JOURNAL OF BACTERIOLOGY, Sept. 1990, p. 5135–5139
0021-9193/90/095135-05$02.00/0

Vol. 172, No. 9

Phosphorylated Tyrosine in the Flagellum Filament Protein of *Pseudomonas aeruginosa*

KIMBERLY KELLY-WINTENBERG, TINA ANDERSON, AND THOMAS C. MONTIE*

Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996

Received 4 April 1990/Accepted 15 June 1990

Purified flagella from two strains of ^{32}P-labeled *Pseudomonas aeruginosa* were shown to be phosphorylated. This was confirmed by autoradiography of flagellin protein in polyacrylamide gels. Thin-layer electrophoresis and autoradiography of flagellin partial hydrolysates indicated that phosphotyrosine was the major phosphorylated amino acid. High-pressure liquid chromatographic analysis confirmed the presence of phosphotyrosine in flagellum filament protein. Preliminary data indicated that less than one tyrosine per subunit was phosphorylated. No evidence was found for phosphorylation of serine or threonine. A function related to tyrosine phosphorylation has not been determined.

Pseudomonas aeruginosa is a successful competitor and survivor with the capacity to respond to environmental changes quickly at a variety of biochemical and metabolic levels. This gram-negative soil bacterium is also a plant pathogen, an invasive human pathogen, and can cause chronic colonization of lungs in cystic fibrosis patients. Success of this organism in certain niches apparently depends on highly regulated syntheses (8, 16) of extracellular products, including proteases, toxins, phospholipases, polysaccharides, and pigments as well as siderophores to chelate iron. Cell components of importance that contribute to survival include environmentally sensitive porins, regulators of phosphate concentration (1), flagella (4, 15), and motility and chemotaxis (4), and adhesins such as pili (15).

Evidence has been provided implicating motility as an invasive virulence factor in *P. aeruginosa* (6), and in related research, flagellum filaments are a vaccine source for active or passive immunization (5). *P. aeruginosa* possesses a single polar flagellum which in certain strains is dissociated into subunits of 53 kilodaltons (kDa) (designated b type) or in other strains exhibits a repeating a-type subunit ranging in weight from 45 to 52 kDa (12). Typically, as with other characterized bacterial flagellins, amino acid compositions of flagellum filaments show no histidine, no cysteine, low amounts of aromatic amino acids, and a common N-terminal sequence (T. C. Montie, F. Dorner, J. C. McDonel, and A. Mitterer, U.S. patent 4,831,121, May 1989; (T. C. Montie, unpublished data).

Interest in investigating possible posttranslational modification of flagellin protein occurred after a block at N-terminal amino acid 10 was detected several years ago, and subsequently an unidentified phenylthiocarbamyl (PTC) derivative was detected by more sensitive techniques (Montie et al., U.S. patent 4,831,121, May 1989; Montie, unpublished data). Certain observations prompted us to investigate whether flagella of *P. aeruginosa* are phosphorylated. These included the finding that several different modes exist for posttranslational modification of bacterial flagella and pili (11) and a recent suggestion that *Campylobacter* flagellum filaments are phosphorylated (9).

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and chemicals. *P. aeruginosa* wild-type strain PAO1 was graciously supplied by A. Kropinski, Queen's University, Kingston, Ontario, Canada. Strain M-2, originally isolated from the small intestine of a CF-1 mouse, was kindly provided by I. A. Holder, Shriner's Burn Institute, Cincinnati, Ohio (5).

Cells were grown in 2 liters of modified mineral salts medium [4×10^{-4} M K_2HPO_4, 2.2×10^{-4} M KH_2PO_2, 7.5×10^{-3} M $(NH_4)_2SO_4$, 2.0×10^{-4} $MgSO_4$, 0.4% sodium succinate]. The total phosphate concentration was decreased to approximately twofold over the limiting levels. [^{32}P]phosphoric acid (ICN Biomedical, Inc., or Dupont Biotechnical Systems) was added at a concentration of 5 μCi/1 ml, and the cells were grown at 30°C for 22 h. Phosphoamino acid standards were from Sigma Chemical Co., St. Louis, Mo.

