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Isolation and Characterization of Isogenic Pairs of Domed Hemolytic and Flat Nonhemolytic Colony Types of *Bordetella pertussis*

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Four different serotype strains of *Bordetella pertussis*, 3779BL_2S_4, Tohama I, 353/Z, and 2753, were plated on Bordet-Gengou agar, where they grew as domed, hemolytic (D^+H^+) wild-type colonies. Cloned D^+H^+ colony types of all four strains were passed onto modified Stainer-Scholte medium solidified with 1% Noble Agar. Colonies were selected from Stainer-Scholte agar, and these subsequently grew as flat, nonhemolytic (D^-H^-) colonies when transferred back onto Bordet-Gengou agar. The frequency of D^-H^- organisms within a population of cloned D^+H^+ was determined to be between 5×10^{-5} and 5×10^{-6}. The D^-H^- colony types maintained their flat, nonhemolytic characteristics for over 80 single-colony passages on Bordet-Gengou agar. The isogenic pairs of D^+H^+ and D^-H^- colony types from the four strains were compared for hemagglutination titer, lymphocytosis-promoting activity, adenylate cyclase activity, and presence of agglutinogens by agglutination. In all cases the D^-H^- colony types showed reduced activities or amounts of antigen compared with their D^+H^+ parents. Freely diffusible antigens were markedly different between the two phenotypes as noted by double diffusion of antisera added to plates on which colonies of the variants were growing. Antigens solubilized from the two colony types by Triton X-100 were also markedly different as judged by radial immunodiffusion with antifimbrial hemagglutinin, antilymphocytosis-promoting factor, and anti-353/Z adsorbed with autoclaved 353/Z. In addition, autoradiographs of ^{125}I-surface-labeled whole cells separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed unique banding patterns for each colony type. Since all organisms, regardless of colony type, were grown on Bordet-Gengou agar, the differences reported could not be due to medium composition. Differences between phenotypes were also independent of passage number on Bordet-Gengou agar. By analogy to previous studies, the D^-H^- organisms appear to fulfill the criteria for phase III or phase IV in the system of Leslie and Gardner (P. H. Leslie and A. D. Gardner, J. Hyg. **31**:423–434, 1931) or phase III in the system of Kasuga et al. (T. Kasuga, Y. Nakase, K. Ukishima, and K. Takatsu, Kitasato Arch. Exp. Med. **26**:121–134, 1954).

Wild-type (e.g., clinically isolated) *Bordetella pertussis* are known to change phenotypically during cultivation in vitro (5, 20, 32). Even when grown on blood-rich Bordet-Gengou agar (BGA), organisms that have been repeatedly passaged can lose many of the biological activities and surface components commonly found in fresh isolates (15, 20, 26, 32). These physical and biological differences between wild-type versus laboratory-passaged organisms have been exploited to study the surface properties of *B. pertussis* and their relation to pathogenicity (1, 8, 17, 20, 25, 26, 30).

Three major difficulties have obscured the interpretation of results obtained in several of the studies cited above. First, most studies have compared freshly isolated and laboratory-passaged organisms from heterogenous strains (1, 8, 27). This approach may lead one to overlook intrastrain similarities among phenotypic variants of the same genotype and is inadequate for understanding the genetic mechanism of variation. However, in the past it has been difficult to obtain stable isogenic pairs of the representative phenotypes for such intrastrain and interstrain comparisons (1).

Second, the descriptions of phenotypic variation in *B. pertussis* have been complicated by the use of different maintenance media in different laboratories. Reversible phenotype variants can result from adaptation of wild-type *B. pertussis* to media other than BGA (5, 26). More specifically, Lacey (18) showed that alteration of BGA by certain salts, for example, magnesium, resulted in reversible "antigenic modulation" of virulent "X-mode" organisms to avirulent "C-mode" organisms. The removal of added magnesium would allow C-mode organisms to grow back their X-mode characteristics. The relationship of such reversible variants to stable, defined isogenic mutants grown on standard BGA is presently poorly understood.

