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Tyrosine Phosphate in a- and b-Type Flagellins of *Pseudomonas aeruginosa*

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Both a- and b-type purified flagellins from a number of *Pseudomonas aeruginosa* strains grown in radiolabeled phosphate were shown to be phosphorylated. Analysis of partial acid-hydrolyzed flagellar filaments revealed that $^{32}P_i$ was in phosphotyrosine. Three ^{32}P-phosphopeptides apparently are common to a- and b-type flagellins, but a fourth peptide was found only in b-type hydrolysates. *P. aeruginosa* PAK flagellin, containing only two tyrosines, both in the variable region, was readily labeled and gave the same peptide pattern as flagellins containing additional tyrosines. Data showing that a- and b-type flagellins gave positive immunoblots with antiphosphotyrosine monoclonal antibody and that release of P_i by alkaline phosphatase occurred indicated that unmodified tyrosine phosphate exists in flagellin.

Pseudomonas aeruginosa exhibits a single polar flagellum which contains a single type of repeating filament subunit (flagellin) characteristic of the individual strain. The flagellins have been designated type b (53 kDa) or type a (45 to 52 kDa) on the basis of molecular mass (1) and predominance of a cross-reacting antigen (2, 19). A primary antigenic site presumably resides in the variable region of the subunit protein, as is the case with most flagellins (19, 19a, 22, 26). The a-type flagellins are more heterogeneous than b-type flagellins, exhibit a predominant cross-reacting antigen, and may show some minor subantigen reactivity (1, 2, 19).

It has been known for some time that certain surface protein structures, such as flagella (12–14, 16) and pili (20, 25), are posttranslationally modified. The biological significance of these modifications remains unknown. Although thought to be of little significance until recently, posttranslational phosphorylation in bacteria is now recognized as playing a vital role in individual-enzyme regulation (11, 21), as well as in global control mechanisms (5, 9, 23, 24).

Tyrosine phosphorylation, although somewhat less common than modifications of serine and threonine, contributes to specific and highly important regulatory events in eukaryotic cells (4, 6). Tyrosine phosphorylation in prokaryotes remains controversial (10); however, evidence demonstrating the presence of tyrosine kinases and phosphorylation of several bacterial proteins at tyrosine has now accumulated (3, 8, 17).

In an initial report, we demonstrated the presence of phosphotyrosine (P-Tyr) in b-type flagellin (15). In research presented here, we have extended these findings (i) to show that P-Tyr is present in a-type flagellins, (ii) to locate and compare distribution of phosphate in phosphopeptides of both flagellin types, and (iii) to substantiate the presence of unmodified tyrosine phosphate. Several lines of evidence presented support the conclusion that *P. aeruginosa* native flagellar filaments, as well as isolated flagellin, contain unmodified tyrosine phosphate.

Isolation and purification of ^{32}P-labeled a- and b-type flagellins. *P. aeruginosa* strains used in this study were common laboratory strains. The b-type strains used included M-2 and PAO1 (both containing seven tyrosines; M_r, 53,000). The a-type strains used were PAK (M_r, 43,000; two Tyr), 170018 (M_r, 45,000; seven Tyr), 5940 (M_r 47,000; five Tyr), and 2993 (M_r, 50,000; two Tyr). Bacteria were grown in modified sodium succinate mineral salts medium (MSM) (1, 15). To achieve maximum radiolabeling, the total phosphate concentration was decreased from that in MSM to approximately twofold above growth-limiting levels (0.62 mM phosphate). [^{32}P]phosphoric acid was added at a concentration of either 5 or 10 μCi/ml, and the cells were grown at 30°C for 22 h. Labeled flagellar preparations from the a- and b-type cells were isolated by mechanical shearing and differential centrifugation and were further purified by several steps of molecular sieving, as previously described (15). Since cells were not broken, flagellar purification procedures were designed primarily to remove excessive lipopolysaccharide (LPS).