Isolation and purification of flagellum filaments. The cells were concentrated by repeated centrifugation at $5,000 \times g$ at 4°C. After concentration, the cells were suspended in 10 mM phosphate buffer (pH 7.0) (35 ml/liter of cells) containing 0.1 mM phenylmethylsulfonyl fluoride, and the flagella were removed from the cells by shearing in a Waring blender for 30 s on low speed. The sheared cell suspension was centrifuged at $5,000 \times g$ for 30 min at 4°C. The supernatant containing flagellum filaments was further centrifuged at $16,000 \times g$ to remove any contaminating cellular debris. The supernatant was retained, and the flagellum filaments were pelleted by ultracentrifugation at $40,000 \times g$ for 3 h. After ultracentrifugation, the pellets were suspended in 30 mM Tris chloride (pH 8.0), dialyzed overnight, lyophilized, and stored at −20°C.

Isolated flagellum filaments were purified primarily to remove lipopolysaccharide by gel filtration. The partially purified sample (approximately 2 mg) was dissolved in 1 ml of sample buffer (15 mM Tris [pH 8.0], 0.1 M EDTA [pH 8.0], 0.1 mM phenylmethylsulfonyl fluoride, 1% sodium azide, 0.1% sodium deoxycholate) and was loaded onto a Sephacryl S-300 superfine column containing 30 mM Tris chloride (pH 8.0) and 2 mg of deoxycholate buffer per ml. Flagellum filaments emerge as undissociated protein at the void volume. After lyophilization, the combined flagellar fractions were passed through a Sephadex G-25 column equilibrated with a 30 mM Tris chloride (pH 8.0) buffer to remove excess deoxycholate and EDTA. The void fraction

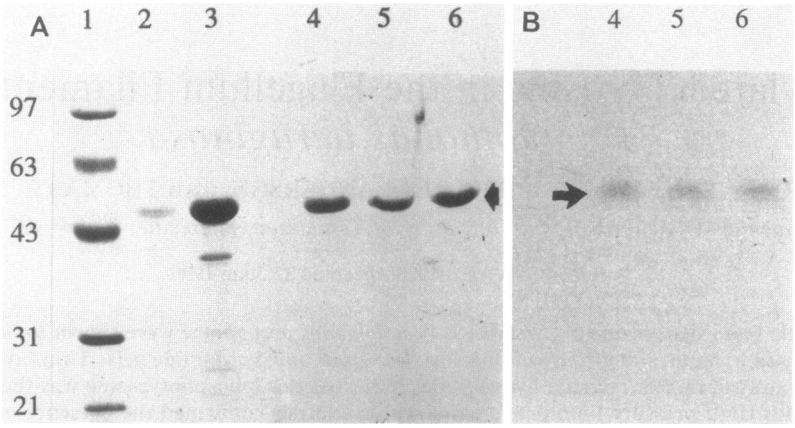

FIG. 1. Phosphorylation of *P. aeruginosa* flagellum filament protein. Flagellum protein b-type standards (1.0 μg) and 2 to 4 μg of labeled, purified flagellum protein from PAO1 were separated by electrophoresis in a 10 to 15% polyacrylamide gradient. (A) Coomassie blue-stained polyacrylamide gel. Lanes: 1, molecular mass markers (kilodaltons); 2 and 3, standards of 53-kDa flagellin from PJ108 and PAO1; 4, 5, and 6, isolated labeled PAO1 flagellin (4, 2, and 3 μg, respectively). (B) Autoradiogram of panel A, lanes 4, 5, and 6, as described above. Arrow denotes 53-kDa band.

sample was passed through a second Sephadex G-25 column with 0.1% NH_4OH as the running buffer to desalt the preparation.

