INFECTION AND IMMUNITY, Jan. 1984, p. 224–232
0019-9567/84/010224-09$02.00/0

Vol. 43, No. 1

Two Physically and Serologically Distinct Lipopolysaccharide Profiles in Strains of *Bordetella pertussis* and Their Phenotype Variants

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Received 25 May 1983/Accepted 7 October 1983

The lipopolysaccharide (LPS) from nine strains representing 18 phenotype variants of *Bordetella pertussis* could be grouped into one of two distinct profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. One group, representing the wild-type LPS profile of *B. pertussis*, consisted of two silver-staining bands: a dominant brown-amber *a* band and a faster-migrating, minor, black-staining *b* band. The second group, representing a variant LPS profile, consisted of a single black-staining band of similar mobility to the *b* band in the wild-type profile. By electrophoretic transfer (Western) blot analysis, mouse antiserum raised against whole cells of Tohama I (prototype wild-type LPS strain) recognized only the *a* band from all strains/phenotypes possessing the wild-type LPS profile. In contrast, mouse antiserum raised against whole cells of 134 (prototype variant LPS strain) recognized all *b* bands, regardless of strain/phenotype, and could be shown to cross-react weakly with the *a* band from Tohama I. These results and results from cohemagglutination and immunodiffusion analyses support the classification of *B. pertussis* into one of two physiologically and serologically distinct LPS phenotypes: Lps AB for the wild-type profile and Lps B for the variant profile. The relationship of LPS type and phenotypic, or "phase," variation is discussed.

Lipopolysaccharide (LPS) is an integral component of the gram-negative bacterial outer membrane (9, 16). It is the molecule attributed with the multiple heat-stable (100°C, 1 to 2 h) biological activities of gram-negative bacterial endotoxin as well as the heat-stable O-antigenicity of gram-negative bacteria (31).

The LPS of *Bordetella pertussis* exhibits endotoxic activities similar to those of other gram-negative bacterial LPSs (11, 23, 27). In addition, *B. pertussis* LPS sensitizes mice to histamine (5), may affect lymphocytosis by lymphocytosis-promoting toxin (20), and may account for some of the reactogenicity associated with vaccines containing whole cells of *B. pertussis* (7, 13, 26).

The serology of *B. pertussis* LPS, however, is confusing, especially the association of LPS serotypes with certain phenotypic variants (PVs) or phases of *B. pertussis*. At one extreme, Kasuga et al. (17, 18) show (putative) LPS serotype to be phase specific. Two other groups, Ackers and Dolby (1) and Aprile and Wardlaw (2) report both intraphase and interphase differences in LPS serotype. At another extreme, Le Dur et al. (22) report no difference at all in the composition of LPS from any serological phase of *B. pertussis*.

More specifically, Kasuga et al. show that their phase I, intermediate phase, and phase III variants of *B. pertussis* all possess the same heat-stable (100°C, 2 h) O antigen. Only their rough phase organisms possess a serologically distinct heat-stable ϕ antigen(s).

The other groups based their studies on LPS extracted by the phenol-water (PW) method (35). Ackers and Dolby used double diffusion and bactericidal analysis to define two distinct serotypes of *B. pertussis* LPS. Aprile and Wardlaw also described two LPS serotypes in *B. pertussis* but did so using immunodiffusion and passive immune lysis of LPS-coated erythrocytes. In both of these typing systems a single phase I strain (strain 134) was shown to possess the same LPS serotype as certain strains labeled phase IV. The remainder of phase IV strains, observed by both groups, possessed the LPS serotype found in all other (i.e., non-134) phase I strains.

LeDur et al. describe two chemically distinct LPSs in the PW extracts of *B. pertussis* (8). The ratio of LPS I to LPS II, they report, is 2:3, regardless of whether the LPS is extracted from a phase I or a phase IV.

As we show in the accompanying paper (30), *B. pertussis* which is capable of growth on nutrient agar (i.e., putative phase III or phase IV) can possess either a wild-type two-band LPS pattern or a single, fast-migrating LPS band when observed by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

I was interested in the relationships of these two SDS-PAGE LPS types with the consistent observation of two serological LPS types as described above and with the LPS I and LPS II described by LeDur et al. (21, 22).

Nine strains of *B. pertussis* representing 18 PVs, including most of those tested by Ackers and Dolby (1) and Aprile and Wardlaw (2, 3), were compared by SDS-PAGE and Western blotting. By these two techniques all 18 PVs could be assigned to one of two LPS phenotypes, either wild-type (Lps AB) or variant (Lps B). The data reported here identify the two immunological specificities of LPS in the systems of Aprile and Wardlaw (2, 3) and Ackers and Dolby (1) as lying within the *a* and *b* bands of *B. pertussis* LPS.

MATERIALS AND METHODS

Organisms, phenotype designations, and growth conditions. The strains of *B. pertussis* used in this study, their sources, phase designations, colonial phenotypes, and LPS serotypes based on hemagglutination inhibition (3) and bactericidal antigen (10) are listed in Table 1. Unless otherwise stated, organisms were grown on Bordet-Gengou agar (BGA) as previously described (29).

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TABLE 1. Strains of *B. pertussis* and their PVs, including LPS serotypes, where tested

Strain	Source[a]	Phase designation	Original BGA colonial phenotype(s) received[b]	Previously reported LPS serological type	
				By hemagglutination inhibition[c]	By bactericidal antibody production[d]
Tohama I	Munoz	I	Dom^+ Hly^+ Gna^-		
Tohama I			Dom^- Hly^- Gna^-[e]		
Tohama I			Dom^- Hly^- Gna^+[e]		
134	Wardlaw	I	Dom^+ Hly^+ Gna^-	BD	Low
134			Dom^- Hly^- Gna^-[e]		
134			Dom^- Hly^- Gna^+[e]		
11089 (L84)	NCTC	I	Dom^+ Hly^+ Gna^-	AE	High
10902 (L84)	NCTC	IV	Dom^- Hly^- Gna^+ Mil^-[f]	AE	High
10902 (L84)			Dom^- Hly^- Gna^+ Mil^+		
D3148	Dolby	I	Dom^+ Hly^+ Gna^-		Low
D3148		IV	Dom^- Hly^- Gna^+		Low
11615	ATCC	IV	Dom^- Hly^- Gna^+ Mil^+	BD	
11615			Dom^- Hly^- Gna^+ Mil^-		
10901 (364)	NCTC	IV	Dom^- Hly^- Gna^+ Mil^-	BD	Low
10901 (364)			Dom^- Hly^- Gna^+ Mil^+		
Sakairi	Sekiya	III	Dom^- Hly^- Gna^+		
Tohama III	Weiss	III	Dom^- Hly^- Gna^+		

[a] Munoz, J. J. Munoz, Rocky Mountain Laboratories, Hamilton, Mont.; Wardlaw, A. Wardlaw, Department of Microbiology, University of Glasgow, Glasgow, United Kingdom; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdon; Dolby, J. M. Dolby, Clinical Research Centre, Harrow, Middlesex, United Kingdom; ATCC, American Type Culture Collection, Rockville, Md.; Sekiya, K. Sekiya, Kitasato University, Tokyo, Japan; Weiss, A. Weiss, Department of Medical Microbiology, Stanford University School of Medicine, Stanford, Calif.