Third, the terminology and criteria used to describe phenotypic variants of *B. pertussis* vary among investigators. One problem stems from misuse of the poorly defined classification scheme of Leslie and Gardner (20), who described four serologically distinct "phases" in *B. pertussis*. The phases, they believed, were a continuum of degradative adaptations from phase I, which represented the most "smooth" fresh clinical isolates, to phase IV, the most "rough" laboratory-passaged phenotype. Although their serological classification made an important distinction between protective and nonprotective phenotypes of *B. pertussis*, no description of colony morphology or other nonserological criteria was presented for subsequent researchers to accurately select phase variants and repeat these observations (32).

In a separate serological classification scheme, Kasuga et al. (15, 16) did describe colonial phenotypes of their *B. pertussis* variants on BGA, but retained the term phase to name them. However, their phase I organisms (29) represent only one of several serological types termed phase I in the system of Eldering et al. (9). The very term phase may itself be a misnomer since phenotypic variation in *B. pertussis* on BGA (6, 29) does not appear to occur with nearly the frequency described for flagellar phase variation of *Salmonella typhimurium* (34).

The present paper uses several techniques to resolve some of the problems mentioned above. First, *B. pertussis* phenotypes are shown to be readily distinguishable by colonial morphology on BGA with the aid of special lighting (28) and a stereomicroscope. Colonies of a defined phenotype are routinely cloned on BGA to maintain described characteristics (35). Second, it has previously been reported that primarily only phase IV cultures of *B. pertussis* will grow on Stainer-Scholte agar (SSA) containing 1% Casamino Acids (8). In the present paper, therefore, a modified SSA has been used to select for flat, nonhemolytic (D^-H^-) variants, at a calculable frequency, from a cloned population of domed, hemolytic (D^+H^+) wild-type *B. pertussis*. Third, the isogenic pairs of D^+H^+ and D^-H^- phenotypes obtained by this method are both shown to be stable on BGA. Comparisons (e.g., of biological activity, etc.) between the two phenotypes are therefore independent of differences in growth medium and passage number on BGA. Last, the isogenic pairs of D^+H^+ and D^-H^- organisms from four strains of different serotypes are characterized and compared by their respectively different biological and antigenic properties. In addition, their sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) whole-cell lysate profiles are shown to be respectively different by either Coomassie brilliant blue staining or ^{125}I surface labeling. The relationship of D^+H^+ and D^-H^- colony types with phase variation is discussed.

MATERIALS AND METHODS

Organisms and media. The following strains of *B. pertussis* used in this study were obtained from John J. Munoz (Rocky Mountain Laboratories): $3779BL_2S_4$ (referred to as 3779 here), serotype 1,0,3,0,0,6 (2, 3); Tohama I, serotype 1,2,0,4,0,0 (3); 353/Z, serotype 1,0,0,0,0,0 (30); and 2753, serotype 1,2,3,4,5,0 (the mouse challenge strain 18323 of P. Kendrick obtained through Merck Sharpe & Dohme) (30). Master cultures were received as suspensions frozen at −70°C in sterile skim milk (Difco Laboratories, Detroit, Mich.). Organisms cloned from the master cultures were stored at −70°C, suspended in 1 to 2 ml of 5% (wt/vol) sodium glutamate–1.75% bovine serum albumin (modified Greaves solution [12]). Other strains were obtained as lyophiles from the National Collection of Type Cultures, London, England, 10905, 10906, 10907, 10908, 10909, 10910; from Charles R. Manclark (Food and Drug Administration Bureau of Biologics, Bethesda, Md.), BB103; and from Kachiko Sekiya (Kitasato University, Tokyo, Japan), Maeno (prototype "phase I" of Kasuga et al. [cf. 29]) and Sakairi (prototype "phase III" of Kasuga et al. [cf. 29]). Lyophiles were reconstituted with isotonic sodium chloride solution, grown up on BGA, cloned, subcultured, and stored at −70°C as described above. All cultures except Sakairi yielded colonies which were domed and hemolytic on BGA. Sakairi yielded flat, nonhemolytic colonies on BGA.