Electrophoresis and immunoblots. Flagellum samples were dissolved in sodium dodecyl sulfate-EDTA dissociating buffer (2% sodium dodecyl sulfate, 20 mM EDTA, 10% glycerol, 0.05% β-mercaptoethanol, 0.003% bromophenol blue, 60 mM Tris, pH 6.8) and heated at 100°C for 5 min. Samples containing 1 to 4 μg of flagellum protein were applied to sodium dodecyl sulfate-10 to 15% polyacrylamide gels and separated by the Phast system (Pharmacia Diagnostics, Piscataway, N.J.). After electrophoresis, the gels were stained with Coomassie brilliant blue. Immunoblotting was done as previously described (17). Proteins were resolved by electrophoresis as described above and transferred onto nitrocellulose paper by diffusion. After blocking with bovine albumin, the blots were incubated with antiflagellin rabbit antiserum previously adsorbed with heated b-type bacteria (diluted 1:1,000), followed by application of peroxidase conjugated to goat antirabbit immunoglobulin G (diluted 1:10,000). A yellow band color was positive. Location of ^{32}P-labeled proteins was determined by autoradiography with Kodak X-Omat R film (Eastman Kodak Co., Rochester, N.Y.) in darkness at −70°C for 48 to 72 h.

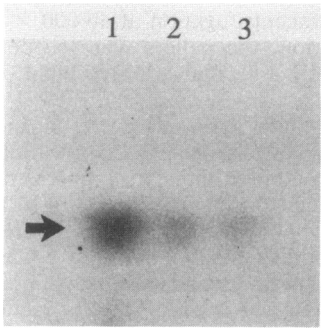

FIG. 2. Autoradiogram of immunoblot of ^{32}P-labeled flagellin. Gels were run as described in the legend to Fig. 1A, and the immunoblots were obtained as described in Materials and Methods. Lanes 1, 2, and 3, Labeled PAO1 flagellin. Arrow denotes 53-kDa band.

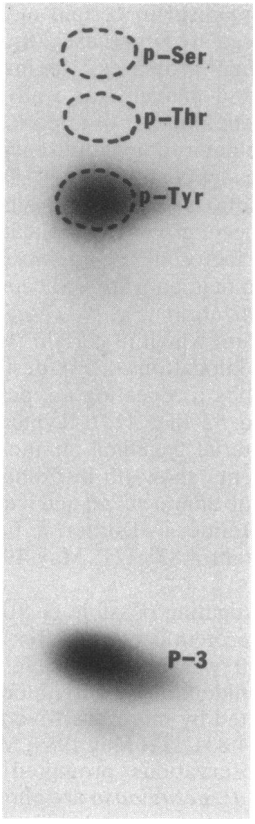

FIG. 3. Autoradiogram of TLE of hydrolyzed filament protein. Approximately 15 μl of the hydrolysate and 60 μg of phosphoamino acid standards were spotted onto a cellulose thin-layer chromatography plate and separated as described in Materials and Methods. Dried plates were sprayed with ninhydrin, and spots developed upon spraying with a stream of hot air. Migration of unlabeled standards was determined by ninhydrin staining, and labeled amino acids were detected by autoradiography. p-Ser, Phosphoserine; p-Thr, phosphothreonine; p-Tyr, phosphotyrosine.

TABLE 1. Analysis by TLE of radiolabeled compounds in partial acid hydrolysates (45 min) of M2 flagellum filaments[a]

Compound	Separation buffer		HPLC fraction in pH 3.5 buffer[b]	
	pH 1.9[c]	pH 3.5	Fraction 5	Fraction 12
Phosphotyrosine	7 (57)	34 (2,870)	60 (1,850)	<1 (4)
Phosphoserine	—[d]	—[d]	<1 (23)	0 (0)
Phosphothreonine	—[d]	—[d]	<1 (2)	0 (0)
P-2[e]	3 (30)	13 (1,100)	13 (400)	<1 (5)
P-3[e]	41 (335)	37 (3,148)	23 (724)	99 (4,096)
P-4[e]	38 (314)	4 (400)	2 (86)	<1 (8)
P-8[d,e]	11 (87)	12 (1,000)	<1 (20)	(0)

[a] Analogous results were seen with flagella of strain PAO1. Results are shown as percent total radioactivity excluding free P_i; numbers in parentheses are counts per minute.

