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Nature of the Carbohydrate and Phosphate Associated with ColB2 and EDP208 Pilin

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Studies were carried out to elucidate the nature of phosphate and sugar linkages to F-like conjugative pili encoded by the ColB2Fdr (compatibility group FII) and EDP208 (compatibility group FV) plasmids. Both types of pili, when in the intact undissociated state, were found to contain approximately 3 mol of phosphate and 3 mol of sugar per mol of pilin. However, further purification of the two types of pilin by gel filtration chromatography in the presence of sodium dodecyl sulfate (SDS) removed all of the carbohydrate from EDP208 pilin and approximately 65% of the carbohydrate from ColB2 pilin. Approximately 0.8 to 1.0 mol of glucose per mol of protein remained associated with ColB2 pilin after SDS gel filtration chromatography, but it was not possible to determine whether this was covalently linked to the pilin, or tightly associated in an SDS-resistant manner. SDS-gel chromatography did not remove phosphate from either ColB2 or EDP208 pilins. ^{31}P nuclear magnetic resonance studies indicated that the pilin-associated phosphate is involved in a phosphodiester linkage. Acetone precipitation or chloroform-methanol extraction of the purified pilin material reduced the phosphate associated with EDP208 pilin to less than 0.04 molecule per pilin monomer. ColB2 pilin, under the same conditions, retained approximately 0.5 phosphate per pilin monomer. The extracted phosphate-containing moieties were identified as phosphatidylglycerol and phosphatidylethanolamine by thin-layer chromatography. Since the ^{31}P nuclear magnetic resonance spectra for both ColB2 and EDP208 were identical and no signal other than that of a phosphodiester was detected in the ColB2 spectrum, the phosphate remaining with the ColB2 pilin after chloroform-methanol extraction is most likely due to a tightly bound noncovalent residue.

Conjugative pili are required for initiating cell-to-cell contact during bacterial conjugation (1) and act as the attachment site in the infectious process of several bacteriophages (23). These pili are composed of a single subunit, pilin, arranged in a helical fashion to give thin tubular filaments 8 nm in diameter and approximately 1 μm in length (16).

The biosynthesis of a functional F-like conjugative pilus requires the action of at least 13 genes identified on the F transfer operon (2). Possible roles for these genes could involve synthesis and modification of the pilin protein, transport of the pilin through the bacterial cell envelope, and assembly and disassembly of the pilus during outgrowth and retraction (2). Previous reports have suggested that F-like pili are both glycosylated and phosphorylated (6, 11). Thus at least two *tra* gene functions required for pilus production could involve post-translational modification. However, since the nature of the sugar and phosphate linkages to pilus protein have not yet been determined, it is possible that these moieties are not associated with pilus protein in a covalent manner.

The present study was carried out, therefore, to elucidate the nature of phosphate and sugar linkages to F-like conjugative pili. The work was performed on pili encoded by ColB2Fdr (18) and EDP208 (3) plasmids since *Escherichia coli* K-12 strains carrying these plasmids generally produce greater quantities of conjugative pili than strains carrying other F-like plasmids and therefore facilitate the purification of relatively large amounts of pili material (our unpublished studies). Although the compatibility group of ColB2Fdr (FII) is different from that of the F plasmid (FI), ColB2 pili are closely related to F on the basis of sensitivity to RNA phages, amino acid composition, and immunogenicity (our unpublished studies). On the other hand, EDP208 (compatibility group FV) pili differ from F on the basis of these same criteria (3). However, the filamentous DNA phage F1 is plated with

equal efficiency on F- and EDP208-carrying cells (15), suggesting possible structural similarities at the tips of these pili.

In what follows it is shown that both sugar and phosphate residues can be removed quantitatively from EDP208 pili by nonhydrolytic procedures. These same procedures only remove approximately 65 and 85%, respectively, of the sugar and phosphate moieties of ColB2 pili. It was concluded that EDP208 pilin is neither phosphorylated nor glycosylated. ColB2 pilin, on the other hand, may possess one covalently bound glucose per pilin subunit, although it is probably not phosphorylated.

MATERIALS AND METHODS

Bacterial strains and plasmids. The ColB2 Fdr and EDP208 plasmids were carried by the host strain JC6256 (*E. coli* K-12 F^- *trp lac*). They are the derepressed forms of ColB2 (17, 18) and Folac (15) plasmids, respectively, and were kindly donated by N. S. Willetts, Department of Molecular Biology, University of Edinburgh. F pili were prepared from *E. coli* ED2692 which is the *traD8* mutant of F (26) in the host strain WP1000 (*E. coli* B/r Fla^- Fim^-).

Purification of pili. The purification of F-like pili has been described previously (3). To remove contaminating flagella and membranes, ColB2 and F(*traD8*) pili preparations were treated with 4 M guanidine hydrochloride before cesium chloride gradient centrifugation (3). Usually 1 to 2 mg of F(*traD8*) pili, 10 mg of ColB2 pili, and 50 mg of EDP208 pili could be purified from 250 g of cells (wet weight).

Gel filtration. Pili were dissolved in 50 mM Tris-hydrochloride (pH 8.3) containing 1 mM EDTA and 1.0% sodium dodecyl sulfate (SDS), at a concentration of 1 to 2 mg/ml. The sample was applied to a Sephadex G-200 column (2.5 by 39 cm) equilibrated with the same buffer. The flow rate of the column was maintained at 20 ml/h, and 1-ml fractions were collected. The fractions were monitored for absorbance at 280 nm, and the peaks containing protein were extensively dialyzed against water adjusted to pH 8.0 with ammonium hydroxide at room temperature. The pilin was then lyophilized and stored at −20°C.