Selected phenotypes were maintained on the BGA medium described by Munoz and Bergman (23). Briefly, BGA base (Difco) was formulated and autoclaved according to the manufacturer's instructions in 1% glycerol–1% (wt/vol) Neopeptone (Difco). After the medium was cooled to 45 to 50°C, freshly drawn and defibrinated horse blood at ambient temperature was added to give a final concentration of 13.0% (vol/vol). After thorough mixing by swirling, 15 to 20 ml of medium was distributed into 100- by 15-mm plastic petri dishes (Falcon Plastics, Oxnard, Calif.; no. 1001). In my experience, the way BGA plates were dried influenced colony morphology. Thus, stacks of 20 plates were allowed to gel and dry for 20 h at

ambient temperature before inverting and storing them at 4°C in a loosely covered stainless steel box. Medium prepared in this manner was used for at least 1 month, or until drying or darkening prompted its disposal.

For propagation of desired phenotypes, single colonies grown for 3 to 5 days on BGA were selected on the basis of colony morphology and hemolysis at each passage. The selected colony was then streaked onto BGA to again obtain isolated colonies for further cloning. Throughout this study, the selection of desired phenotypes was aided by the use of a stereomicroscope (StereoZoom 4; Bausch & Lomb, Inc., Rochester, N.Y.) at ×7 to ×15 magnification. Colonies were illuminated by reflected light from a universal illuminator (AO 653; American Optical Corp., Buffalo, N.Y.) as described by Penn et al. (28) for obtaining reflection highlights. Colony measurements were made with a reticle (no. 3-16-44; Scientific Products Div., McGaw Park, Ill.) fitted to the eyepiece of the stereomicroscope.

For the selection of D^-H^- phenotypes, the synthetic broth medium of Stainer and Scholte (33) as modified and prepared by the method of Hewlett et al. (13) was solidified with 1% wt/vol Noble agar (no. 1042, Difco).

Assays for biological activity. Fimbrial hemagglutinin (FHA) was assessed in round-bottomed microtiter plates. A 24-h BGA-grown culture was harvested with a swab and suspended to 1.2×10^{10} colony-forming units (CFU) per ml in 50 mM mono- and disodium phosphate-buffered 0.85% sodium chloride–0.02% sodium azide, pH 7.5 (PBSA). Fifty microliters was serially twofold diluted in the microtiter plate, and to each well was added 50 μl of 0.5% (packed vol/vol) chicken erythrocytes in PBSA. The plate was sealed with tape, and the suspensions were mixed thoroughly by tapping the plate and then allowed to stand for 1 to 2 h before reading. Endpoints were recorded as the last dilution to show disruption of erythrocyte settling.

Lymphocytosis-promoting factor (LPF) was kindly assayed by John J. Munoz and associates at Rocky Mountain Laboratories as previously described (23). The whole organisms used for intravenous injection were grown and harvested as described for the adenylate cyclase assay (see below) and were incubated at 56°C for 15 min to inactivate the heat-labile toxin.

Serotyping was conducted with antisera obtained from Grace Eldering (Michigan State Health Department). Agglutination titers for different organisms were measured in flat-bottomed microtiter trays (no. 76-003-05; Linbro, Hamden, Conn.). Sera were serially twofold diluted in 50 μl of PBSA 1/10 to 1/1,280, and 50 μl of a 1.2×10^{10}-CFU/ml suspension of 24-h BGA-grown organisms in PBSA was added to each well. Trays were covered with tape and shaken for 15 min at 200 rpm on an incubator-gyratory shaker with a one-in. (ca. 2.5-cm) stroke (model G-25; New Brunswick Scientific Co., New Brunswick, N.J.). Endpoints were expressed as the last dilution to show agglutination visible by ×10 magnification.

Adenylate cyclase activity was quantitated by a cAMP competitive binding assay (no. 171 689; Boehringer Mannheim Corp., Indianapolis, Ind.).