[b] No radioactivity was detected in HPLC fractions 13 and 14, which seemed to correspond to phosphothreonine and phosphoserine fractions.

[c] 2-h hydrolysates showed similar results.

[d] In some experiments, P-8 overlapped with phosphoserine and phosphothreonine, obscuring accurate determinations of the compounds. Preliminary experiments with pronase digestion of the hydrosylate and TLE analysis showed a 51% reduction in radioactivity, indicating that a peptide contained most of the label.

[e] P denotes peptide and number denotes migration from origin in centimeters.

TLE of acid hydrolysates. The labeled flagellum protein (0.5 mg) was hydrolyzed in 200 μl of 6 N HCl at 110°C for 45 min in a sealed tube. The sample was then lyophilized and suspended in 400 μl of 0.3 N HCl. Approximately 15 μl of the flagellar hydrolysate samples and 60 μg of phosphoamino acid standards were spotted onto thin-layer chromatography cellulose paper (Eastman Kodak) as described by Cooper et al. (2) and separated in either pH 3.5 buffer (10:100:1,890 pyridine-glacial acetic acid-distilled H_2O) or pH 1.9 buffer (50:156:1,794 88% formic acid–glacial acetic acid–distilled H_2O) at 18 mA and 350 V for 1.5 or 3 h, respectively. After drying, the plates were sprayed with a ninhydrin solution (0.5% ninhydrin in acetone–4.5% collidine), and spots were developed by exposure to a stream of hot air. Locations of unlabeled phosphoamino acid standards and unknown peptides were determined by ninhydrin staining, and labeled amino acids and peptides were detected by autoradiography and scintillation counting.

HPLC analysis. For high-pressure liquid chromatographic (HPLC) analysis, 100 μl (100 to 125 μg) of hydrolyzed flagella in 0.3 N HCl was mixed with 400 μg each of phosphotyrosine, phosphothreonine, and phosphoserine in 300 μl of 0.1 M Tris chloride (pH 8.0). This sample was injected into a VYDAC C_{18} column connected to a Waters HPLC system with a solvent system consisting of 0.1% trifluoracetic acid–acetonitrile (10). One-minute fractions were collected, and 20-μl samples were spotted on glass fiber filter discs and dried, and the radioactivity was assayed by scintillation counting.

Quantitation of phosphorylated compounds. The relative amount of each sample containing phosphate radioactivity was determined by scraping each lane, which had been divided into 1-cm squares, and quantitating the radioactivity present by scintillation counting. The relative location of labeled phosphoamino acids was determined by comparing them with comigration of ninhydrin-stained phosphoamino acid standards. Results were expressed as a percentage of radioactivity recovered in the phosphoamino acids and phosphopeptides.

RESULTS AND DISCUSSION

In separate experiments, two b-type strains, PAO1 and M2, were grown in the presence of $^{32}P_i$ overnight (5 μCi/ml).