Labeling of flagellar protein was then demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The presence of phosphate-labeled b-type flagellins has previously been shown (15). A purified preparation of a representative a-type flagellin (PAK) was separated by electrophoresis in 10% homogeneous gels and stained with Coomassie brilliant blue (Fig. 1A). As expected, the M_r of strain PAK (Fig. 1A, lane 1) was 43,000 to 45,000 (26). The autoradiogram shown in Fig. 1B showed radioactivity associated with a-type flagellin, as previously shown with b-type flagellin. Similar results were seen with a-type strains 170018 and 2993 (data not shown). The additional unresolved labeled bands above the flagellin band (Fig. 1B), which were not visible in the Coomassie blue-stained gel (Fig. 1A), corresponded to phosphate in LPS, which is more difficult to remove from a-type flagellar preparations.

Immunoblotting with antiphosphotyrosine monoclonal antibody. To obviate problems associated with determining the form of ^{32}P existing in flagellin, a direct measure of unmodified P-Tyr was employed. Flagellar protein blotted on nitrocellulose was incubated with antiphosphotyrosine monoclonal antibody (MAb). An improved immunoblotting assay coupled to chemiluminescent detection was employed to increase sensitivity. By this method, the presence of unmodified P-Tyr was confirmed for both flagellin types (Fig. 2). PAK (type a) and PAO1 (type b) both reacted positively with the antiphosphotyrosine MAb (Fig. 2B, lanes 2 and 3, respectively). No reactivity of bovine serum albumin (BSA)

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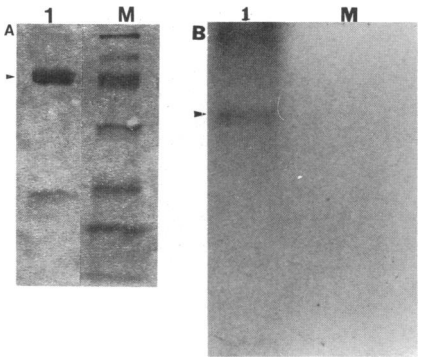

FIG. 1. Demonstration of ^{32}P labeling in the flagellar filament. Purified PAK a-type flagellin (10 μg) was separated by electrophoresis in a 10% homogeneous polyacrylamide gel. (A) Coomassie blue-stained gel; (B) autoradiogram of the gel. Lanes 1, PAK; lanes M, molecular mass standards of 97, 66, 45, 31, 22, and 14 kDa. The location of the a-type flagellin is indicated (arrowhead) for each panel. The lower band in lane 1 of panel A probably represents the 15-kDa pilin protein, which is not phosphorylated.

(Fig. 2B, lane 1) or any other protein was seen. A replica assay in which the primary antibody was preincubated with 1 mM P-Tyr prior to use in the blotting experiment was performed. As shown in Fig. 2C, an obvious decrease in reactivity was associated with the addition of the preincubation step. The MAb no longer reacted with PAK flagellin, and its recognition of PAO1 flagellin was significantly reduced. These immunoblotting results were confirmed in experiments substituting ^{125}I-conjugated secondary antibody.

Comparative phosphopeptide analysis of flagellins in partial acid hydrolysates. The primary objective of these experiments was to demonstrate that the radioactivity was associated with phosphotyrosine and not with phosphoserine or phosphothreonine (15). To demonstrate the presence of phosphoamino acids in proteins, the standard procedure involves partial acid hydrolysis (1 h) to avoid destruction of the phosphoester bond (7). This approach not only facilitated identification of P-Tyr, since degradation was reduced, but also allowed comparison of phosphotyrosine-containing peptides which were incompletely hydrolyzed in both flagellar types (15). The partial acid hydrolysates were examined by thin-layer electrophoresis (TLE) in a pH 3.5 buffer. As shown by the autoradiograms of the electropherograms in Fig. 3, a substantial portion of the total radioactivity migrated to a spot corresponding to P-Tyr in both a-type flagellins and b-type flagellins. No other phosphoamino acids were observed. The remaining label in both types was associated with ninhydrin-positive phosphopeptides, which were numbered relative to the distance (in centimeters) migrated from the origin (P-2, P-3, and P-4). An interesting finding was that peptide P-8, observed with the b-type hydrolysates examined (15), was not present in any of the a-type strains (Fig. 3). Although the hydrolysate of PAK flagellin was not shown in Fig. 3, upon TLE analysis it displayed the identical a-type peptide pattern. This variation reflects some expected sequence differences between the b-type and a-type variable regions. However, although there is variable tyrosine content among the flagellins, these data suggest that a certain conserved tyrosine-containing sequence(s) is phosphorylated.