SDS polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Lugtenberg et al. (22) with the following modifications. The running gel contained 15% acrylamide-0.27% methylene bisacrylamide, whereas the stacking gel contained 7.5% acrylamide-0.2% methylene bisacrylamide. The gels were stained using the method of Fairbanks et al. (14). Autoradiography was performed using Kodak Blue Brand Royal X-omat X-ray film.

Acetone precipitation of EDP208 from SDS. The concentration of EDP208 pilin dissolved in 1.0% SDS was adjusted to approximately 2 mg/ml before the addition of 4 volumes of spectral-grade acetone. After 30 min, the precipitate was collected by centrifugation at 3,000 × *g* for 20 min.

Chloroform methanol extraction. Chloroform methanol extraction was carried out by a modified cation of the method of Bligh and Dyer (5). Five volumes of chloroform-methanol (2:1, vol/vol) was added to 1 volume of pili dissolved in water in a concentration of 2 mg/ml. The solution was blended vigorously in a Vortex mixer and centrifuged at 3,000 × *g* for 20 min. The chloroform layer was removed and evaporated to dryness in a stream of nitrogen.

Phosphate determination. Samples (0.5 to 1.0 mg) for phosphate determination were hydrolyzed for 72 h under vaccuum in constantly boiling 6 M HCl containing 1.0% phenol. The samples were then dried over NaOH pellets and assayed for total inorganic phosphate by the method of Chen et al. (9).

Carbohydrate analysis. Samples of pili (80 to 800 nmol) were hydrolyzed with 4 N HCl for 2 to 4 h at 110°C. The samples were dried and suspended in 0.5 ml of water (resultant pH 4.0). The hydrolysate was passed through a Dowex AG50W-X8 cation-exchange column (9 mm by 3 cm), and the neutral sugars were collected in 5 ml of water. The eluate was freeze-dried, and the sugars were separated by ascending thin-layer chromatography, using 0.25-mm silica gel plates (Brinkmann Instruments, Inc.) and isopropanol-ammonium hydroxide-water (7:2:1, vol/vol/vol) as the solvent. The sugars were detected using a silver nitrate spray (24) or an anisaldehyde-sulfuric acid spray (24) which gives a characteristic color for each sugar. In addition, 1 to 2 mg (80 to 160 nmol) of pili were treated with 1.5 M HCl in anhydrous methanol at 80°C for 24 h in sealed ampoules. After separating the resulting methylated sugars by thin-layer chromatography as described above, the chromatograms were sprayed with 3% potassium chromate in 60% sulfuric acid and heated at 350°C to visualize the sugars (24). Sugar quantitation was achieved by gas chromatography. The dried samples were trimethylsilylated with Trisil reagent (Pierce Chemicals Inc.) and were run on a Hewlett Packard 5710 gas chromatograph equipped with a flame ionization detector. The glass column (2 mm by 4 feet [2 mm by 1.22 m]) was packed with 3% OV-1 on 100/2100/120 Supelcoport according to the manufacturer's directions. The gas chromatograph was programmed from 100 to 230°C at 8°/min. When these procedures were evaluated for carbohydrate recovery using ovalbumin as a standard glycoprotein, approximately 80% of the ovalbumin carbohydrate was removed from the protein and identified by gas chromatography. The limit of detection for glucose was 2 nmol per sample, which allowed the detection of as little as 0.02% sugar in 1- to 2-mg pilin samples.

Phospholipid analysis. Phospholipids were separated by thin-layer chromatography using silica gel plates (see above) and the following solvent system: chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, vol/vol). The phospholipids were visualized with iodine vapors and were quantitated by assaying for total P_i in the spots scraped off the plate.

Amino acid and protein estimation. Protein was estimated by the method of Lowry et al. (21). Amino acid analyses were performed as previously described (3).

^{31}P NMR spectroscopy. ^{31}P nuclear magnetic resonance (NMR) spectra were obtained at a frequency of 109.3 MHz on a Bruker HXS-270 spectrometer, interfaced with a Nicolet-1080 computer and operating

in the Fourier transform mode. The spectrometer was locked on the deuterium resonance of the 20% D_2O included in all of the samples. All chemical shifts are referenced to an external standard of 85% phosphoric acid; downfield shifts are given a positive sign. All spectra were recorded at 28 ± 1°C in 1.5-ml samples in 10-mm NMR tubes equipped with Vortex plugs. A typical setting to obtain a spectrum was: pulse angle, 60°; acquisition time, 0.4 s; relaxation delay, 1.6 s; sweep width, 5,000 Hz. When ^1H decoupling was used, the decoupler was on during acquisition with 3 W of power irradiating an area corresponding approximately to 3.0 to 7.0 ppm in a proton NMR spectrum.

RESULTS

Compositional analysis of ColB2 and EDP208 pili. Purified pili preparations were subjected to amino acid analysis; the results of these studies are summarized in Table 1. For comparison purposes, the amino acid analysis of F pili is also included. The table indicates that F and ColB2 pilin were very similar and somewhat different from EDP208 pilin. Generally speaking, however, all three types of pilin contained a fairly high proportion (about 53%) of hydrophobic amino acids (Ala, Leu, Ile, Val, Phe, Trp, Met). All of the proteins lacked histidine and proline, possibly indicating a high α-helical content in each. The F and ColB2 pilins were also found to lack arginine and cysteine, and EDP208 pilin lacked tryptophan.