[b] The Dom^- Hly^- Gna^- PV was isolated from the Dom^+ Hly^+ Gna^- PV of the same strain as previously described (29). The Dom^- Hly^- Gna^+ PVs were isolated from the Dom^- Hly^- Gna^- PV as previously described (30).

[c] Data from Aprile and Wardlaw (3).

[d] Data from Dolby and Ackers (10).

[e] Additional phenotypes isolated.

[f] Colonial morphology variants on BGA: Mil^+, milky; Mil^-, nonmilky.

SDS-PAGE. The discontinuous buffer system of Laemmli was used as previously described (29). For certain comparisons, however, the total acrylamide concentration in the separating gel was varied from 12.5 to 16.0% as noted in the figure legends.

When the profiles of whole-organism lysates were compared for both LPS and protein profiles in the same gel, the LPS was visualized by the silver stain technique of Hitchcock and Brown (15). The gel was then counterstained with 0.2% Coomassie brilliant blue R-250 as described previously to visualize protein bands (29). To visualize only the LPS in whole-organism lysates, BGA-grown organisms were washed, suspended in lysing buffer, and boiled for 5 min as previously described to observe protein profiles (29). Ten micrograms of proteinase K (Boehringer Mannheim Corp., Indianapolis, Ind.) in 10 μl of digestion buffer was then added per 50 μl of organism suspension (approximately 150 μg of organism protein), and the mixture was incubated in a water bath at 56°C for 2 h (15) with occasional vortexing. The samples were boiled for 5 min, and 10 μl of the digested organisms was applied per lane and electrophoresed as before. For gels run with proteinase K-treated organisms or for chemically extracted LPS, the silver stain of Tsai and Frasch was used to visualize LPS (33). Color differences in all silver-stained LPS were observed after equilibration of the polyacrylamide gel in either 7% (vol/vol) glacial acetic acid or 25% (vol/vol) isopropanol–7% (vol/vol) glacial acetic acid.

Isolation of LPS. Some of the phase IV organisms would not grow in Stainer-Scholte broth (SSB) (32), so for pilot experiments on interstrain comparisons, LPS was extracted from organisms grown for 5 days at 37°C on 10 to 20 BGA plates. Initially, the hot PW procedure described by Westphal and Jann (34) was used with a minor modification. It was necessary to increase the volume of 45% (vol/vol) phenol to 10 times that recommended to adequately suspend the organisms. Aqueous extracts were precipitated with 2 volumes of ice-cold acetone overnight, and then washed once at 10,000 × *g* for 15 min with cold 70% (vol/vol) aqueous acetone to remove the phenol. The pellets were resuspended in 10 ml of distilled water and ultracentrifuged at 100,000 × *g* for 2 h. The pellets were washed twice more with distilled water and then lyophilized.

The aqueous phenol-chloroform-petroleum ether extraction procedure (PCP) of Galanos et al. (12) was used to more efficiently extract the variant LPS from *B. pertussis*. Organisms grown on BGA as described above or in SSB as described below were harvested and washed twice with distilled water. The washed organisms were then extracted by the original procedure (12), except that 45 ml of PCP was used per 2.5 g of organisms (dry weight) to facilitate mixing. For bulk preparation of LPS, organisms were grown in 15 liters of sterile-filtered original SSB (32) in 5-gallon plain Pyrex solution bottles. The bottles were fitted with rubber stoppers possessing a short stainless steel air exit tube and a long stainless steel air inlet tube (approximately 8 mm outer diameter, 6 mm inner diameter each). The long inlet tube was threaded at the bottom to accept a threaded gas diffuser stone (VWR Scientific, Seattle, Wash.; catalog no. 32573-007). When assembled, the chromic acid-cleaned bottles were autoclaved and cooled, and sterile medium (0.2 μm membrane filtered) was infused through the bottle's inlet tube by attaching the exit tube to a house vacuum. The bottles were then placed in a 37°C incubator, and the medium was aerated by connecting the exit tube to a house vacuum and the inlet tube to a 0.2-μm sterile filter (Pall no.

DFA 3001AC; Pall Trinity Corp., Cortland, N.Y.). A KOH trap and flow meter were connected in line with the exit tubing. Air flow was set at 3 to 4 liters/min. This flow rate was adequate to affect good mixing as well as good aeration of the medium. The 15 liters of medium was inoculated with 100 ml of an SSB starter culture which had itself been inoculated with a single 5-day-old colony of BGA-grown organisms and then incubated with gyratory shaking at 37°C for 2 to 3 days. The 15-liter cultures were grown for 3 days, and samples were plated for purity. One and one-half grams of merthiolate was dissolved in a small volume of saline and added to the bottles, and the bottles were placed at 4°C overnight. Organisms were collected by centrifugation at 10,000 × *g* for 10 min at 4°C, and the LPS was extracted by one of the methods described above.

Two reference LPSs from *Salmonella typhimurium* were kindly supplied by C. McLaughlin, Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, Hamilton, Mont. One was a PW-extracted smooth LPS from strain LT_2, and the other was a PCP extract of an Re mutant strain, SL1102.

Chemical and biological assays. The protein content of the bulk LPS preparations was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.) which uses the Coomassie brilliant blue G-250 binding procedure of Bradford (6). RNA was assayed by the orcinol reaction adapted for whole bacterial cells (14). The ability of the LPS preparations to gel a *Limulus* amoebocyte lysate was quantitated by using a kit from Haemachem, Inc. (St. Louis, Mo.) which included reference endotoxin from *Escherichia coli* O55:B5.

Immunization. Whole-cell *B. pertussis* vaccines were prepared by harvesting the growth from single colonies streaked onto BGA and grown for 3 days at 37°C. The organisms were suspended in 10 ml of 54 mM disodium hydrogen phosphate–13 mM potassium dihydrogen phosphate–73 mM sodium chloride (pH 7.4) (PBS) and centrifuged at 8,000 × *g* for 10 min. The pellets were washed once in 10 ml of PBS and then resuspended in PBS to an absorbance at 540 nm of 0.2 in 13- by 100-mm test tubes (approximately 2×10^{10} organisms per ml). The organisms were killed by incubation at 56°C for 30 min and cooled, merthiolate was added to a final concentration of 0.01% (wt/vol), and the vaccine was stored at 4°C. Ten 7- to 8-week-old female CFW mice were inoculated intraperitoneally with 0.2 ml of vaccine every 3 days for a total of four inoculations. Immunization was stopped for a period of 4 weeks and then resumed with the same regimen as before for three further inoculations. Ten days after the last inoculation, the mice were exsanguinated, and their sera were pooled, filter sterilized, and stored at 4°C.