For quantitative estimations of the distribution of radiolabel in various peptides, chromatograms were sectioned and the radioactivities in all fractions were compared. In the b-type hydrolysates, 24 to 34% of the label was associated with P-Tyr, while 37 to 49% was associated with P-Tyr in the a-type strains tested. The remaining label was in the peptides.

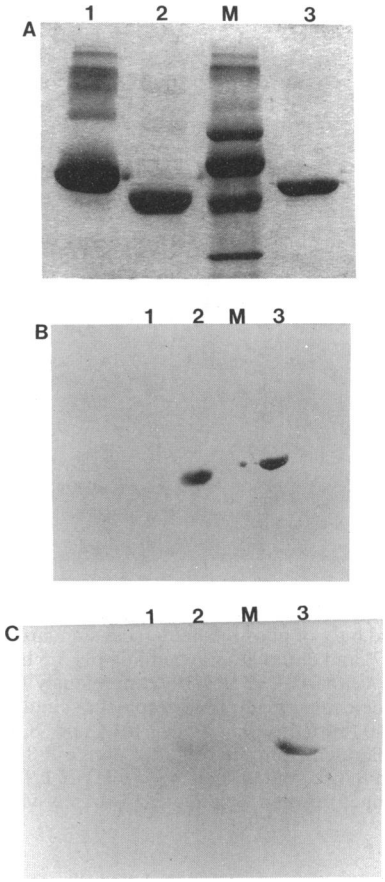

FIG. 2. Immunoblotting with antiphosphotyrosine MAb. After electrophoresis of 10 μg of each of the samples, the proteins were transferred to nitrocellulose. After being blocked with a 3% BSA-Tris-buffered saline solution (TBS) (10 mM Tris-Cl [pH 7.4], 0.9% NaCl), the blots were incubated with antiphosphotyrosine MAb (Upstate Biotechnology, Inc.) at 1:200 (5 μg/ml) for 2 h at 37°C. Goat anti-mouse immunoglobulin G conjugated to peroxidase (Bio-Rad) was added at a dilution of 1:5,000 (0.2 μg/ml), and the blots were incubated for 1.5 h at 37°C. Enhanced chemiluminescence reagents (Amersham) were added, and the blots were exposed to ECL-Hyperfilm (Amersham) according to the manufacturer's instructions. (A) Coomassie blue-stained gel; (B) chemiluminescence-exposed film ("lumogram") of immunoblot from gel; (C) lumogram of immunoblot in which antiphosphotyrosine MAb was preincubated with 1 mM P-Tyr prior to use in the blotting experiment. Lanes 1, BSA; lanes 2, PAK (a-type flagellin); lanes 3, PAO1 (b-type flagellin); lanes M, molecular mass markers of 97, 66, 45, and 31 kDa.

To further demonstrate that labeled spots other than P-Tyr present on autoradiograms were peptides, the spots were eluted and subjected to pronase digestion. The counts per minute for peptides P-2, P-3, and P-4 were decreased upon treatment with pronase by 41, 87, and 52%, respectively. These data showed that these spots, while not susceptible to the partial acid hydrolysis conditions employed, were digested by pronase and, therefore, were peptide in nature.