Phosphate and carbohydrate analysis indicated the presence of both phosphate and carbohydrate in purified pili preparations. Determination of total phosphate by the method of Chen et al. (8) indicated two to three phosphates per pilin in each case. Colorimetric assays using the phenol sulfuric acid (4) and anthrone (4) procedures indicated that all three pili types possessed 2 or 3 mol of hexose per mol of protein. These results were consistent with those for F of Brinton (6) and Date et al. (11).

Carbohydrate analysis of ColB2 and EDP208 pilin. As stated above, colorimetric assays of ColB2 and EDP208 pili using glucose or galactose as a standard indicated that there were approximately two to three glucose equivalents per pilin subunit. Brinton (6) had reported one glucose residue per F pilin. However, hydrolysis and/or methanolysis of EDP208 and ColB2 pili samples revealed the presence of four different sugars as identified by thin-layer chromatography. These include glucose, galactose, and an unidentified pentose and dideoxyhexose as deduced from their mobilities on a thin-layer chromatogram and reaction with silver nitrate or anisaldehydesulfuric acid spray which produces colors characteristic for each sugar (24). Since it seemed possible that some of the carbohydrate moieties detected might represent contamination from cell envelope material such as lipopolysaccharide, an effort was made to separate the carbohydrate from pilin by gel filtration chromatography.

TABLE 1. *Amino acid composition of ColB2 pili*[a]

Amino acid	EDP208		ColB2		F(*traD*)	
	Residues/ monomer	Mol %	Residues/ monomer	Mol %	Residues/ monomer	Mol %
Lys	7	7.4 ± 1.06	9	8.6 ± 0.34	10	7.6 ± 0.50
His	0	0.0	0	0.0	0	0.15 ± 0.05
Arg	2	2.0 ± 0.44	0	0.0	0	0.30 ± 0.15
Asp	9	10.0 ± 0.82	8	6.8 ± 0.71	8	6.6 ± 0.60
Thr	10	13.6 ± 0.43	9	6.3 ± 0.23	8	6.4 ± 0.40
Ser	2	2.5 ± 0.15	9	6.5 ± 0.50	11	9.3 ± 0.90
Glu	2	2.06 ± 0.22	8	4.7 ± 1.00	4	3.6 ± 0.40
Pro	0	0.0	0	0.0	0	0.0
Gly	11	10.6 ± 0.70	13	10.5 ± 0.65	15	11.6 ± 0.30
Ala	10	9.3 ± 1.02	16	14.0 ± 0.45	15	12.4 ± 0.30
1/2 Cys	2	2.1 ± 0.50	0	0.0	0	0.0
Val	12	10.5 ± 0.20	20	16.4 ± 0.91	21	16.7 ± 1.70
Met	5	4.4 ± 0.69	8	6.6 ± 0.36	8	6.9 ± 0.20
Ile	10	8.6 ± 0.05	7	3.6 ± 0.24	4	3.1 ± 0.40
Leu	14	12.2 ± 0.30	9	8.2 ± 0.23	9	7.3 ± 0.50
Tyr	2	1.7 ± 0.42	2	1.9 ± 0.13	2	1.5 ± 0.20
Phe	6	6.1 ± 0.41	7	6.5 ± 0.31	7	5.3 ± 0.30
Trp	0	0.0	1	.75 ± 0.25	2	1.4 ± 0.20

[a] All amino acid analyses were an average value of three hydrolyses at 24, 48, and 72 h. Serine and threonine were estimated by extrapolating to zero time. Cysteine and methionine were determined after performic acid oxidation (19). Tryptophan was estimated spectrophotometrically using the method of Edelhoch (12). Molecular weight: EDP208, 11,500; ColB2, 12,800; F (*traD*), 12,100.

ColB2 and EDP208 pili were therefore dissociated into monomers by dissolving them in 1% SDS, and then passed through Sephadex G-200 gel filtration columns. Figure 1 shows typical elution profiles obtained from these columns and demonstrates that the material obtained from pure ColB2 or EDP208 pili was resolved into three peaks. In each case, amino acid analysis and SDS-PAGE demonstrated that the fractions in the first peak (I) contained large aggregates of EDP208 or ColB2 pilin plus minute amounts of high-molecular-weight contaminants. Fractions from the second peak (II) from each of the columns contained EDP208 or ColB2 pilin monomers. Fractions from the third peak (III) contained traces of protein, probably peptides, which were not detected by SDS-PAGE.

After acid hydrolysis or methanolysis of 80- to 800-nmol samples of column-purified EDP208 pilin (peak II material), thin-layer and gas chromatography indicated that all of the sugars had been removed from the protein. Further examination of peak III material for sugars revealed the presence of the same four sugars originally seen in intact pili. It was concluded therefore that EDP208 pilin does not contain covalently bound carbohydrate. The sugars which are removed from intact pili by SDS column gel filtraiton probably represent contamination from cell surface components such as lipopolysaccharide.