Cohemagglutination technique. Anti-LPS titers of immune sera were determined by a modification of the indirect hemagglutination technique of Neter et al. (28) as described by McCabe (24). Rabbit erythrocytes were sensitized with saponified LPS and adjusted to 0.5% packed volume in PBS containing 0.02% (wt/vol) sodium azide (PBSA). Sera were diluted serially twofold in 50-μl volumes in PBSA containing 0.1% (wt/vol) bovine serum albumin (BSA) (Cohen fraction V; Miles Laboratories, Inc., Elkhart, Ind.) in round-bottomed polystyrene microtiter trays. To each well was added 50 μl of LPS-sensitized erythrocytes. The suspensions were mixed gently, and the trays were covered with tape and incubated for 0.5 h at 37°C with mixing at 200 rpm on a gyratory shaker. The trays were then centrifuged at 1,000 × *g* for 10 min, the supernatant fluids were aspirated, and the cells were resuspended in PBSA with BSA and centrifuged again. Each well of washed cells was resuspended in 50 μl of PBSA with BSA containing 0.25% (wet wt/vol) *Staphylococcus aureus* Cowan I prepared by the method of Kessler (19) by S. Stewart, Laboratory of Microbial Structure and Function. The trays were reincubated for 15 min with shaking, and the endpoints were read when the cells settled.

Electrophoretic transfer (Western) blotting. Whole *B. pertussis* cells were subjected to slab gel SDS-PAGE as described above. Electrophoresis was stopped when the dye front was 5 mm from the end of the gel. The gel was removed and transferred to a polypropylene tray containing 50 mM sodium phosphate buffer (pH 7.5). A nitrocellulose membrane (NCM) large enough to cover the entire gel (Millipore Corp., Bedford, Mass.; catalog no. HAHY 00010; 0.45 μm pore size) was carefully wetted in the phosphate buffer and laid on the acrylamide slab. The gel was then assembled into a Bio-Rad Trans-blot apparatus (Bio-Rad Laboratories) according to the manufacturer's instructions and electrophoresed at 27 V 1.8 A for 2 h in 50 mM sodium phosphate buffer (pH 7.5) (4).

The NCM was measured and cut into strips, inserted into a heat-sealable bag, and blocked with 10 ml of 3% BSA in 10 mM Tris–150 mM sodium chloride–0.02% sodium azide (pH 7.0) (BTAS) for 1 h at ambient temperature on a rocking platform. The BTAS was poured off, 10 ml of a 10^{-4} dilution of antiserum in BTAS was then added, and the NCMs were further incubated for 2 h at ambient temperature with rocking. The NCMs were then removed from their bags, placed in polypropylene boxes, and washed five times with 250 ml of 0.15 M sodium chloride. The washed NCMs were placed into fresh heat-sealable bags, and 10^5 cpm of ^{125}I-labeled protein A (specific activity, approximately 8 μCi/μg) was added in 10 ml of BTAS. The NCMs were incubated with rocking as before for 2 h, washed five times with 0.15 sodium chloride as described above, and dried at 37°C between fine-pore sponges. The dried NCMs were mounted on stiff paper, and Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) was overlaid with a Cronex lightning-plus screen (E. I. duPont de Nemours and Co., Wilmington, Del.). The film was exposed for 5 h at −70°C and then developed with a Kodak X-Omat M20 processor.

Hydroxylapatite chromatography of LPS. The method used was essentially that of LeDur et al. (22), except that spheroidal hydroxylapatite was used (BDH; Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y.), and after the LPS was applied to the column in 0.01 M sodium phosphate–0.1% SDS (BDH; specially pure; product no. 44244) (pH 6.4), a linear gradient of 0.1 to 1.0 M sodium phosphate (pH 6.4) containing 0.1% SDS was applied to elute the component LPS bands. To accommodate the high molarities of sodium phosphate and to keep the SDS in solution, chromatography was done at 37°C. Fractions were collected dropwise in approximately 1-ml volumes. The phosphate gradient was monitored by measuring the conductivity of each fraction. To monitor elution of LPS, a sample of each fraction was added to an equal volume of Laemmli (29) sample preparation mix and heated in a boiling water bath for 5 min. Ten microliters was applied per lane for SDS-PAGE. The silver stain of Tsai and Frasch (33) was used to visualize the LPS bands in the gel.

RESULTS

Pilot experiments: qualitative and quantitative differences in various LPS extracts. Yields of 0.4, 0.3, and 0.5% of bacterial dry weight were obtained when the PW procedure was used to obtain LPS from the Dom^+ Hly^+ Gna^-, Dom^- Hly^-

Gna$^-$, and Dom$^-$ Hly$^-$ Gna$^+$ PVs, respectively, of strain Tohama I. In the same pilot experiment, however, strain 11615, Dom$^-$ Hly$^-$ Gna$^+$, which bears the variant LPS (30), yielded only 0.1%. Equal dry weight samples of each of these preparations were compared to each other and to smooth and Re LPS from *S. typhimurium* by SDS-PAGE and silver staining.

Figure 1 shows that the LPS profiles from all three PVs of Tohama I were similar and consisted of a major slower-migrating, brown-amber band (band *a*) and a faster-migrating, minor, black-staining band (band *b*). A single band is seen in strain 11615 which appears identical to the *b* band both in mobility and in black coloration with the silver stain. Differences in the amount of band *b* can be seen in three Tohama I PVs. Several minor bands above band *a* in the Tohama I PVs are also visible (brackets). These bands appeared inconsistently and may reflect artifactual aggregation due to variations in solubilization.