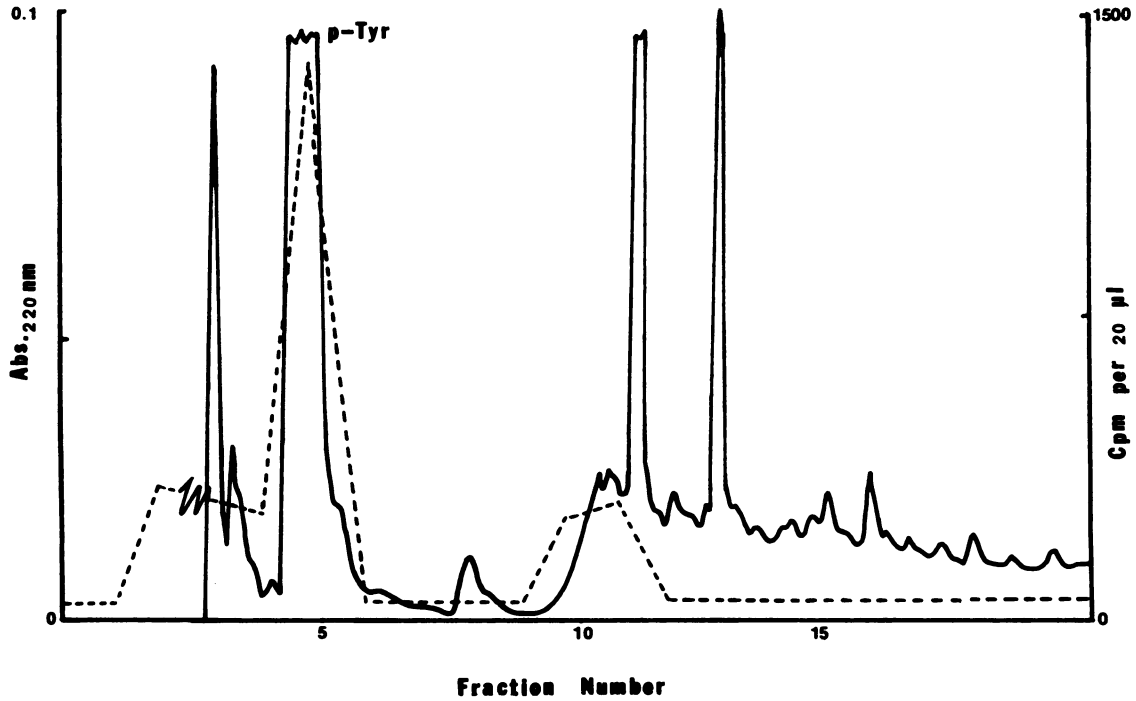

FIG. 4. HPLC profile and fraction radioactivity of hydrolyzed filament protein. One-minute fractions were collected, and 20-μl samples were spotted onto glass fiber filter discs and dried, and the radioactivity assayed by scintillation counting as described in Materials and Methods. Solid line represents A_{220} of fractions; dashed line represents counts per fraction.

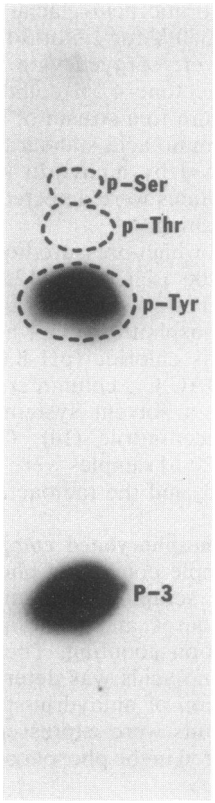

FIG. 5. Autoradiogram of TLE of the peak fraction (no. 5) of HPLC-purified hydrolyzed filament protein. Samples (15 μl) of the peak fractions were spotted onto cellulose thin-layer chromatography medium along with standards, and the components were separated by TLE as described in the text. Migration of unlabeled standards was determined by ninhydrin staining, and labeled amino acid components were detected by autoradiography. p-Ser, Phosphoserine; p-Thr, phosphothreonine; p-Tyr, phosphotyrosine.

Flagellum filaments were sheared from intact cells, and flagellin protein was purified as previously noted (13) and modified as described in Materials and Methods. Isolation of b-type 53-kDa subunits from purified preparations is shown on stained gels in Fig. 1A. Autoradiograms obtained from stained gels showed that 2 to 4 μg (dry weight) of protein applied to mini-Phast gels readily gave positive banding (Fig. 1B). Immunoblot bands also gave radioactive bands corresponding to 53-kDa flagellin when subjected to autoradiography (Fig. 2). Since purified ^{32}P-labeled PAO1 flagellin was readily detectable, we repeated these experiments with another b-type strain, M-2, to eliminate the possibility of any unusual properties associated with a single strain. Analogous results were obtained with M-2 flagellin at all levels of purification. We concluded from these data that phosphate was covalently bound to flagellin.