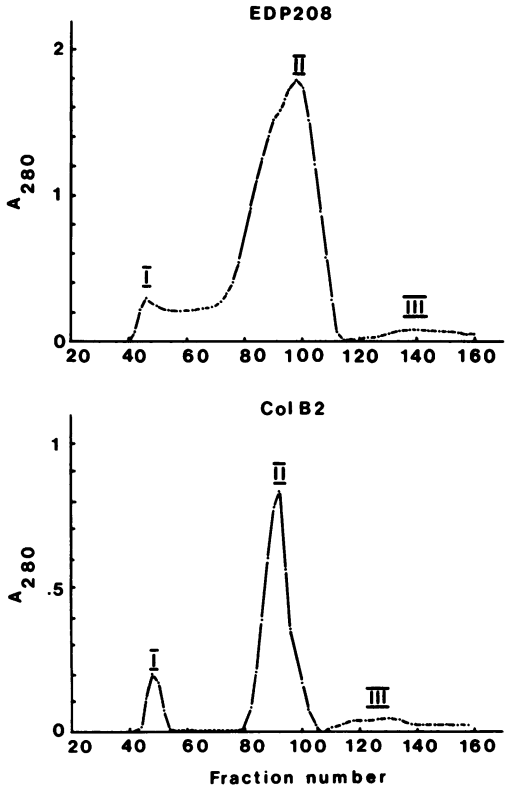

FIG. 1. *Sephadex G-200 gel filtration chromatography of EDP208 and ColB2 pili. The pili were dissociated into monomers in 1.0% SDS and eluted from the column using 0.1 M Tris-hydrochloride (pH 8.3), 1 mM EDTA, and 1.0% SDS. Fractions (1 ml) were collected at a flow rate or 20 ml/h. Column dimensions, 2.5 cm by 39 cm.*

When the same procedures were employed on purified ColB2 pilin, glucose was detected (Fig. 2). In Fig. 2A, standard glucose was resolved into both the α and the β anomers (peaks 1 and 2). This has been observed by others (9, 25). The same two peaks (3 and 4) were detected on a gas chromatogram of the ColB2 hydrolysate (Fig. 2B). Furthermore, quantitation of the glucose revealed approximately 0.8 to 1.0 mol of glucose per mol of pilin. Peak 1 in Fig. 2B was probably due to a derivatized amino acid, since standard amino acids were detected in this region. The peak 2 material in Fig. 2B is probably due to non-carbohydrate material since it was not detected as a reducing sugar on thin-layer chromatograms after aqueous hydrolysis nor as a methylated sugar after methanolysis.

Characterization of EDP208- and ColB2-associated phosphate. Although the SDS-gel filtration procedure resulted in the removal of most of the sugars from pilin, this treatment did not affect the level of pilin-associated phosphate. Determination of the total phosphate in column-purified EDP208 and ColB2 pilin revealed the presence of phosphate (Table 2). Therefore, the pilin-associated phosphate was either covalently bound to the protein or associated through some noncovalent SDS-resistant interaction.

To distinguish between these possibilities, EDP208 and ColB2 column-purified pilin was precipitated from the SDS solutions with acetone or extracted with chloroform-methanol as described in Materials and Methods. Phosphate analysis of the acetone-precipitated pilin indicated that 97% of the phosphate was removed from EDP208 and 86% was removed from ColB2 pilin. Moreover, the missing phosphate could be quantitatively accounted for in the acetone supernatant solutions (Table 2). The failure of acetone precipitation to remove all of the ColB2-associated phosphate as efficiently as EDP208 phosphate may reflect a tighter binding of the phosphorylated moiety to ColB2 pilin. However, the existence of one covalently bound phosphate in ColB2 pilin was an alternative possibility.

^{31}P NMR of EDP208 and ColB2 pilin in SDS. With a view to elucidating the nature of the phosphate linkages in EDP208 and ColB2

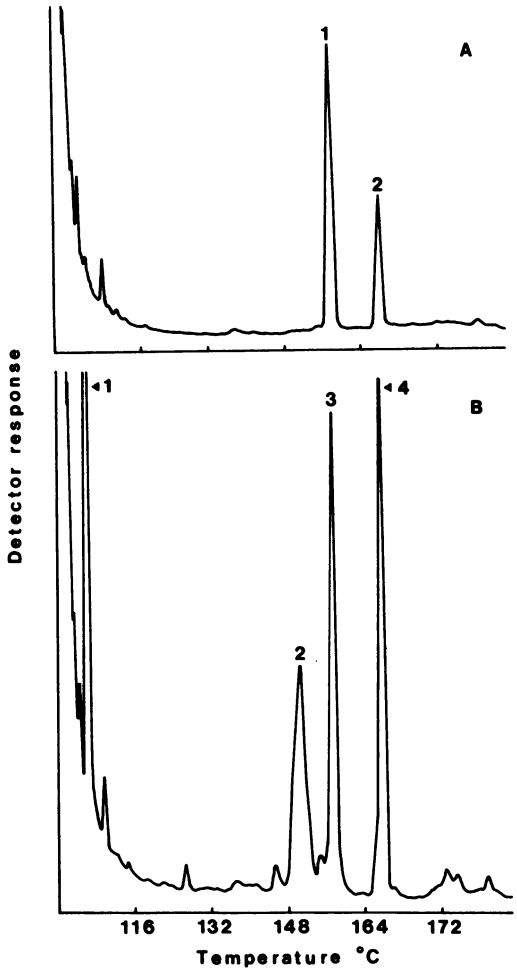

FIG. 2. *Gas chromatograms of standard glucose (A) and ColB2-associated sugars (B) removed by mild acid hydrolysis. The dried samples were trimethylsilylated with Trisil reagent (Pierce Chemical Co.) according to the manufacturer's directions, and the derivatized sugars were subjected to gas chromatography on a gas chromatograph equipped with programmable temperature control and a flame ionization detector. The sugars were resolved by increasing the temperature from 100°C to 230°C at a rate of 8°C per minute. The two glucose peaks represent the α and β anomers which are both generated by acid hydrolysis.*