Individual colonies of desired phenotype which had grown for 3 days on BGA were transferred to fresh BGA, streaked heavily across the plate, and grown for 24 h at 37°C. Cells were collected with a cotton swab and suspended in Stainer-Scholte broth (SSB). The suspensions were centrifuged at 8,000 × *g* for 10 min at ambient temperature, and the supernatant fluid was discarded. The pellets were resuspended in SSB and adjusted to an absorbancy at 540 nm of 0.12 in a Coleman Jr. spectrophotometer, using 13- by 100-mm test tubes. Volumes of 1 ml were warmed to 37°C in a water bath, and at zero time 100 μl of 10 mM ATP (Sigma Chemical Co., St. Louis, Mo.) was added per tube. Samples of 100 μl were taken in triplicate at 20 min and 40 min. The amount of cAMP produced in a given culture was assayed by its ability to inhibit the binding of [^{3}H]cAMP to a cAMP-binding protein. Counts were measured in 10 ml of Aquassure (New England Nuclear Corp., Boston, Mass.) in polypropylene-capped glass scintillation vials for 1 min in a Beckman LS 8100 liquid scintillation counter. Values were expressed in picomoles of cAMP produced per minute based on a standard curve.

SDS-PAGE. Organisms grown for 3 days on BGA were suspended to an absorbancy at 540 nm of 0.12 (Coleman Jr.) in 13- by 100-mm test tubes containing 4 ml of 50 mM Tris–43 mM sodium glutamate–90 mM sodium chloride, pH 7.5 (TGS). A 1.5-ml sample of the suspension was centrifuged for 3 min in a Beckman Microfuge B, and the supernatant fluid was discarded. Pellets were suspended in 25 μl of the SDS (sodium lauryl sulfate, specially pure; BDH Chemicals Ltd., Poole, England) solubilizing solution of Laemmli (19) and heated in a boiling water bath for 5 min. A 10-μl sample was applied to each lane of a 12- by 14-cm slab gel composed of the following: a separating gel 12.8% (wt/vol) total acrylamide of which 2.6% was *N,N'*-methylenebisacrylamide and a stacking gel 4% (wt/vol) total acrylamide of which 2.6% was *N,N'*-methylenebisacrylamide. Samples were electrophoresed at 10 W constant power for approximately 2.5 h in the SDS–Tris-glycine buffer system of Laemmli (19).

Gels were fixed overnight in 7% acetic acid–25% isopropanol and stained the next day with 0.2% Coomassie brilliant blue R-250 dissolved in the acetic acid-isopropanol fixing solution. After staining for 20 to 30 min, gels were destained to a light background in the above acetic acid-isopropanol fixing solution and then transferred to 7% acetic acid until the background stain was gone. Low-molecular-weight protein standards (Bio-Rad Laboratories, Richmond, Calif.) were used as a reference.

^{125}I iodination of whole organisms. The procedure used to surface label whole cells of *B. pertussis* was adapted from Fraker and Speck (10) and Markwell and Fox (21). Dram screw-cap vials (no. 15045; Brockway Glass Co., Parkersburg, W.Va.) were coated with 1,3,4,6-tetrachloro-3α,6α,diphenylglycouril (Iodogen; Pierce Chemical Co., Rockford, Ill.). One hundred microliters of 10-μg/ml Iodo-gen dissolved in chloroform was evaporated to dryness under a stream of nitrogen. Organisms were suspended and centrifuged as for SDS-PAGE. Each pellet was suspended in 50 μl of TGS and transferred to a single Iodo-gen-coated vial. Ten microliters of Na^{125}I (New England Nuclear Corp., high specific activity) at 1.5 mCi/ml was added, and the reaction was allowed to proceed for 10 min with occasional gentle swirling. The sample was then transferred to 1.0 ml of TGS and centrifuged for 3 min in a Beckman Microfuge B. The pellet was washed once with 1.5 ml of TGS and then resuspended

in 25 μl of SDS solubilizing solution and prepared for SDS-PAGE as described above. Autoradiograms of gels dried on a Hoefer model SE 1140 slab gel drier were made on Kodak X-Omat-R film with a Lanex regular intensifying screen (Eastman Kodak Co., Rochester, N.Y.) by 16 to 20 h of exposure at −70°C.

