HCl (pH 7.4) containing 1 M KCl and 5 mM EDTA. Cellular fractions were incubated with [γ-^{32}P]ATP, and proteins were resolved by electrophoresis on an SDS-9% polyacrylamide gel. Additional experimental details are given in the text. Molecular size standards (in kilodaltons) are given to the left of the photograph. (B) Lanes: 1, in vitro phosphorylation in low-density membrane fraction by using [γ-^{32}P]ATP; 2, same as lane 1 except that [α-^{32}P]ATP was substituted for [γ-^{32}P]ATP. Phosphorylation reactions were run simultaneously, and proteins were separated by electrophoresis on the same gel.

membranes were phosphorylated in vitro by using [γ-^{32}P] ATP and proteins were separated by SDS-gel electrophoresis. The 85-kDa phosphoprotein was cut from a Western blot (immunoblot) on an Immobilon-P membrane (Millipore Corp. Bedford, Mass.), hydrolyzed in KOH, and neutralized

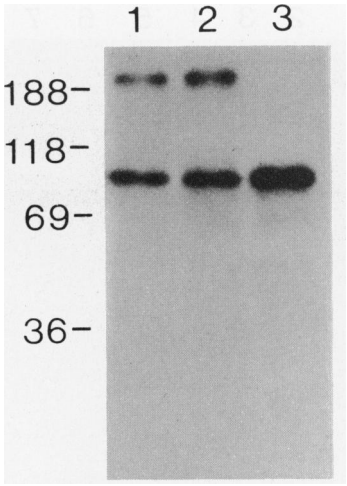

FIG. 2. Effect of solubilization conditions on apparent molecular weight of the phosphoprotein. Conditions for phosphorylation and electrophoresis of membrane proteins were the same as those described for Fig. 1A, lane 3, except that the temperatures for solubilization of membranes in Laemmli sample buffer were as follows: lane 1, 25°C; lane 2, 45°C; lane 3, 90 to 100°C.

as previously described (27). The hydrolysate was combined with phosphoamino acid standards and chromatographed on Silica Gel 60 with ethanol-water (63:37 [vol/vol]) in the first dimension and with chloroform–methanol–17% (wt/wt) ammonia (2:2:1 [vol/vol/vol]) in the second dimension. Phosphoamino acid standards were detected by spraying thin-layer chromatography plates with 0.2% ninhydrin in ethanol and then heating at 75°C.

For immunoanalysis with antiphosphotyrosine antibody, membrane proteins (20 μg of protein per lane) were separated on an SDS–8% polyacrylamide gel and transferred to nitrocellulose. Where indicated, in vitro phosphorylation was carried out as described above except that the 15-min incubation period with radiolabelled ATP (10 μM) was followed by a 1-h incubation period with 100 μM unlabelled ATP. Nitrocellulose membranes were blocked and incubated with ICN PY-20 monoclonal antibody (ICN Biochemicals, Costa Mesa, Calif.) as previously described (28), and antiphosphotyrosine antibodies were detected by enhanced colloidal gold (8). Phosphorylated epidermal growth factor receptor and protein 4.1 were used as positive controls (28).

RESULTS AND DISCUSSION

When total membrane fractions isolated from *P. solanacearum* were incubated with [γ-^{32}P]ATP, an 85-kDa protein was radiolabelled (Fig. 1A). Labelling of this protein reflected protein phosphorylation rather than adenylation or ATP binding since it did not occur in the presence of [α-^{32}P]ATP (Fig. 1B). The cytosolic fraction did not contain this phosphoprotein although it contained a weakly radiolabelled 45-kDa protein. To determine whether the 85-kDa protein was in the inner or outer membrane, we fractionated the total membrane preparation by sucrose gradient centrifugation. This yielded two bands. The specific content of 2-keto-3-deoxyoctonic acid, an outer membrane marker (19), was two to three times greater in the high-density band than in the low-density band. However, the two bands exhibited similar specific activities of NADH oxidase, a cytoplasmic membrane marker (20), and of the tyrosine kinase (data not