pilin, both proteins were examined by ^{31}P NMR. Since no ^{31}P NMR resonances were observed with intact pili, probably because the lines were too broad to be observed, the pili were dissociated into monomers by treating with 1% SDS. This allowed for a greater mobility of the pilin subunits and gave rise to ^{31}P NMR resonances sufficiently narrow to be readily observed. The spectra of EDP208 and ColB2 pilins were essentially the same in terms of chemical shift and displayed only a broad downfield resonance at 1 ppm and a minor resonance at 0.6 ppm (Fig. 3A and B). Titration of the samples in the 6 to 10 pH range did not result in any significant shifts of peak positions. Moreover, the addition of a trace of manganese chloride caused severe, EDTA-reversible, peak broadening, indicating that the phosphorus is highly accessible to solvent and therefore not internally bound. Such shielding is therefore unlikely to be the cause of the nonshifting behavior upon pH titration. The preceding observations, combined with our observation that proton decoupling leads to line narrowing of the spectra (data not shown), indicate that the observed resonances arise from a phosphodiester moiety in both proteins (7, 10, 13).

TABLE 2. *Fate of pili-associated phosphate after acetone precipitation*

Fraction	Type of pilin	Amt of pilin[a] (nmol)	Amt of phosphate[b] (nmol)	Phosphate per pilin
G-200 purified peak II pilin	EDP208	112	325	2.9
	ColB2	25.0	75.0	3.0
Acetone[c] precipitate	EDP208	97.0	<8.0	<0.08
	ColB2	20.8	8.1	0.39
Acetone supernatant solution	EDP208	<8.0	270	
	ColB2	<8.0	53	

[a] Determined by amino acid analysis of samples hydrolyzed in 6 N HCl for 24 to 48 h.

[b] Determined by the method of Chen et al. (8) for P_i after hydrolyzing samples for 72 h in 6 N HCl. The detection limit of the assay was 5 nmol.

[c] The precipitate was collected by centrifugation at 10,000 × *g* for 20 min after the addition of 4 volumes of spectral-grade acetone to 1- to 2-mg/ml solutions of column-purified pilin in 1.0% SDS.

The acetone supernatant solution of EDP208 pilin resuspended in the SDS buffer appeared at a similar chemical shift position as that of unprecipitated EDP208 pilin (Fig. 3C). Again, we showed that no change of the chemical shift position occurred upon titration from pH 6 to 10. This suggests that the phosphodiester linkage is still intact and therefore could not have involved a covalent linkage with the protein. Identical results were obtained with the ColB2 pilin. Since no other resonances were observed in any of these spectra, it is unlikely that other types of phosphate linkage are involved. The remaining 0.5 mol of phosphate per mol of ColB2 pilin after chloroform methanol extraction is therefore most probably a noncovalent but very tightly bound moiety.

Identity of pili-associated phosphate. Col-

umn-purified EDP208 and ColB2 pilins were also extracted with chloroform-methanol as described in Materials and Methods. This procedure removed essentially all of the phosphate from EDP208 and about 80% from ColB2 pilin (Table 3). Furthermore, silica gel thin-layer chromatography of the chloroform-methanol extracts and visualization of the phospholipids with iodine vapors revealed the presence of both phosphatidylglycerol and phosphatidylethanolamine (Fig. 4). Phosphatidylethanolamine was also detected on thin-layer chromatograms using the cadmium-ninhydrin spray. As indicated in Table 3, all of the EDP208-associated phosphate and about 77% of that associated with ColB2 pilin could be accounted for in these two phospholipids in a 2:1 ratio of phosphatidylglycerol to phosphatidylethanolamine. No phosphate was detected in the minor spots seen on the chromatogram in Fig. 4.

^{32}P-labeling of EDP208 pili. To gain further insight into the nature of the phospholipid associated with EDP208 pili, attempts were made to label EDP208 pili with ^{32}P. However, since small liquid cultures do not contain sufficient pili to permit efficient precipitation with PEG 6000, unlabeled pili were added to the cell-free supernatant solutions to facilitate pilus precipitation. As a control, unlabeled pili were also precipitated from cell-free supernatant solutions of ^{32}P-labeled cultures of the plasmidless strain, JC6256. Surprisingly, when these pili were dissolved in SDS and passed through G-200 columns, the ^{32}P elution profiles in each case displayed the same three peaks (Fig. 5). Moreover, autoradiography of an SDS-polyacrylamide slab gel of the peak fractions (Fig. 6) indicated that the ^{32}P in peak I was mostly associated with the high-molecular-weight pilin aggregates, although some migrated at the same position as monomeric EDP208 pilin. The ^{32}P compounds in peak III moved with the dye marker. The ^{32}P in peak II was associated with the Coomassie blue-stained pilin band as well as with a band of material (probably phospholipid) that moved slightly faster than the pilin, but did not stain with Coomassie blue. The latter observation further demonstrated that the phospholipids remain bound to the pilin in the presence of SDS. However, it was evident that the phospholipids which are released into the growth medium are efficiently absorbed to previously purified pilus preparations. It was not determined, however, whether the newly absorbed phospholipids exchange with those already present on the pili or whether they are simply added to them.