The phosphoryl linkage hypothesized was most likely an *O*-ester linkage to serine, threonine, or tyrosine since there is no histidine in the flagellin and acyl residues are very unstable (3). We suspected phosphoserine or phosphothreonine as likely candidates because these amino acids are the ones most commonly phosphorylated in *Escherichia coli* (2, 3). However, limited acid hydrolysis (2 to 4 h) of flagellin and examination of extracts by TLE in pH 1.9 buffer (2.5% formic acid, 7.8% glacial acetic acid in H_2O) gave very little to no activity in these amino acids, which are acid stable (Table 1). To avoid degradation of labeled phosphoamino acids, the hydrolysis time was reduced to 45 min and extracts were examined by TLE in a pH 3.5 buffer (0.5% pyridine, 5% glacial acetic acid in H_2O). Hydrolysate samples gave approximately 250 cpm/μg of protein. Results from autoradiography of chromatograms unexpectedly showed that most of the amino acid label appeared in phosphotyrosine (Fig. 3). For quantitative estimations, TLE plates were sectioned and radioactivity in all fractions was compared. Results showed 34% radiolabel in phosphotyrosine and negligible activity in phosphoserine and phosphothreonine, with most of the remaining radiolabel associated with putative ninhydrin-positive peptides (Fig. 3 and Table 1). The phosphotyrosine was clearly resolved in the single-dimension electrophoresis at pH 3.5 (2). Three slowly migrating peptides, designated P-2, P-3, and P-4 (Table 1), retained over 50% of the total amino acid radiolabel. Two similarly migrating components designated (P-3, P-4) were observed in TLE separations at pH 1.9 (Table 1). In some experiments, small amounts of phosphotyrosine were seen in pH 1.9 separations, but overall, the combination of acid hydrolysis and pH 1.9 buffer apparently degraded phosphotyrosine and reduced peptide radioactivity. Consistent with these results, the counts in P_i increased in the pH 1.9 buffer compared with the pH 3.5 buffer, indicating hydrolysis of the phosphoryl linkage.

Confirmation of these results was obtained by HPLC separations of 45-min hydrolysates on C_{18} columns (Fig. 4). A major amount of activity was associated with an early appearing 5-min peak corresponding to phosphotyrosine and a peptidelike component. The presence of phosphotyrosine in fraction 5 was confirmed by its absorption spectrum with a peak at 265 nm and by TLE assay (Table 1 and Fig. 5). No evidence was obtained for the presence of any other labeled amino acid. However, fraction 12 contained a single ninhydrin-positive component that migrated on TLE assays as a ninhydrin-positive peptide and was responsible for all the radioactivity in that fraction.

We conclude that *P. aeruginosa* flagellin filament protein is phosphorylated in vivo and that a substantial portion of the phosphate is bound to tyrosine. Previous amino acid analyses have consistently shown the presence of four to seven tyrosine residues per 53-kDa flagellin subunit (Montie et al., U.S. patent 4,831,121, May 1989). It will be of interest to determine whether all the tyrosines present per subunit are phosphorylated and whether every 53-kDa subunit is phosphorylated. Based on preliminary data we estimated that there is less than 1.0 mol of ^{32}P per mol of flagellin protein.

We have not completely excluded phosphorylation of serine or threonine, but these compounds were not readily detectable in acid hydrolysates. Experiments employing alternative hydrolysis procedures are under way to examine the components of the incompletely digested peptides to determine whether phosphotyrosine is the phosphorylated amino acid present in the peptides.