Figure 1 shows the typical (15, 25) ladder profile of smooth-type LPS from *S. typhimurium* and the abbreviated profile of an Re mutant from the same species. In comparison, the mobility and profile of *B. pertussis* LPS appeared to resemble mutants of salmonellae with intermediate-length oligosaccharide cores (15, 25). Similarly, the *b* band in *B. pertussis* migrated like an LPS with a shorter core than the LPS represented by the *a* band. From the data in Fig. 1, it seemed reasonable that the low yields of strain 11615 LPS by PW might be increased by using the PCP extraction procedure of Galanos et al. (12). To test this, another strain bearing only the *b* band, strain 10901 (30), was grown on BGA, harvested, washed in distilled water, and lyophilized. The dried organisms were divided into four equal samples: two were extracted by PW and two by PCP. The percent bacterial dry weight yields were 0.2 and 0.3% for PW extracts, whereas the PCP extracts gave yields of 0.8 and 0.9%. Five micrograms of each of the four preparations were compared to each other and to the PW preparations from strains 11615, Dom$^-$ Hly$^-$ Gna$^+$, and Tohama I, Dom$^+$ Hly$^+$ Gna$^-$, by SDS-PAGE and silver staining. As shown in

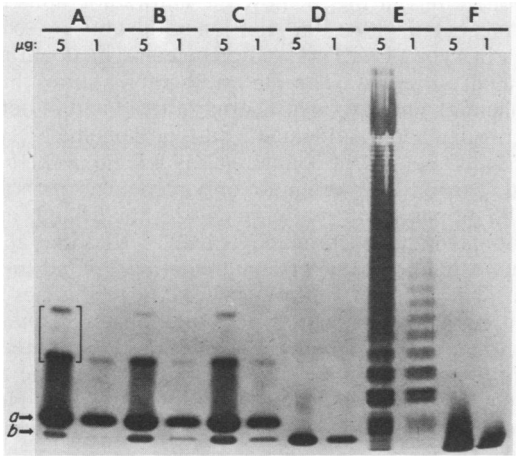

FIG. 1. SDS-PAGE (12.5% [wt/vol] total acrylamide, Tsai and Frasch silver stain, [33]) of PW extracts from *B. pertussis* Tohama I, PVs Dom$^+$ Hly$^+$ Gna$^-$ (A), Dom$^-$ Hly$^-$ Gna$^-$ (B), Dom$^-$ Hly$^-$ Gna$^+$ (C), and strain 11615, PV Dom$^-$ Hly$^-$ Gna$^+$ Mil$^+$ (D) are compared with a PW extract of *S. typhimurium* wild-type strain LT_2 (E) and a PCP extract of *S. typhimurium* Re mutant strain SL1102 (F). Samples of 5 and 1 μg (dry weight) were applied for each strain as indicated. The *a* and *b* bands of the *B. pertussis* LPS are marked by arrows.

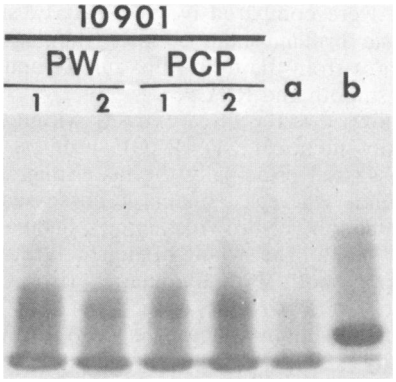

FIG. 2. Qualitative comparison of 5 μg of the extracts from *B. pertussis* 10901, PV Dom$^-$ Hly$^-$ Gna$^+$ Mil$^+$, as prepared by the PW and PCP methods. Duplicate extractions by each procedure are represented by lanes 1 and 2. These samples are further compared with PW extracts from *B. pertussis* 11615, PV Dom$^-$ Hly$^-$ Gna$^+$ Mil$^+$ (lane a) and Tohama I, PV Dom$^+$ Hly$^+$ Gna$^-$ (lane b). Conditions of SDS-PAGE and gel staining and the PW extracts of 11615 and Tohama I are the same as for Fig. 1.

Fig. 2, the method of extraction made little difference in the SDS-PAGE profiles of the preparation from strain 10901. All four preparations of 10901 showed the same *b* band type mobility and black coloration as the PW extract of strain 11615, although more tailing material was seen in the preparations from 10901. As in Fig. 1, the PW extract from Tohama I, Dom$^+$ Hly$^+$ Gna$^-$, showed a prominent brown-amber *a* band and a minor *b* band, a profile distinctly different from that of strain 10901.

Bulk isolation of *B. pertussis* LPS. The strains and PVs of *B. pertussis* used for bulk preparation of LPS were selected for their ability to grow well in SSB and for absence of virulence-associated characteristics (29). The Dom$^-$ Hly$^-$ Gna$^-$ PV of strain Tohama I was selected as a source of wild-type LPS. However, because 11615 and 10901 are both Dom$^-$ Hly$^-$ Gna$^+$ PVs which possess considerable heterogeneity, including growth characteristics (30), a more reliable Dom$^-$ Hly$^-$ Gna$^-$ PV was selected from the Dom$^+$ Hly$^+$ Gna$^-$ PV of strain 134 as previously described (29).

Three separate 15-liter bulk cultures were grown from strain 134, Dom$^-$ Hly$^-$ Gna$^-$, and Tohama I, Dom$^-$ Hly$^-$ Gna$^-$. To optimize yields as reported above, the variant LPS from strain 134 was extracted with PCP; the wild-type LPS from Tohama I was extracted by PW. Yields of crude LPS from the three bulk cultures were 1.4, 3.8, and 3.8% from strain 134 and 2.0, 3.5, and 2.0% from Tohama I. These yields were overall greater than the yields from the pilot experiments described above. This probably reflects greater efficiency in extraction from bulk (5 to 10 g [dry weight] of organisms) versus extraction and recovery from the small (100 mg [dry weight] of organisms) samples in the pilot experiments. It is also possible that SSB-grown organisms from the bulk cultures extracted more efficiently than the BGA-grown organisms used for the pilot experiments or that agar artifactually inflated the yields of organisms from the BGA plates.

By weight, all the bulk LPS preparations had less than 3.0% contaminating RNA and less than 5.0% protein. Endpoints for gelation of *Limulus* amoebocyte lysate were between 15 and 30 pg/ml for all preparations; the *E. coli* reference endotoxin gave an endpoint of 30 pg/ml in the same test.

Five-microgram samples of the extracts from each of the

bulk cultures were compared by SDS-PAGE, silver stain, and Coomassie brilliant blue counter staining to whole-organism lysates from the third bulk culture and also to *S. typhimurium* smooth and Re LPS.

Figure 3 shows that the three extracts within each strain give essentially identical SDS-PAGE profiles. However, strain 134 possessed principally the faster-migrating black-staining *b* band, whereas Tohama I possessed both the slower-migrating, amber-brown-staining *a* band and a lesser amount of *b* band. The whole-organism lysate from the respective strains both show the characteristic Coomassie-stained profile of a *B. pertussis* of the Dom$^-$ Hly$^-$ Gna$^-$ PV (29). Based on colonial morphology of BGA, no reversion to the Dom$^+$ Hly$^+$ Gna$^-$ PV was noted in any fermentor culture at frequencies as low as 3×10^{-4}. The SDS-PAGE profiles of the whole-cell lysates also show different staining patterns by silver, characteristic of the type of LPS each strain bears. These whole-cell LPS profiles are more distorted than previously shown (30) and may be an artifact of reboiling the samples after 2 weeks of storage at −20°C in sample digestion mix. Regardless, both the extracts and their respective whole-cell lysates show silver-staining profiles of slower mobility than *S. typhimurium* Re chemotype LPS, but considerably simpler than the "ladder" of the smooth type LPS from the same species.