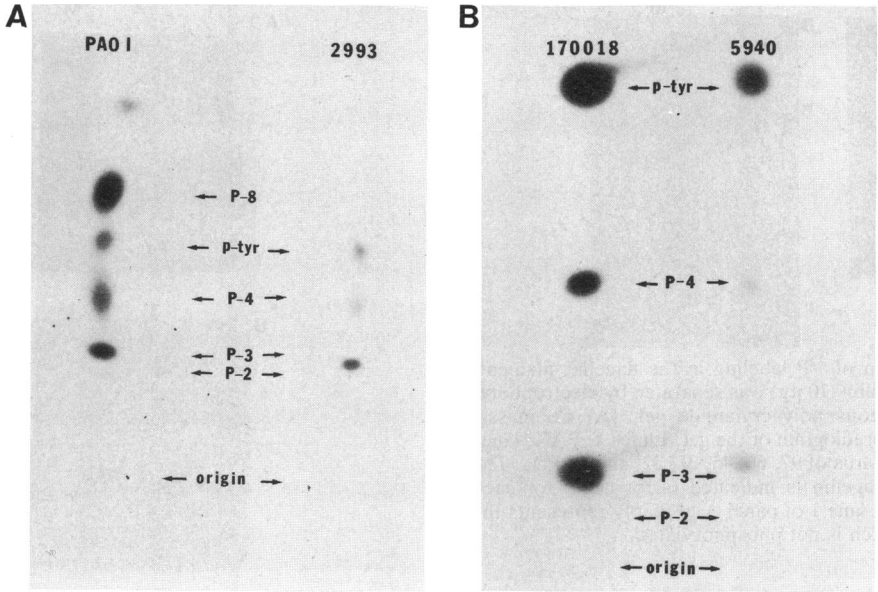

FIG. 3. Autoradiogram of TLE of partial-acid-hydrolyzed flagellar filament protein (types a and b). Approximately 20 μg of labeled hydrolysate and 6 μg of phosphoamino acid standards were spotted onto thin-layer plastic-back cellulose for electrophoresis as described by Cooper et al. (7) and chromatographed in a pH 3.5 buffer (pyridine-glacial acetic acid-distilled H_2O [10:100:1,890, vol/vol/vol]) at 18 mA and 300 V for 2 to 4 h at 4°C, as described previously (15). Standard amino acids were detected with ninhydrin, and labeled amino acids and peptides were detected by autoradiography or scintillation counting of sectioned electropherograms. (A) PAO1 (type b) and 2993 (type a); (B) 170018 and 5940 (both are type a). P-2 to -4 and -8, partial acid hydrolysis-resistant phosphopeptides (the numbers represent distances [in centimeters] migrated from the origin). Peptide P-8 routinely migrated close to phosphoserine and phosphothreonine in the pH 3.5 buffer; however, when the solvent was changed to a pH 1.9 buffer, a different migration pattern, which clearly resolved P-8 from phosphoserine and phosphothreonine, resulted. Peptide P-2 is barely visible in the autoradiograms, but its presence was confirmed by scintillation counting.

Phosphatase treatment of labeled flagella and tryptic peptides. Phosphotyrosine is not a common *O*-phosphate modification in prokaryotic systems except when attached by a phosphodiester bond to a nucleoside (10). Thus, it was important to establish whether the P-Tyr modification on the flagellin protein of *P. aeruginosa* was generated by a phosphorylation reaction or a nucleotidylation reaction. Phosphoprotein isolated from cells grown in the presence of ^{32}P could contain phosphodiester linkages (e.g., nucleotidylation) to tyrosine, and partial acid hydrolysis of these phosphodiester linkages could produce *O*-phosphates (18).

To demonstrate the presence of *O*-phosphomonoester linkages, the labeled flagellar filaments were treated with two types of phosphatase enzymes (18). Calf intestinal alkaline phosphatase (CIP) and bacterial alkaline phosphatase (Sigma) were used to treat labeled whole flagella and trypsinized samples of labeled flagella. For trypsin digests, purified lyophilized flagellin protein was suspended in 0.1 M Tris-Cl (pH 8.0) containing 0.1% deoxycholate (5:1, wt/vol). After a 30-min incubation at 70°C, trypsin was added (trypsin/protein ratio, 1:50). The suspension was incubated for 30 min at 37°C. The reaction was terminated by heating at 100°C, and the trypsinized flagellar peptides were lyophilized. Tryptic digests of labeled flagella (20 μg each) were treated with 0.06 to 0.1 U of CIP and incubated for 30 to 60 min at 37°C. Labeled flagella (40 μg) were treated with 0.3 U of CIP at 37°C for 40 min. The samples were spotted on cellulose paper for TLE separation. An increased enzymatic release of $^{32}P_i$, which migrates farthest on the chromatogram, was readily observed in all cases. Representative autoradiograms of TLE analyses of CIP-treated flagella and peptides are shown in Fig. 4. Large amounts of $^{32}P_i$ were released from M-2 (b-type) flagella by the phosphatase in contrast to control preparations (untreated), for which almost no $^{32}P_i$ was detected (Fig. 4A). In Fig. 4B are shown results for tryptic peptides from M2 flagella treated with CIP. The labeled peptides of M2 flagellin treated with CIP released considerably more $^{32}P_i$ than untreated M2 peptides. After