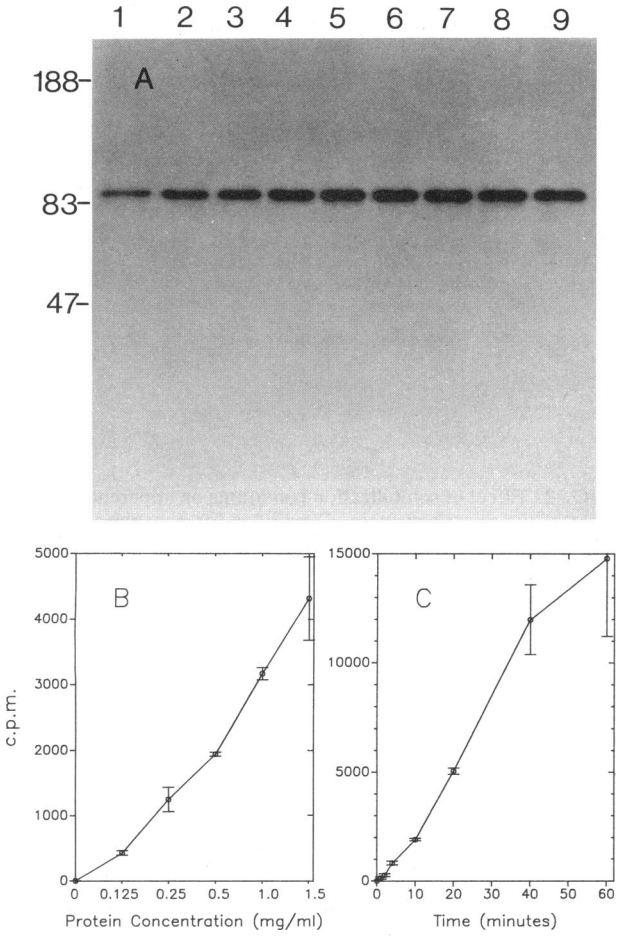

FIG. 3. Kinetics of protein phosphorylation in membranes of *P. solanacearum*. (A) Autoradiogram of phosphoprotein after electrophoresis. Lanes: 1 to 7, reactions were stopped after 0.5, 1, 2, 4, 16, 32, and 60 min, respectively; 8 and 9, 32-min reaction followed by a 30-min chase with 1 mM unlabelled ATP and ADP, respectively. Phosphorylation and electrophoresis conditions were the same as those described for Fig. 1A, lane 3, except that proteins were separated on an 8% gel. (B) Dependence of phosphoprotein (TCA-precipitable ^{32}P) formation on protein concentration. (C) Time course of phosphoprotein formation at 0.4 mg of protein per ml. For panels B and C, in vitro phosphorylation reactions were stopped by spotting onto glass fiber filters and immersion in 10% TCA. Data are averages of triplicate determinations ± 1 standard deviation.

shown). These data suggest that the kinase and its substrate may be associated with the inner membrane. However, determination of their membrane localization will require additional investigation. Washing membranes with 1 M KCl and 5 mM EDTA did not eliminate phosphorylation activity, suggesting that the kinase and its substrate are either integral membrane proteins or tightly membrane associated.

When the reaction mixture was solubilized in sample buffer at 25 to 45°C instead of 100°C, an additional phosphoprotein of about 190 kDa was revealed by electrophoresis (Fig. 2). Since the presence of this band was accompanied by reduced intensity of the 85 kDa band, the phosphoprotein may exist as a multimer in vivo. These phosphoproteins must be present in small quantities since silver staining of gels did not resolve corresponding protein bands.

Phosphorylation of the 85-kDa protein increased with time

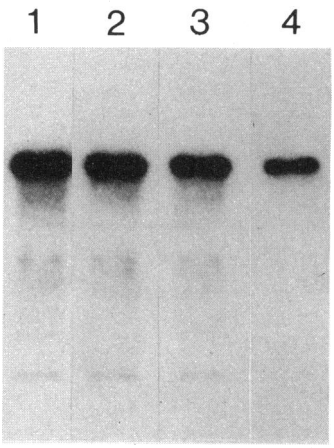

FIG. 4. Acid and base stabilities of the phosphoprotein. In vitro protein phosphorylation and SDS-polyacrylamide gel electrophoresis of membrane proteins were performed as described for Fig. 1A, lane 3. Proteins were transferred to an Immobilon P membrane, and identical blots were treated as follows. Lanes: 1, water for 2 h at 25°C; 2, 1 N KOH for 2 h at 55°C; 3, 0.1 N HCl at 55°C for 2 h; 4, 16% TCA at 90 to 100°C.

(Fig. 3A). Dephosphorylation did not occur when reaction mixtures were chased with unlabelled ATP or ADP. The kinase activity was exceptionally cold tolerant: assays were generally carried out at 10°C (to slow down the reaction), and we observed significant enzyme activity even at 0 to 4°C (data not shown). To acquire more quantitative data, we also measured TCA-precipitable phosphate. These data indicated that phosphoprotein formation was roughly proportional to both protein concentration and length of incubation (Fig. 3B and C). The rate of phosphorylation was relatively constant at pH values of 6 to 8, with an optimum between 7.25 and 7.50 (data not shown).