Serological reactions of mouse antisera and *B. pertussis* LPS. Polyclonal mouse antisera were raised to whole cells of strain 134, Dom$^-$ Hly$^-$ Gna$^-$, and Tohama I, Dom$^-$ Hly$^-$ Gna$^-$, as described above. To specifically test for anti-LPS titers in these whole-cell antisera, the extracted LPS from 134 and Tohoma I were attached to rabbit erythrocytes and tested by cohemagglutination as described above. The result showed a "one-way" cross-reactivity of the sera to the LPS of the heterologous strain: anti-134 recognized both its homologous LPS and the LPS of Tohama I to similar high reciprocal titers of approximately 10,240, but anti-Tohama I recognized only the LPS from Tohama I at 10,240; the LPS from 134 was recognized weakly at 160. Double diffusion analysis gave a similar pattern of one-way cross-reactivity (Fig. 4). Precipitin lines are visible between anti-Tohama I (A) and Tohama I LPS (a) but not between anti-Tohama I and 134 LPS (b). In contrast, anti-134 (B) forms a precipitate with both Tohama I LPS and 134 LPS.

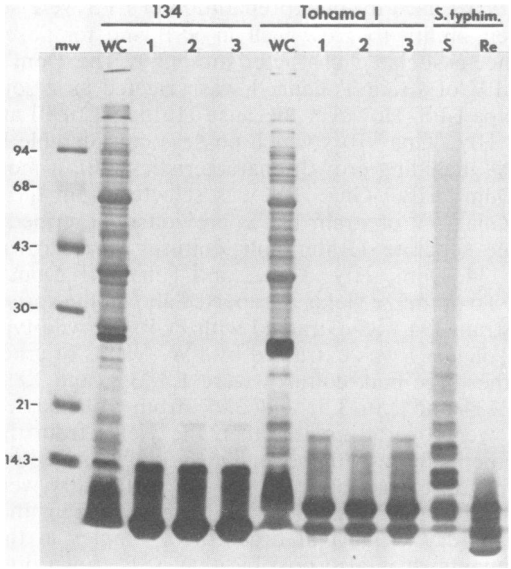

FIG. 3. Qualitative comparison of 5 μg of LPS from three separate fermentor cultures (1, 2, and 3) of *B. pertussis* 134, PV Dom$^-$ Hly$^-$ Gna$^-$, extracted by PCP, and Tohama I, PV Dom$^-$ Hly$^-$ Gna$^-$, extracted by PW. Whole-cell lysates (WC) of the third fermentor culture of each strain are included for comparison with their respective LPS. Five micrograms of the same *S. typhimurium* LPS preparations in Fig. 1 is also shown, as well as protein molecular weight markers (mw). The SDS-PAGE contained 16% [wt/vol] total acrylamide to improve the resolution of the LPS bands. The gel was silver stained by the Ag-LPS method of Hitchcock and Brown for LPS (15) and counter stained for protein by Coomassie brilliant blue R-250.

I next investigated the relationship of the one-way cross-reactions in double diffusion and cohemagglutination to the banding patterns seen on silver-stained SDS-PAGE. First, the *a* and *b* bands from Tohama I LPS were separated from each other and from residual nucleic acid and protein (22) by hydroxylapatite chromatography in SDS. The fractionation was monitored by SDS-PAGE and silver staining (Fig. 5). The linear 7 to 32 mmhos/cm (0.1 to 1.0 M) sodium phosphate gradient (pH 6.4) with 0.1% SDS was started at fraction 55 (not shown). Until about fraction 80, the principal band eluted was the slower-migrating, brown-amber-stained *a* band. Starting around fraction 80, or about 15 mmhos/cm on the gradient, however, an increasing amount of the faster-migrating, black-staining *b* band was eluted. A 5-μg sample of the same Tohama I LPS applied to the hydroxylapatite column was included in the SDS-PAGE gel as an unfractionated control. 134 LPS was also included in the gel for reference.

Fractions 76 and 95 were selected as reasonably pure (by silver stain) preparations of *a* and *b* bands, respectively, for antigenic analysis by Western blotting. The unfractionated LPS from Tohama I and 134 were included for reference. The top photograph in Fig. 6 shows one of the silver-stained SDS-PAGE gels after blotting. Sufficient material was retained in the gel to visualize the characteristic patterns of wild-type LPS from Tohama I (lane 1), its component *a* and *b* bands (lanes 2 and 3, respectively), and the variant LPS control from 134 with its single *b* band (lane 4). The bottom two photographs in Fig. 6 show the autoradiographs of the NCMs incubated with a 10^{-4} dilution of the antisera to either Tohama I (A) or 134 (B) and radiolabeled by incubation with ^{125}I-labeled protein A. The autoradiographs show that the anti-Tohama I serum recognizes only the *a* band in both the unfractionated and fractionated wild-type Tohama I LPS and detects faint amounts of contaminating *a* band in the *b* band fraction. The anti-134 serum not only recognizes its homologous LPS preparation but also recognizes the *b* bands in both unfractionated and fractionated Tohama I LPS and appears to detect a faint amount of contaminating *b* band in the *a* fraction. The anti-134 serum also weakly recognizes the *a* band in both unfractionated and fractionated Tohama I. Longer exposures of film on these blots or blots treated with 10^{-3} dilutions of the respective sera show the same cross-reactions but more intensely (data not shown). Normal mouse sera showed no reaction at a 10^{-2} dilution (not shown).