When the ^{32}P-labeled EDP208 pilin was precipitated with acetone or extracted with chloroform-methanol, about 75% of the counts were removed into the acetone supernatant solutions. Autoradiography of thin-layer chromatograms of these revealed the presence of four phospholipids. Quantitation of the ^{32}P on these chromatograms indicated that 53% of the EDP208-associated phospholipid was phosphatidylglycerol, 30% was phosphatidylethanolamine, 4% was phosphatidylcholine, and 13% was phosphatidylserine. This result was the same regardless of the cell-free supernatant solution from which the pili were obtained. It was, therefore, concluded that EDP208 readily absorb phospholipids present in the cell-free bacterial growth medium, and that the phospholipids probably represent contaminants of the pili preparations. On the other hand, quantitation of the phospholipids in membranes of the *E. coli* K-12 strain used in these studies revealed that 90% of the membrane phospholipid was phosphatidylethanolamine and only about 10% was phosphatidylglycerol. It therefore seemed unusual that the major phospholipid associated with EDP208 pilin was phosphatidylglycerol.

Since acetone precipitation failed to remove all of the radioactivity from EDP208 pilin (i.e., approximately 2 to 3% radioactivity remained), the possibility existed that other phosphate moieties, perhaps nucleic acids or nucleotides, were also present in the preparations. Accordingly, column-purified pilin, obtained from either of the labeled cell-free supernatant solutions, was extracted with formic acid. Ascending chromatography of the formic acid supernatant solutions on DEAE-cellulose revealed the presence of all four ribonucleotides after autoradiography of the chromatograms. However, quantitation of these by high-pressure liquid chromatography indicated that their concentrations were extremely low (<0.1 mol per 8 mol of pilin). These components were, therefore, considered to be minor pilus contaminants.

DISCUSSION

Previous chemical analyses of EDP208 and F pilin indicated the presence of 1 to 2 mol of phosphate and about 1 to 2% by weight of carbohydrate (3, 6, 11). However, these investigations failed to determine whether these components were covalently bound to the protein.

The present investigation shows that neither the phosphate nor the carbohydrate moieties associated with intact EDP208 pili are covalently bound to the protein. The carbohydrate component was found to comprise four different monosaccharides which were removed from pilin almost quantitatively by SDS-gel filtration chromatography. The identify of the four sugars

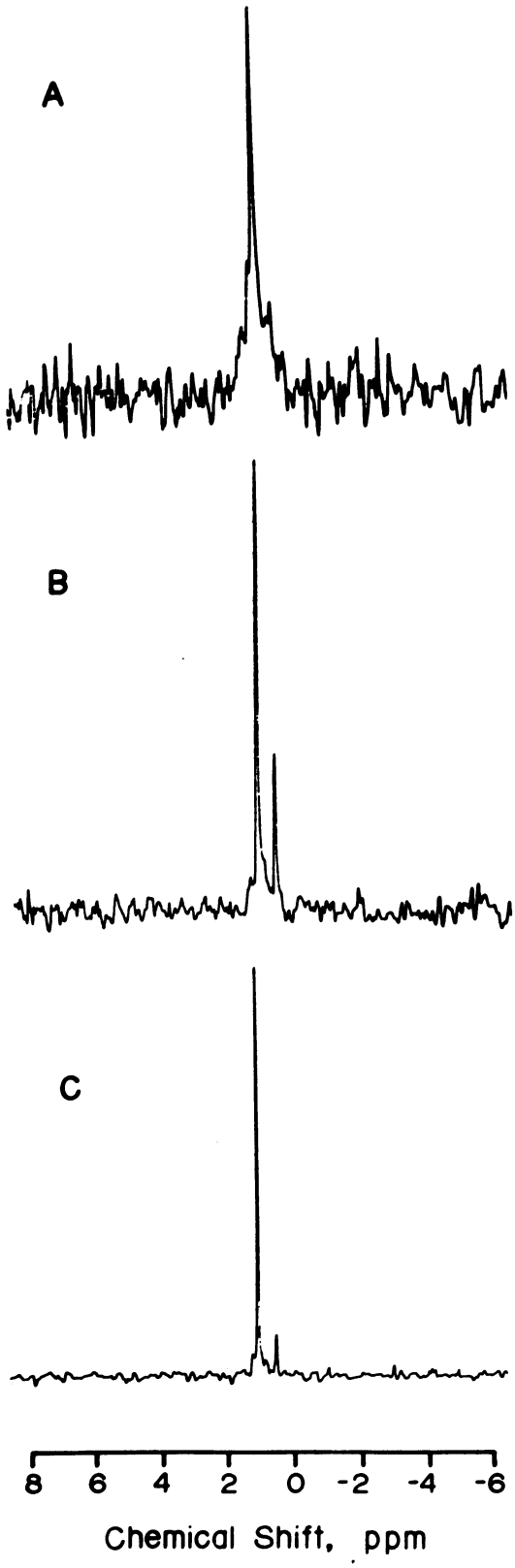

FIG. 3. *Proton decoupled ^{31}P NMR spectra of whole column-purified EDP208 (A) and ColB2 (B) pilin dissolved in 1.0% SDS. For spectra (A) and (B), 2,500 scans were accumulated on samples containing 2 mg of pilin per ml. Spectrum C shows the acetone supernatant of EDP208 pilin redissolved in 1% SDS buffer.*

TABLE 3. *Quantitation of chloroform-methanol-extracted phospholipids from EDP208 and ColB2 pilins*

Fraction	Type of pilin	Amt of pilin present (nmol)	Amt of phosphate (nmol)	Phosphate/pilin
G-200 purified peak II pilin	EDP208	166	473	2.9
	ColB2	161	499	3.1
Chloroform layer	EDP208	ND[a]	340	
	ColB2	ND	370	
Chloroform-precipitated pilin pellet	EDP208	135	<5	<0.04
	ColB2	131	47	0.60
Phosphatidylglycerol	EDP208		220	
	ColB2		150	
Phosphatidylethanolamine	EDP208		120	
	ColB2		135	

[a] ND, None detected.