Immunodiffusion tests. Antigens produced by living colonies of D^+H^+ and D^-H^- phenotype variants of pertussis were compared on SSA containing 1% (wt/vol) bovine serum albumin fraction V (Miles Laboratories, Inc., Elkhart, Ind.) (SSA-BSA). Organisms grown for 24 h on BGA were suspended to an absorbancy at 540 nm of 0.12, and 10 μl was added to a single well of a sterile conical-bottomed microtiter plate (Linbro no. 76-321-05). A stainless steel replicating device (7) made of 0.5-mm adjustable pins was used to transfer small volumes of the suspensions in a fixed pattern to 10 SSA-BSA plates. At 24-h intervals, two plates were removed, and 2.5-mm wells were cut approximately 1 cm diagonal to the growing colonies. Five microliters of horse anti-353/Z serum which had been adsorbed with autoclaved 353/Z was added per well, and the plates were incubated for 3 days at ambient temperature in a humid chamber. A 0.85% (wt/vol) sodium chloride solution containing 0.02% (wt/vol) sodium azide (saline-azide) was added to each plate, and the colonies were washed off. The agar was soaked overnight in saline-azide and then washed repeatedly for 2 h with distilled water. The washed agar was floated onto a 50- by 75-mm glass slide and dried. Dried slides were stained for 10 min in the Coomassie stain described for SDS-PAGE. The slides were destained to give a colorless background in 7% glacial acetic acid–25% isopropanol.

Certain pertussis antigens are not freely soluble in physiological solutions (23). To qualitatively test for FHA and LPF on D^+H^+ and D^-H^- organisms, the extraction procedure of Munoz and Cole (24) was used.

Single colonies isolated from a 3-day growth on BGA were streaked broadly onto fresh BGA. After growth for 3 days at 37°C, the entire plate was harvested into 10 ml of TGS and centrifuged at 8,000 × *g* for 10 min at 4°C. The pellets were suspended in 1 ml of 0.5% Triton X-100–1 M sodium chloride–50 mM sodium pyrophosphate–0.02% sodium azide, pH 8.0 (extraction buffer), and gently agitated for 16 h at 4°C. The extracted organisms were centrifuged at 8,000 × *g* for 5 min, and the pellets were discarded. The supernatant fluid was ultracentrifuged at 100,000 × *g* for 1 h. Pellets were suspended in 30 μl of extraction buffer. Supernatants were concentrated 10-fold by vacuum concentration in collodian dialysis tubes with a 10K molecular weight cutoff (Schleicher & Schuell, Inc., Keene, N.H.). Radial immunodiffusion plates were made by adding 1 to 2% (vol/vol) hyperimmune serum (horse anti-353/Z adsorbed with autoclaved 353/Z, rabbit anti-LPF, or rabbit anti-FHA) to extraction buffer containing molten 2% (wt/vol) agarose (Sigma; type II) at 45°C. The antiserum-agarose (4 ml) was poured onto 25- by 75-mm microscope slides and allowed to gel. Wells (2.5 mm) were cut approximately 1 cm apart, and 5 μl of the antigen preparations was added per well. Slides were incubated, washed, dried, and stained as described above for the colony plates.

Reference antigens and antisera. Reference antigens FHA and LPF and hyperimmune sera used for immunodiffusion were obtained from John J. Munoz, Rocky Mountain Laboratories. The anti-agglutinogen antiserum had been raised in a horse by repeated intravenous injections of BGA-grown 353/Z organisms; antibody directed against heat-stable antigens was removed by adsorption with autoclaved 353/Z organisms. The specificity and preparation of the following materials have been described previously: FHA (3), rabbit anti-FHA (22), LPF (22), and rabbit anti-LPF (22).

Efficiency of plating. The relative ability of the four strains and their two colony types to grow on various modifications of SSA medium was quantitated by viable counts of serially diluted organisms.