Eighteen PVs representing nine strains were tested by Western blotting to determine whether the seemingly identical mobilities of the *a* and *b* LPS bands from different isolates (30) were also antigenically similar. For simplicity in observing LPS patterns of reactivity, the whole-cell lysates of the test organisms were digested with proteinase K (15) instead of extracting each organism with PW or PCP. The chemically extracted LPS of Tohama I and 134 were includ-

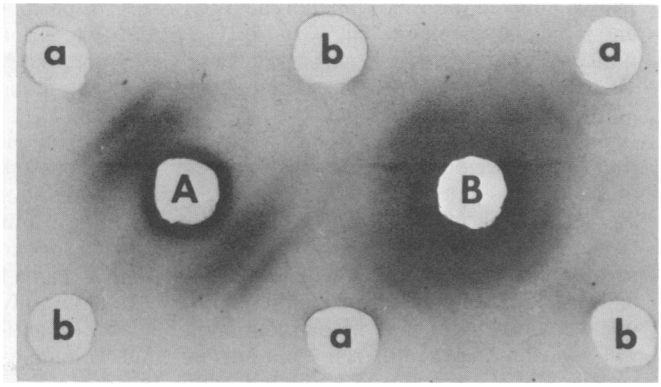

FIG. 4. Ouchterlony double diffusion of mouse hyperimmune sera raised against 56°C-killed *B. pertussis* whole cells versus their homologous and heterologous LPS preparations. The 0.5% agarose gel contained 0.1% sodium deoxycholate in $\mu = 0.04$ barbital buffer (pH 8.6). (A) 40 μl of a 1/2 dilution of mouse anti-Tohama I, PV Dom$^-$ Hly$^-$ Gna$^-$; (B) 40 μl of undiluted mouse anti-134, PV Dom$^-$ Hly$^-$ Gna$^-$; (a) 40 μl of 100 μg of PW-extracted LPS from strain Tohama I PV Dom$^-$ Hly$^-$ Gna$^-$ per ml; (b) 40 μl of 50 μg of PCP-extracted LPS from strain 134 PV Dom$^-$ Hly$^-$ Gna$^-$ per ml. Diffusion proceeded for 3 days at ambient temperature, and the gel was cut out of its petri dish, compressed onto a glass slide, washed in saline and then distilled water, and finally dried before staining with 0.2% (wt/vol) amido black in 5% (vol/vol) glacial acetic acid–40% (vol/vol) methanol.

ed in the analysis as positive controls. The top photograph in Fig. 7 shows the silver-stained SDS-PAGE gel of the test organisms after blotting. All the strains and PVs shown possess either the wild-type *a*, *b* or the variant *b* profiles described above. In addition, the profiles of the solvent-extracted control LPS are indistinguishable from the proteinase K-treated whole organisms of the homologous strain and PV (lanes 1 and 4 and 2 and 7, respectively). The bottom two photographs in Fig. 7 show the autoradiographs of the NCMs after blotting and incubation with a 10^{-4} dilution of mouse antisera to Tohama I (A) or 134 (B) and ^{125}I-labeled protein A as before. Regardless of the strain or PV of origin, anti-134 recognizes the *b* band, whereas anti-Tohama I recognizes only the *a* band. Thus, for the strains and PVs shown here, all the *a* bands appear antigenically related to each other. Similarly, all the *b* bands appear antigenically related to each other.

DISCUSSION

The *B. pertussis* strains and PVs studied here possess one of two LPS profiles after SDS-PAGE and silver staining. The wild-type pattern possesses mainly the slower-migrating, brown-amber-staining *a* band but also has lesser amounts of the faster-migrating, black-staining *b* band. This pattern is termed wild type because it was found in 12 strains in the Dom$^+$ Hly$^+$ Gna$^-$ (wild-type) PV of *B. pertussis* (M. S. Peppler, Abstr. XIII Int. Cong. Microbiol. 1982, P51:10, p. 135), and to date the only Dom$^+$ Hly$^+$ Gna$^-$ PV not to possess the wild-type LPS profile is from strain 134, as shown here. Strain 134, regardless of PV, and three other strains of the Dom$^-$ Hly$^-$ Gna$^+$ PV (11615, 10901, and D3148) show what I have called the variant LPS pattern on SDS-PAGE and silver staining. It consists of a single faster-migrating and black-staining band, indistinguishable from the *b* band of the wild-type LPS. As the Western blotting analysis suggests, the *b* bands of both wild-type and variant LPS are also antigenically very similar. Thus, the concept of two LPS phenotypes in *B. pertussis* is supported not only by their respective SDS-PAGE/silver-staining profiles but also by their unique antigenicities as determined by Western blotting. For simplicity, the two LPS phenotypes can be defined by the bands they possess on SDS-PAGE and silver stain: the wild-type pattern (e.g., strain Tohama I) being Lps AB and the variant pattern (e.g., strain 134) being Lps B.

Classification of *B. pertussis* LPS into two major groups is not without precedent. Aprile and Wardlaw (2, 3) used passive immune lysis, immune lysis inhibition, and double diffusion with rabbit anti-whole-cell sera to analyze the antigenic determinants of *B. pertussis* LPS. By testing with antisera to several strains (including two non-*B. pertussis*), as many as six antigenic determinants (A, B, C, D, E, and F) could be assigned to the isolated LPS from various *B. pertussis* strains (2). Antiserum raised to the homologous strain, however, showed specificity to, at most, two of these determinants (e.g., AC, AE, or BD). As a result, these authors could classify 10 strains of *B. pertussis* by passive hemagglutination inhibition into two exclusive groups, those

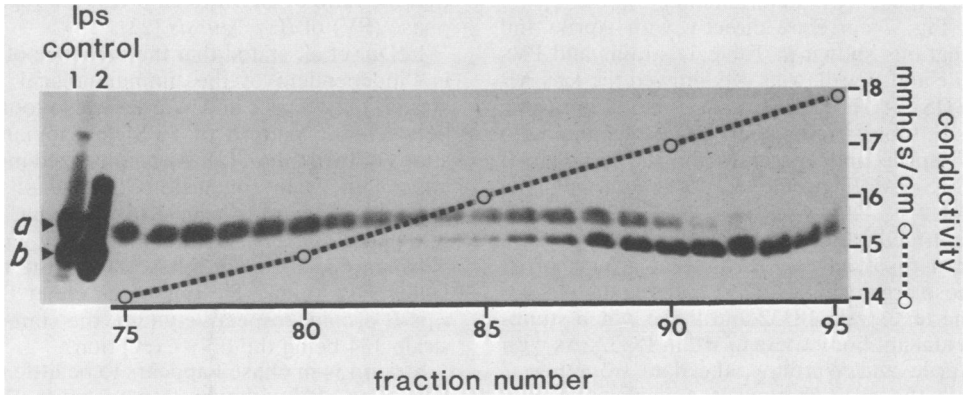

FIG. 5. SDS-PAGE (12.5% [wt/vol] total acrylamide, silver stained by the Tsai and Frasch technique) showing the resolution of the *a* and *b* bands of PW-extracted LPS from Tohama I, PV Dom$^-$ Hly$^-$ Gna$^-$, by hydroxylapatite chromatography. Five-microliter samples of fractions 75 through 95 are shown, and the conductivity (dotted line superimposed on gel) of the 0.1% (wt/vol) SDS (pH 6.4)–sodium phosphate gradient is expressed in mmho/cm. Unfractionated control LPS from Tohama I, PV Dom$^-$ Hly$^-$ Gna$^-$, is shown in lane 1, and its component *a* and *b* bands are designated by arrowheads. Lane 2 contains the PCP-extracted LPS of strain 134, PV Dom$^-$ Hly$^-$ Gna$^-$, showing its dominant *b* band for comparison.