We used several methods to identify the phosphorylated residue in the 85-kDa phosphoprotein. Since phosphate linkages to serine, threonine, arginine, histidine, and tyrosine residues exhibit characteristic stabilities under alkaline and acidic conditions (18), we tested the stability of the phosphate linkage in the 85-kDa protein. The phosphate linkage was highly resistant to base hydrolysis, which is characteristic of both phosphohistidine and phosphotyrosine

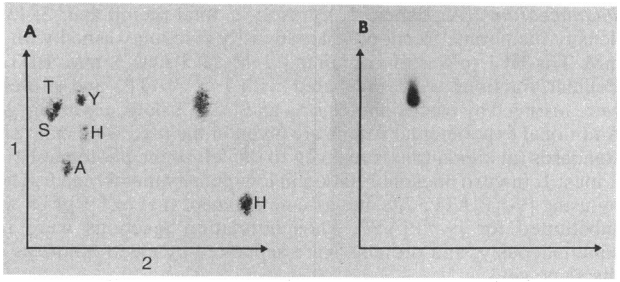

FIG. 5. Phosphoamino acid analysis of the 85-kDa phosphoprotein. (A) Ninhydrin-reactive spots after two-dimensional thin-layer chromatography of ^{32}P-phosphoprotein hydrolysis products and authentic phosphoamino acid standards. Abbreviations: A, phosphoarginine; H, phosphohistidine (phosphorylated at N1 or N3); S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. The unlabelled spot represents unidentified products of protein hydrolysis. (B) Autoradiogram of the thin-layer chromatography plate.

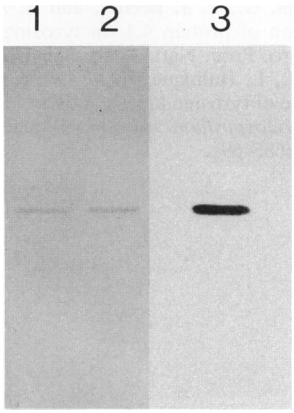

FIG. 6. Immunodetection of phosphotyrosine-containing proteins in the low-density membrane fraction by antiphosphotyrosine immunoblot. Lanes: 1, *P. solanacearum* membrane proteins; 2, same as lane 1 except that membranes were preincubated with 100 μM ATP containing 1 μCi of [γ-^{32}P]ATP before electrophoresis; 3, autoradiogram of Western blot.

residues (Fig. 4). Since phosphohistidine is rapidly destroyed by 0.1 N HCl at 55°C (25), the acid resistance of the 85-kDa phosphoprotein is most consistent with the presence of a phosphotyrosine residue. These experiments also demonstrated two weakly radiolabelled protein bands with acid and base stabilities similar to those of the 85-kDa protein. These proteins may be degradation products of the 85-kDa protein or unrelated proteins that are weakly phosphorylated.

The identity of the phosphorylated residue was determined by two-dimensional thin-layer chromatography of the products of base hydrolysis of the 85-kDa phosphoprotein. A ^{32}P-labelled hydrolysis product comigrated with authentic phosphotyrosine and was well resolved from authentic phosphoarginine, phosphoserine, phosphothreonine, and phosphohistidine (Fig. 5). The presence of phosphotyrosine in the 85-kDa protein was confirmed by immunoblot analysis with antiphosphotyrosine antibody (Fig. 6). Detection of this protein by antibody did not require prior incubation of membranes with ATP, indicating that the protein had been phosphorylated in vivo. In vitro incubation of membranes with 0.1 mM ATP for 1 h only slightly increased recognition by the antibody.

In summary, our results demonstrate in vitro and in vivo tyrosine phosphorylation of an 85-kDa protein in membranes from *P. solanacearum*. It remains to be determined whether the kinase activity and the phosphorylation site(s) are located in one or two proteins and whether these proteins function in the pathogenicity of this bacterium on its plant hosts.

ACKNOWLEDGMENTS

We thank B. Ankenbauer, J. Cangelosi, and E. Nester for their generous gift of phosphohistidine standards and Krishna Ella and Gosukonda Subrahmanyam for phosphorylated epidermal growth factor receptor and protein 4.1.

This work was supported by grant DCB-8917582 from the National Science Foundation.