Phosphorylation of flagellin and particularly at tyrosine has not been previously reported. The presence of phosphotyrosine in bacteria seems limited (7), although it has been identified as a component of the light-harvesting complex of the purple photosynthetic bacterium *Rhodospirillum rubrum* (14). At present, the function(s) of posttranslational modifications of flagellum filament protein is unknown. It may reflect a necessary structural benefit or be related to a signal for monomer export or filament assembly.

ACKNOWLEDGMENTS

We thank J. Joshi and D. Roberts for discussion and advice, K. Morgan for HPLC analysis, and C. Osborne for help with acid hydrolysis; we also thank P. Keaton and A. Williams for manuscript preparation.

LITERATURE CITED

1. **Bell, A., and R. E. Hancock.** 1989. Outer membrane protein H1 of *Pseudomonas aeruginosa*: purification of the protein and cloning and nucleotide sequence of the gene. J. Bacteriol. **171**:3211–3217.
2. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantitation of phosphotyrosine in proteins. Methods Enzymol. p. 387–402.
3. **Cozzone, A. J.** 1988. Protein phosphorylation in prokaryotes. Annu. Rev. Microbiol. **42**:97–125.
4. **Craven, R. C., and T. C. Montie.** 1985. Regulation of *Pseudomonas aeruginosa* chemotaxis by the nitrogen source. J. Bacteriol. **164**:544–549.
5. **Drake, D., and T. C. Montie.** 1987. Protection against *Pseudomonas aeruginosa* infection by passive transfer of antiflagellar serum. Can. J. Microbiol. **33**:755–763.
6. **Drake, D., and T. C. Montie.** 1988. Flagella, motility, and invasive virulence of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **134**:43–52.
7. **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.
8. **Hindahl, M. S., D. W. Frank, and B. H. Iglewski.** 1987. Molecular studies of a positive regulator of toxin A synthesis in *Pseudomonas aeruginosa*. Antibiot. Chemother. (Basel) **39**: 279–289.
9. **Logan, S., T. J. Trust, and P. Guerry.** 1989. Evidence for posttranslational modification and gene duplication of *Campylobacter* flagellin. J. Bacteriol. **171**:3031–3038.
10. **Lottspeich, F., and A. Henschen.** 1985. Amino acids, peptides, proteins, p. 176–177. *In* A. Henschen, K. Hupe, F. Lottsperch, and W. Voelter (ed.), High performance liquid chromatography in biochemistry. VCH Publishers, Deerfield Beach, Fla.
11. **Macnab, R.** 1987. Flagella, p. 70–73. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
12. **Montie, T. C., D. Doyle-Huntzinger, R. C. Craven, and I. A. Holder.** 1982. Loss of virulence associated with absence of flagellum in an isogenic mutant of *Pseudomonas aeruginosa* in the burned mouse model. Infect. Immun. **38**:1296–1298.
13. **Montie, T. C., D. Drake, H. Sellin, O. Slater, and S. Edmonds.** 1987. Motility, virulence, and protection with a flagellar vaccine against *Pseudomonas aeruginosa* infection. Antibiot. Chemother. (Basel) **39**:233–248.
14. **Pairoba, C. F., and R. H. Vallejos.** 1989. Protein phosphorylation in purple photosynthetic bacteria. Biochimie **71**:1039–1041.
15. **Totten, P. A., J. C. Lara, and S. Lory.** 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J. Bacteriol. **172**:389–396.
16. **Vasil, M. L., J. W. Ogle, C. C. R. Grant, and A. I. Vasil.** 1987. Recombinant DNA approaches to the study of the regulation of virulence factors and epidemiology of *Pseudomonas aeruginosa*. Antibiot. Chemother. (Basel) **39**:264–278.
17. **Wintenberg-Kelly, K., and T. C. Montie.** 1989. Cloning and expression of *Pseudomonas aeruginosa* flagellin in *Escherichia coli*. J. Bacteriol. **171**:6357–6362.