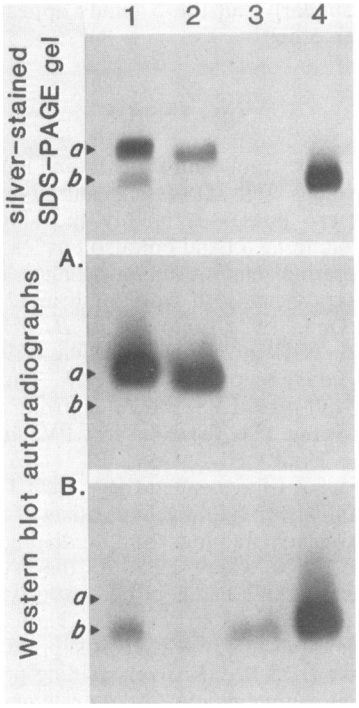

FIG. 6. Western blots and their respective SDS-PAGE profiles (16% [wt/vol] total acrylamide, Tsai and Frasch silver stain) of 5 μg of PW-extracted LPS from Tohama I, PV Dom$^-$ Hly$^-$ Gna$^-$ (lane 1), 5 μl of the hydroxylapatite fractions (76) (lane 2) and 95 (lane 3) of the Tohama I LPS from the separation shown in Fig. 5, and 5 μg of unfractionated PCP-extracted LPS from strain 134, PV Dom$^-$ Hly$^-$ Gna$^-$ (lane 4). The LPS and their fractions were separated in duplicate by SDS-PAGE and then electrophoretically transferred to an NCM. The NCM was cut in two, blocked with BSA, and incubated with a 10^{-4} dilution of hyperimmune mouse serum raised against the 56°C-killed whole cells of either *B. pertussis* Tohama I, PV Dom$^-$ Hly$^-$ Gna$^-$ (A), or 134, PV Dom$^-$ Hly$^-$ Gna$^-$ (B). After washing, the membranes were incubated with ^{125}I-labeled protein A, washed again, and dried, and an autoradiograph was made. The component *a* and *b* bands of the Tohama I LPS are denoted by arrowheads.

bearing LPS with exposed AE determinants and those bearing LPS with exposed BD determinants (3).

The results in Fig. 7 correlate directly with Aprile and Wardlaw's designations shown in Table 1: strains and PVs classified by Aprile and Wardlaw as AE showed the Lps AB phenotype by SDS-PAGE profile and *a* band antigenic specificity in Fig. 7; those strains and PVs corresponding to the BD class of Aprile and Wardlaw showed the Lps B phenotype by SDS-PAGE profile and *b* band antigenic specificity.

The complement-mediated bactericidal assay of Ackers and Dolby (1) also resolved two serological groups of *B. pertussis* LPS, those organisms which stimulated high bactericidal titers to the test strain 18332 and those which stimulated low bactericidal antibody titers to strain 18332. As with the system of Aprile and Wardlaw, the data from Fig. 7 correlate well with the classification of Ackers and Dolby shown in Table 1: those strains and PVs which stimulate high bactericidal titers to 18332, as defined by Ackers and Dolby, showed the Lps AB phenotype by SDS-PAGE profiles and *a* band antigenic specificity in Fig. 7; strains and PVs which elicited low bactericidal titers, as defined by Ackers and Dolby, showed the Lps B phenotype by SDS-PAGE profiles and *b* band antigenic specificity in Fig. 7. The only exception is D3148 phase I, which possessed Lps AB as previously shown (30), but nevertheless stimulated low bactericidal antibody (10).

The one-way cross-reactivity shown by cohemagglutination and double diffusion (Fig. 3) also agrees with the passive immune lysis and Ouchterlony immunodiffusion data of Aprile and Wardlaw (2). The only major difference in our respective results concerns the two precipitin bands seen in my double diffusion (Fig. 3) with anti-134 and both Tohama I and 134 LPS antigens; Aprile and Wardlaw (2) show only one weak precipitin band in their immunodiffusion with anti-134. Considering the differences in our diffusion techniques (Aprile and Wardlaw used undiluted rabbit antisera, 0.75% agarose gels containing unbuffered saline with no detergent, and 5 mg of LPS per ml as antigen), this difference is not surprising. Ackers and Dolby (1), using double diffusion conditions similar to those of Aprile and Wardlaw, also showed the inability of rabbit anti-18332 to precipitate 134 LPS. However, they did not test an anti-134 serum with their various LPS preparations and therefore did not report on one-way cross-reactivity.

LeDur et al. (22) showed that *B. pertussis* LPS could be physically separated into two unique fractions by hydroxylapatite chromatography in 0.1% SDS at 25°C. Using a stepwise elution, the first peak eluted with 0.4 M sodium phosphate and was termed LPS I; the second peak eluted with 0.6 M sodium phosphate and was termed LPS II. When I subjected Tohama I LPS to hydroxylapatite chromatography (Fig. 5) in 0.1% SDS at 37°C, the first band to elute with a linear gradient was the *a* band at 14 mmhos/cm (approximately 0.34 M sodium phosphate), followed by the *b* band at 17 mmhos/cm (approximately 0.42 M sodium phosphate). This suggests that the *a* band is LPS I, whereas the *b* band is LPS II. Although this is likely to be true, further chemical characterization of the wild-type *a* and *b* bands and the variant *b* band is needed to prove the correlation. Nevertheless, it should be noted that by both Western blotting and SDS-PAGE/silver stain, strain 134 (all three PVs) and the Dom$^-$ Hly$^-$ Gna$^+$ PV of strains 11615, 10901, and D3148 are devoid of *a* band. Furthermore, the amount of *b* band appears to vary in relation to the *a* band in the three PVs of Tohama I (Fig. 1A, B, and C; Fig. 7, lanes 3 to 5; and reference 30). These data dispute the claim of LeDur et al. that the ratio of LPS I to LPS II is 2:3 regardless of strain or phase (PV) of *B. pertussis* (22).

LeDur et al. stated that the presence of LPS I and LPS II is "independent of the 'immunological phase' of a given strain" (22). Aprile and Wardlaw also found "no correlation between the pattern of LPS determinants and the phase status of the strain" (3). As emphasized in the accompanying paper (30), such conclusions depend on how phase is defined. If all nutrient agar-growing (Gna$^+$) PVs are lumped into phase IV, this statement is true. If a distinction is made between phase III and phase IV on the basis of whether a Dom$^-$ Hly$^-$ Gna$^+$ PV possesses either the Lps AB or the Lps B profile, respectively (30), the statement is false, with strain 134 being the only exception.