Organisms were cultured on BGA, washed, and suspended to an absorbancy at 540 nm of 0.12 as described for the adenylate cyclase assay above. Serial 10-fold dilutions from undiluted to 10^{-6} were distributed 50 μl to a plate and spread with an alcohol-flamed bent glass rod. The media tested were SSA to which additional Tris or BSA (or both) was added. All media were inoculated in duplicate, except BGA controls, which were inoculated in triplicate. The plates were incubated at 37°C, and colony counts were made at 5 and 10 days of growth. The percent efficiency of plating for a medium Y was calculated as the (CFU/ml on medium Y) × 100 ÷ CFU/ml on BGA.

Frequency of phenotypic variation. The SSA plates (12.5 mM Tris, no BSA) from the efficiency of plating test described above were used to estimate the frequency of D^-H^- colony types within the cloned population of D^+H^+ organisms. After incubation for 10 days at 37°C, SSA plates showing 10 to 15 individual colonies were replica plated to a BGA plate by means of a 9-cm circle of Whatman no. 1 filter paper. The filter paper circles had been previously force fit into the bottom of a standard plastic petri dish and sterilized by ethylene oxide gas (C. R. Bard Inc., Murray Hill, N.J.). D^-H^- colony types appearing on the BGA plates within 3 to 4 days were confirmed by hemolysis and morphology and then counted. The net frequency of variation was equal to $(A/B) \times C$; where A is the CFU of BGA-confirmed D^-H^- colonies per ml appearing in the population of cloned D^+H^+ colonies plated on SSA in the efficiency-of-plating experiment; B is the CFU of the same cloned D^+H^+ per ml plated on BGA in the efficiency-of-plating experiment; and C is the correction factor to account for <100% plating efficiency of D^-H^- organisms on SSA, i.e., the reciprocal of the percent efficiency of plating for the D^-H^- phenotype on SSA × 100 (e.g., if the plating efficiency of a cloned D^-H^- colony from a given strain was 50% on SSA, the correction factor would be 2). The net frequency is therefore expressed as the number of D^-H^- per D^+H^+ in the culture.

RESULTS

Selection of flat, nonhemolytic phenotypes. The four *B. pertussis* strains studied yielded colonies that were domed and exhibited pronounced hemolysis after 3 days of growth on BGA. Two of these strains (3779 and Tohama I) have been reported to grow well in modified SSB (4). However, when 3779 was plated on SSB solidified with Noble agar (SSA), growth of this strain

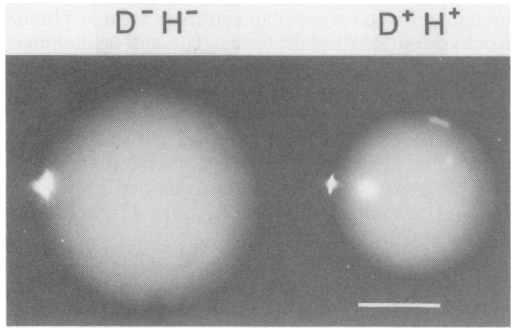

FIG. 1. The two colony types of strain 3779 after growth on BGA for 5 days at 37°C. The colonies are illuminated with reflected light from a universal illuminator, positioned at a 30° angle above the plane of the plate, to illustrate the domed and flat morphologies of the colonies. This lighting does not permit visualization of hemolysis. (See Fig. 3B for hemolysis.) Bar, 1 mm.

was scant. Those organisms which grew well on SSA yielded flat, nonhemolytic colony types when subcultured onto BGA. Figure 1 shows the colonial morphology of the two colony types of strain 3779 after 5 days of growth on BGA. The parent strain was noticeably more domed (D^+) and was hemolytic (H^+) whereas the SSA-passaged colony was flat (D^-) and nonhemolytic (H^-). These two colonial forms, D^-H^- and D^+H^+, have retained their respective characteristics after more than 80 selective passages of single colonies on BGA; no spontaneous changes of D^-H^- to D^+H^+ or D^+H^+ to D^-H^- have been seen on this medium, nor did the passage number on BGA affect the outcome of any of the assays described below. The D^+H^+ colonies were fairly cohesive, and although they were easily suspended in saline, entire colonies could be removed from BGA by touching them with a wire loop. D^-H^- colonies, by comparison, were more emulsifiable and very liquid in texture on BGA. Gram-stained preparations showed no obvious difference between D^+H^+ and D^-H^- colony types grown on BGA; both types showed small coccobacilli with few long forms. Neither colony type would grow on unsupplemented nutrient agar even with inocula of 10^8 CFU/plate.