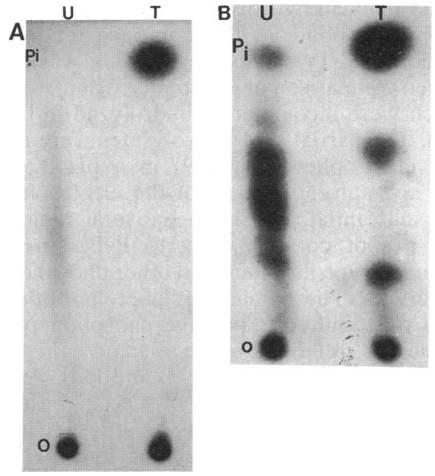

FIG. 4. TLE analysis of phosphatase-treated b-type flagella and tryptic peptides. To demonstrate the presence of an *O*-phosphomonoester linkage, samples of nondenatured M2 flagella and tryptic peptides were treated (T) with CIP and compared with untreated (U) samples by TLE analysis. (A) Nondenatured flagella; (B) tryptic peptides. P_i, free $^{32}P_i$; O, origin. The presence of multiple tryptic peptides is attributed to enzyme-resistant bonds. Longer incubation times (up to 24 h) did not result in further digestion.

CIP treatment, a 2.5-fold decrease in peptide label and a 3.5-fold increase in P_i label released from flagella over the untreated control sample were determined by sectioning and counting the TLE. Treatment with bacterial alkaline phosphatase produced similar results, as did other a-type and b-type CIP- or bacterial alkaline phosphatase-treated strains (data not shown). Since these proteins and peptides were not modified by acid hydrolysis, the release of P_i by phosphatase further substantiates the presence of tyrosine phosphate in native flagella.

Enzymatic assays using bacterial supernatants showed that phosphatase or phosphodiesterase was detected only in late-exponential-phase cells but tyrosine phosphate was found to the same extent in both early- and late-exponential-phase cells. This lack of correlation indicates that the presence of tyrosine phosphate is not the result of an extracellular process which occurs because of a secreted enzyme.

The presence of phosphotyrosine in both antigenic types of flagellin has been confirmed. In agreement with an earlier report investigating b-type flagellin (15), a-type flagellin also exhibits P-Tyr. No phosphothreonine or phosphoserine was detected. The evidence that antiphosphotyrosine MAb reacted with purified flagellin when several methods were used, together with data that show susceptibility of the phosphate bridge to two different alkaline phosphatases, supports the conclusion that phosphate exists as an unmodified monophosphoester in the flagellar filament protein.

Upon completing this work and some preliminary work in which we detected tyrosine phosphokinase activity (23a), we noted the recent report by Atkinson et al. (3) showing tyrosine phosphorylation of a *Pseudomonas solanacearum* 85-kDa membrane protein of unknown function associated with membrane-bound tyrosine kinase activity. Of direct import for our research was the observation that a 45-kDa protein of the cytoplasm was also weakly phosphorylated. We believe that the latter may be *P. solanacearum* flagellin and that tyrosine kinase activity associated with flagellin modification is probably common to all *Pseudomonas* species. We have obtained evidence indicating the presence of enzymatic phosphorylation of flagellin in vitro via a membrane-bound tyrosine kinase in *P. aeruginosa* (23a). The relationship between the membrane-bound kinase and flagellin phosphorylation and the biological significance of this modification remain to be determined.