Strain 134 in phase I appears to be little more than a Dom$^+$ Hly$^+$ Gna$^-$ PV with a mutation from the Lps AB phenotype to the Lps B phenotype. As Fig. 7 indicates, 134 can exist in the same three major PVs, Dom$^+$ Hly$^+$ Gna$^-$, Dom$^-$ Hly$^-$ Gna$^-$, and Dom$^-$ Hly$^-$ Gna$^+$, as strains Tohama I and 3779 (30) while retaining its variant Lps B profile. This suggests that these three major PVs can exist independently of the

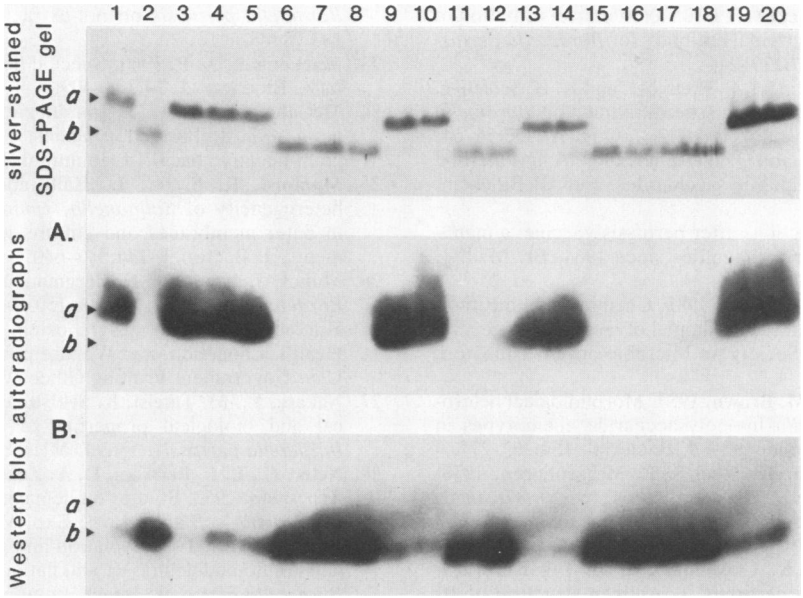

FIG. 7. Western blots and their respective SDS-PAGE profiles (16% [wt/vol] total acrylamide, Tsai and Frasch silver stain) comparing the PW-extracted LPS from *B. pertussis* Tohama I, PV Dom$^-$ Hly$^-$ Gna$^-$ (1), and the PCP-extracted LPS from strain 134, PV Dom$^-$ Hly$^-$ Gna$^-$ (2), with the proteinase K-digested whole cells from 18 *B. pertussis* PVs. Conditions for electrophoresis and blotting are the same as in Fig. 6. Proteinase K-treated organisms in lanes 1 to 3 are from strain Tohama I, PVs Dom$^+$ Hly$^+$ Gna$^-$ (1), Dom$^-$ Hly$^-$ Gna$^-$ (2), and Dom$^-$ Hly$^-$ Gna$^+$ (3). Lanes 4 to 6 are strain 134, PVs Dom$^+$ Hly$^+$ Gna$^-$ (4), Dom$^-$ Hly$^-$ Gna$^-$ (5), and Dom$^-$ Hly$^-$ Gna$^+$ (6). In lanes 9 to 20 are various strains termed phase III and phase IV as listed in Table 1. They are strain L51, PV Dom$^-$ Hly$^-$ Gna$^+$ subtypes Mil$^+$ (9) and Mil$^-$ (10), strain 11615, PV Dom$^-$ Hly$^-$ Gna$^+$ subtypes Mil$^-$ (11) and Mil$^+$ (12), strain L84 (NCTC 10902), PV Dom$^-$ Hly$^-$ Gna$^+$ subtype Mil$^-$ (13), strain Sakairi, PV Dom$^-$ Hly$^-$ Gna$^+$ (14), strain 10901, PV Dom$^-$ Hly$^-$ Gna$^+$ subtypes Mil$^+$ (15) and Mil$^-$ (16), strain D3148, PV Dom$^-$ Hly$^-$ Gna$^+$ subtypes Mil$^+$ (17) and Mil$^-$ (18), and strain Tohama III, PVs Dom$^+$ Hly$^+$ Gna$^+$ (revertant) (19) and Dom$^-$ Hly$^-$ Gna$^+$ (20). In (A) the NCM was incubated with a 10^{-4} dilution of mouse anti-Tohama I, PV Dom$^-$ Hly$^-$ Gna$^-$; in (B) the NCM was incubated with a 10^{-4} dilution of mouse anti-134, PV Dom$^-$ Hly$^-$ Gna$^-$.

LPS phenotype they possess. Better examples of this would be isogenic Lps B mutants of Tohama I or isogenic Lps AB revertants of 134 from each of the three representative PVs. Theoretically, therefore, a given strain could exist as six distinct PVs: Dom$^+$ Hly$^+$ Gna$^-$, Dom$^-$ Hly$^-$ Gna$^-$, and Dom$^-$ Hly$^-$ Gna$^+$, each with either an Lps AB profile or an Lps B profile. We are currently attempting to isolate such mutants and revertants.

It becomes apparent, with at least six clearly defined PVs to contend with, that the phase variation nomenclature currently used is too limited to be of value any longer. The criteria of colonial morphology on BGA (Dom$^+$ Hly$^+$), efficiency of plating on nutrient agar (Gna$^+$), and SDS-PAGE of whole-cell lysates followed by silver and Coomassie brilliant blue staining (Lps AB, Lps B) make the definition of these phenotypic characteristics simple and precise (30). Since these techniques are nonserological, they are, in addition, readily reproducible in most laboratories and should not only foster uniformity in the literature but lead to a better understanding of the genetic mechanisms and the molecular structures involved in phenotypic variation of *B. pertussis*.

ACKNOWLEDGMENTS

I thank Penny Hitchcock of the Laboratory of Microbial Structure and Function at Rocky Mountain Laboratories, Chao-Ming Tsai and Carl Frasch at the National Center for Drugs and Biologics, Bethesda, Md., and Bob Munford, University of Texas Health Science Center at Dallas, Tex., for laying the technical groundwork for this manuscript as well as for their helpful discussions. Thanks also to Merry Schrumpf and Bob Cole for their excellent technical assistance and to Susan Smaus, Heather Mitchell, Chuck Taylor, Bob Evans, and Richard Sherburne for their skill in the preparation of the manuscript.

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