(glucose, galactose, a pentose, and dideoxyhexose) suggested they may represent lipopolysaccharide contamination.

Although SDS-gel filtration chromatography removed trace amounts of phosphate from pilin, the remaining pilin material still contained 3 mol of phosphate per mol of pilin. This was found to be primarily phosphatidylglycerol (about 2 mol/mol of protein) and phosphatidylethanolamine (about 1 mol/mol of protein), which were removed almost quantitatively by chloroform-methanol extraction. Interestingly, extraction of *E. coli* K-12 membrane preparations revealed that 90% of the membrane phospholipid was phosphatidylethanoamine and only 10% was phosphatidylglycerol. Why the latter should be enriched in pilin-associated phospholipids is unclear. The fact that a preponderance of phosphatidylglycerol over phosphatidylethanolamine was also seen in ^{32}P-labeled phospholipids adsorbed by pili from cell-free culture supernatants suggests that either (i) phosphatidylglycerol is excreted preferentially by growing *E. coli* cells, or (ii) the pili have a greater affinity for phosphatidylglycerol than for phosphatidylethanolamine.

When the foregoing studies were performed on ColB2 pili, the results were less clear. Although the same four monosaccharides were found associated with ColB2 as with EDP208 pili, they were not all removed by SDS-gel filtration chromatography. Careful gas chromatography and thin-layer chromatography studies on pilin samples which had been subjected to either mild acid hydrolysis or methanolysis showed that the SDS-G200 Sephadex column-purified ColB2 pilin still contained 0.8 to 1.0 mol of glucose per mol of pilin, even after an additional extraction step with acetone. It was therefore concluded that ColB2 pili may contain one co-

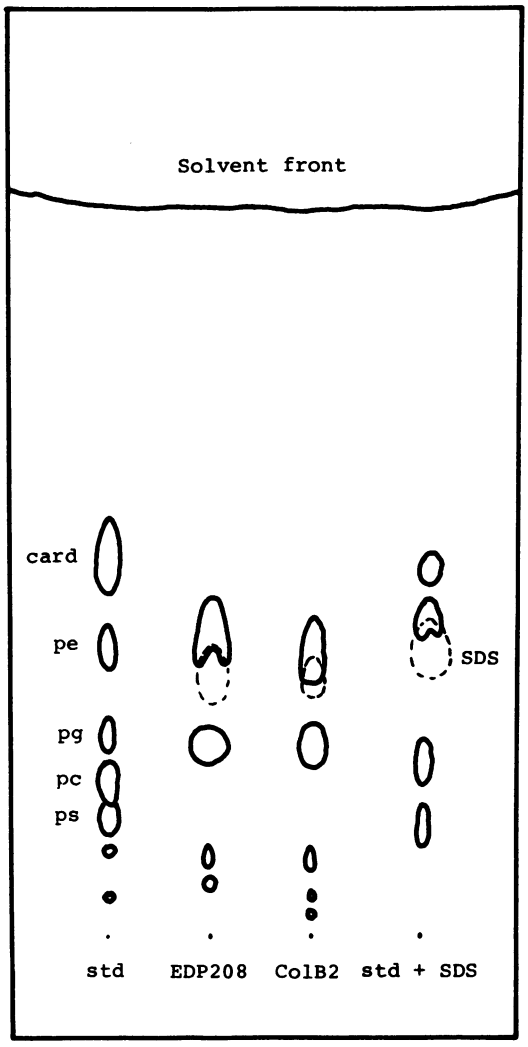

FIG. 4. *Thin-layer chromatography of chloroform-methanol extracts from EDP208 and ColB2 pi. Standard mixtures included 10 μg each of cardiolipin (card), phosphatidylethanolamine (pe), phosphatidylglycerol (pg), phosphatidylcholine (pc), and phosphatidylserine (ps). A second standard mixture also contained 10 gmg of SDS, since this was present in the chloroform-methanol extracts of pilin, and affected the mobilities of pilus-associated lipids. The chromatography solvent system was chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, vol/vol). Phospholipid spots were visualized by iodine vapors or, in the case of phosphatidylethanolamine and phosphatidylserine, by spraying the chromatograms with the cadmium-ninhydrin reagent and heating at 60°C for 5 min. SDS was visualized by charring the chromatograms at 350°C, after spraying with 3% potassium chromate in 60% sulfuric acid.*