REFERENCES

1. **Allen, C., M. Atkinson, and L. Sequeira.** 1990. Characterization of a putative regulatory locus required for endopolygalacturonase production in *Pseudomonas solanacearum*. Phytopathology **80**:982.
2. **Allen, C., Y. Huang, and L. Sequeira.** 1991. Cloning of genes affecting polygalacturonase production in *Pseudomonas solanacearum*. Mol. Plant Microbe Interact. **4**:147–154.
3. **Cook, D., and L. Sequeira.** 1991. Genetic and biochemical characterization of a *Pseudomonas solanacearum* gene cluster required for extracellular polysaccharide production and for virulence. J. Bacteriol. **173**:1654–1662.
4. **Cortay, J. C., C. Rieul, B. Duclos, and A. J. Cozzone.** 1986. Characterization of the phosphoproteins of *Escherichia coli* cells by electrophoretic analysis. Eur. J. Biochem. **159**:227–237.
5. **Cozzone, A. J.** 1988. Protein phosphorylation in prokaryotes. Annu. Rev. Microbiol. **42**:97–125.
6. **Dadssi, M., and A. J. Cozzone.** 1990. Occurrence of protein phosphorylation in various bacterial species. Int. J. Biochem. **22**:493–499.
7. **Dadssi, M., and A. J. Cozzone.** 1990. Evidence of protein-tyrosine kinase activity in the bacterium *Acinetobacter calcoaceticus*. J. Biol. Chem. **265**:20996–20999.
8. **Danscher, G., and J. O. R. Norgaard.** 1983. Light microscopic visualization of colloidal gold on resin-embedded tissue. J. Histochem. Cytochem. **31**:1394–1398.
9. **Dos Santos, R. M. D. B., and J. C. Dianese.** 1985. Comparative membrane characterization of *Xanthomonas campestris* pv. *cassavae* and *X. campestris* pv. *manihotis*. Phytopathology **75**:581–587.
10. **Foster, R., J. Thorner, and G. S. Martin.** 1989. Nucleotidylation, not phosphorylation, is the major source of the phosphotyrosine detected in enteric bacteria. J. Bacteriol. **171**:272–279.
11. **Hayward, A. C.** 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. **29**:65–87.
12. **Hendrick, C. A., and L. Sequeira.** 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. Appl. Environ. Microbiol. **48**:94–101.
13. **Hess, J. F., K. Oosawa, P. Matsumura, and M. I. Simon.** 1987. Protein phosphorylation is involved in bacterial chemotaxis. Proc. Natl. Acad. Sci. USA **84**:7609–7613.
14. **Kelly-Wintenberg, K., T. Anderson, and T. C. Montie.** 1990. Phosphorylated tyrosine in the flagellum filament protein of *Pseudomonas aeruginosa*. J. Bacteriol. **172**:5135–5139.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Londesborough, J.** 1986. Phosphorylation of proteins in *Clostridium thermohydrosulfuricum*. J. Bacteriol. **165**:595–601.
17. **Manai, M., and A. J. Cozzone.** 1982. Endogenous protein phosphorylation in *Escherichia coli* extracts. Biochem. Biophys. Res. Commun. **107**:981–988.
18. **Martensen, T.** 1984. Chemical properties, isolation, and analysis of O-phosphates in proteins. Methods Enzymol. **107**:3–23.
19. **Osborn, M. J.** 1963. Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **68**:499–506.
20. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
21. **Pairoba, C. F., and R. H. Vallejos.** 1989. Protein phosphorylation in purple photosynthetic bacteria. Biochimie **71**:1039–1041.
22. **Roberts, D. P., T. P. Denny, and M. A. Schell.** 1988. Cloning of the *egl* gene of *Pseudomonas solanacearum* and analysis of its role in phytopathogenicity. J. Bacteriol. **170**:1445–1451.
23. **Saier, M. H., L.-F. Wu, and J. Reizer.** 1990. Regulation of bacterial physiological processes by three types of protein phosphorylation systems. Trends Biochem. Sci. **15**:391.
24. **Schieven, G., J. Thorner, and G. S. Martin.** 1986. Protein-tyrosine kinase activity in *Saccharomyces cereviseae*. Science **231**:390–393.
25. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein

phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:**450–490.
26. **Smith, R. A.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
27. **Smith, R. A., R. M. Halpern, B. B. Bruegger, A. K. Dunlap, and O. Fricke.** 1978. Chromosomal protein phosphorylation on basic amino acids. Methods Cell Biol. **19:**153–159.
28. **Subrahmanyam, G., P. J. Bertics, and R. A. Anderson.** 1991. Phosphorylation of protein 4.1 on tyrosine 418 modulates its function in vitro. Proc. Natl. Acad. Sci. USA **88:**5222–5226.
29. **Vallejos, R. H., L. Huluigue, H. A. Lucero, and M. Torruella.** 1985. Evidence of tyrosine kinase activity in the photosynthetic bacterium *Rhodospirillum rubrum*. Biochem. Biophys. Res. Commun. **126:**685–691.