REFERENCES

1. **Allison, J. S., M. Dawson, D. Drake, and T. C. Montie.** 1985. Electrophoretic separation and molecular weight characterization of *Pseudomonas aeruginosa* H-antigen flagellins. Infect. Immun. **49:**770–774.
2. **Ansorg, R.** 1978. Flagellum specific H antigenic schema of *Pseudomonas aeruginosa*. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **242:**228–238.
3. **Atkinson, M., C. Allen, and L. Sequeira.** 1992. Tyrosine phosphorylation of a membrane protein from *Pseudomonas solanacearum*. J. Bacteriol. **174:**4356–4360.
4. **Blacksheer, P. J., A. C. Nairn, and J. F. Kuo.** 1988. Protein kinases 1988: a current perspective. FASEB J. **2:**2957–2969.
5. **Bourret, R. B., K. A. Borkovich, and M. I. Simon.** 1991. Signal transduction pathways involving protein phosphorylation in prokaryotes. Annu. Rev. Biochem. **60:**401–441.
6. **Cadena, D. L., and G. N. Gill.** 1992. Receptor tyrosine kinases. FASEB J. **6:**2332–2337.
7. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantitation of phosphotyrosine in proteins. Methods Enzymol. **99:**387–402.
8. **Dadssi, M., and A. J. Cozzone.** 1990. Evidence of protein-tyrosine kinase activity in the bacterium *Acinetobacter calcoaceticus*. J. Biol. Chem. **265:**20996–20999.
9. **Deretic, V., W. M. Konyecshi, D. C. Mohr, D. W. Martin, and N. S. Hibler.** 1989. Common denominators of promoter control in *Pseudomonas* and other bacteria. Bio/Technology **7:**1249–1254.
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. **Hurley, J. H., A. M. Dean, J. Stohl, D. E. Koshland, Jr., and R. M. Stround.** 1990. Regulation of an enzyme by phosphorylation at the active site. Science **249:**1012–1016.
12. **Jones, C. J., and S. I. Aizawa.** 1991. The bacterial flagellum and flagellar motor: structure, assembly and function. Adv. Microb. Physiol. **32:**109–172.
13. **Joys, T.** 1988. The flagellar filament protein. Can. J. Microbiol. **34:**452–458.
14. **Kanto, S., H. Okino, S. I. Aizawa, and S. Yamaguchi.** 1991. Amino acids responsible for flagellar shape are distributed in terminal regions of flagellin. J. Mol. Biol. **219:**471–480.
15. **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.
16. **Logan, S. M., T. J. Trust, and P. Guerry.** 1989. Evidence for posttranslational modification and gene duplication of *Campylobacter* flagellin. J. Bacteriol. **171:**3031–3038.
17. **Londesborough, J.** 1986. Phosphorylation of proteins in *Clostridium thermohydrosulfuricum*. J. Bacteriol. **165:**595–601.
18. **Martenson, T. M.** 1984. Chemical properties, isolation, and analysis of O-phosphates in proteins. Methods Enzymol. **107:**3–23.
19. **Montie, T. C., and T. R. Anderson.** 1988. Enzyme-linked immunosorbent assay for detection of *Pseudomonas aeruginosa* H (flagellar) antigen. Eur. J. Clin. Microbiol. Infect. Dis. **7:**256–260.

19a. **Montie, T. C., F. Dorner, J. C. McDonel, and A. Mitterer.** May 1989. U.S. patent 9:831,121.

20. **Paranchych, W., and L. S. Frost.** 1988. The physiology and biochemistry of pili. Adv. Microb. Physiol. **29:**53–114.
21. **Robertson, E. F., and H. C. Reeves.** 1989. Phosphorylation of isocitrate lyase in *Escherichia coli*. Biochimie **71:**1065–1070.
22. **Rotering, H., and F. Dorner.** 1989. Studies on a *Pseudomonas aeruginosa* flagellum vaccine. Antibiot. Chemother. (Basel) **42:**218–228.
23. **Roychoudhury, S., K. Sakai, and A. M. Chakrabarty.** 1992. AlgR2 is an ATP/GTP-dependent protein kinase involved in alginate synthesis by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **89:**2659–2663.

23a. **South, S., and T. C. Montie.** Unpublished data.

24. **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.
25. **Strom, M. S., and S. Lory.** 1991. Amino acid substitutions in pilin of *Pseudomonas aeruginosa*. J. Biol. Chem. **266:**1656–1664.
26. **Totten, P. A., and S. Lory.** 1990. Characterization of the type a flagellin gene from *Pseudomonas aeruginosa* PAK. J. Bacteriol. **172:**7188–7199.