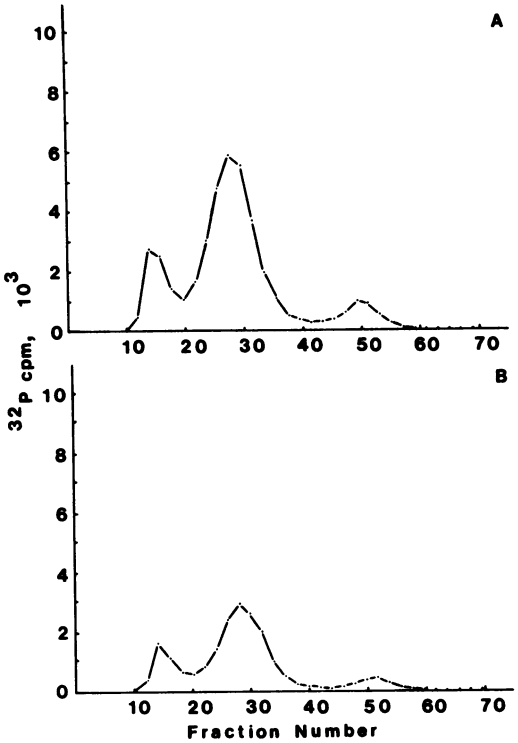

FIG. 5. *Sephadex G-200 column chromatography of ^{32}P-labeled EDP208 pili purified from cell-free supernatant solutions of EDP208/JC6256 (A) and JC6256 (B). The pili were dissociated in 1.0% SDS and then chromatographed on G-200 columns (1 cm by 23 cm) using 0.1 M Tris-hydrochloride (pH 8.3), 1 mM EDTA, and 1.0% SDS. Fractions (0.4 ml) were collected at a flow rate of about 7.5 ml/h. Volumes (0.1 ml) from each fraction were applied to 25-mm 3 MM Whatman filter disks, dried, and then counted in a Beckman LS250 scintillation spectrometer using toluene-based scintillation fluid.*

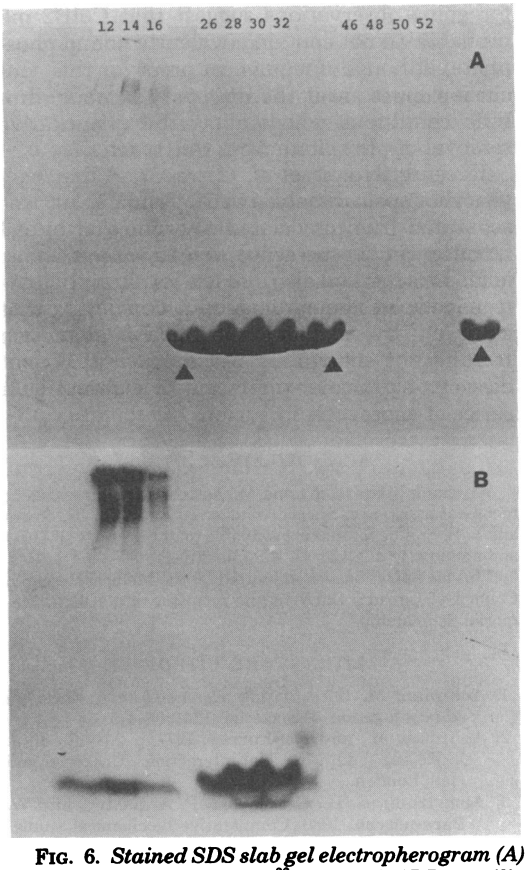

FIG. 6. *Stained SDS slab gel electropherogram (A) and autoradiogram (B) of ^{32}P-labeled EDP208 pilin obtained from the column eluate shown in Fig. 5A. A 50-μl volume of the selected column fractions, indicated by the numbers above each gel slot, was electrophoresed at 50 mA until the bromophenol blue dye marker had migrated approximately 9 cm from the origin. The fraction numbers correspond to the elution profile shown in Fig. 5A. After electrophoresis, the gel was fixed for 12 h in a solution containing 40% methanol and 10% acetic acid and stained by the method of Fairbanks et al. (14). To detect ^{32}P-labeled compounds, the gel was exposed to Kodak X-ray film for 72 h. Unlabeled EDP208 pilin was also electrophoresed in the positions indicated by the darts (▲).*

valently bound glucose per molecule of pili. However, until an appropriate glucose-containing peptide is isolated and characterized, the alternative possibility remains that the glucose may not be covalently bound, but tightly associated with pilin in a manner which makes it resistant to SDS or acetone extraction.

Similarly, single or repeated extractions of ColB2 pilin with acetone and/or chloroform-methanol did not result in the complete removal of phosphate. Approximately 0.4 mol of phosphate per mol of pilin remained after acetone extraction, and 0.6 mol of phosphate per mol of pilin remained after chloroform-methanol extraction. Since these values are significantly lower than unity, it was concluded that the remaining phosphate is more likely to represent tightly bound contaminant material than covalently bound phosphate. This argument was supported by the ^{31}P NMR studies in which there was no evidence of phosphomonoester linkages to amino acid residues. Rather, the only signals detected in the NMR instrument were identical to those obtained with mixtures of phosphatidylglycerol and phosphatidylethanolamine. It might be argued that a covalently bound phosphate with little or no freedom of rotation may not be detected under these conditions. However, the overall rotation time with a sample containing monomeric subunits would be sufficiently short to detect these signals. Thus, the

foregoing observations suggest that ColB2 pili probably do not contain covalently bound phosphate, although unequivocal proof for this conclusion must await the discovery of nonhydrolytic conditions which allow the quantitative removal of phosphate from ColB2 pili.

Recently Huang et al. (2) reported that bacteriorhodopsin dissolved in 5% Triton X-100 was separated from associated phospholipid by gel filtration in the presence of 0.25% deoxycholic acid. This method also dissociates phospholipids from pilin at about the same efficiency as that achieved by chloroform-methanol extraction (results not shown). However, the pili do not dissociate into monomers but are eluted as a series of aggregates of various sizes.

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