To explore the mechanism of selection for D^-H^- types on SSA, single colony clones of D^+H^+ and D^-H^- were suspended in 100 μl of SSB and replica plated onto SSA with and without various supplements. Figure 2A shows that only colonies derived from the D^-H^- phenotype appeared on SSA without additions; both D^-H^- and D^+H^+ suspensions produced colonies on SSA supplemented with 1% normal horse serum (Fig. 2B). SSA-BSA, 0.2% (wet wt/vol) 20 to 50 mesh Dowex 1–×8, 0.1% soluble starch (BBL Microbiology Systems, Cockeysville, Md.) or 0.4% (wt/vol) activated charcoal also supported growth of both phenotypes (data not shown). Transfer of D^-H^- organisms grown on any of the supplemented SSAs onto BGA yielded colonies with D^-H^- phenotypes. Likewise, D^+H^+ clones grown on supplemented SSA retained their D^+H^+ phenotype when transferred back to BGA.

The SSA plates were further used to select for D^-H^- types from the D^+H^+ clones of 11 other strains, except Sakairi, listed above. For the studies which follow, the D^-H^- from 3779, Tohama I, 353/Z, and 2753 were compared with their respective D^+H^+ parents. The data presented are representative of between two and four individual tests for each assay conducted at

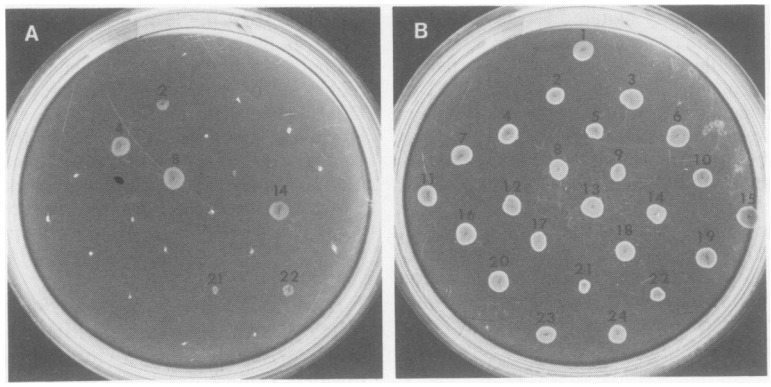

FIG. 2. Replica 9-cm plates of D^+H^+ and D^-H^- colony type clones on (A) SSA and (B) SSA containing 1% normal horse serum. Seventeen D^+H^+ colony clones were plated at positions 1, 3, 5, 6, 7, 9, 10, 11, 12, 13, 16, 17, 18, 19, 20, 23, and 24. Seven D^-H^- colony clones were plated at positions 2, 4, 8, 14, 15, 21, and 22. Plates were grown for 9 days.

TABLE 1. Percent plating efficiency after 10 days of growth on SSA containing various Tris and BSA concentrations versus growth on BGA

Colony type	Strain	CFU/ml on BGA $\times 10^9$	% Plating efficiency at Tris concn:								
			12.5 mM			25 mM			50 mM		
			No BSA	0.1% BSA[a]	1.0% BSA	No BSA	0.1% BSA	1.0% BSA	No BSA	0.1% BSA	1.0% BSA
D^+H^+	3779	0.6	12.0	106	100	75.0	104	111	76.0	101	100
	Tohama I	1.7	0.0003	92	103	0.0006	84	93	0.0004	84	103
	353/Z	1.5	0.69	101	103	44.0	102	98	14.0	90	100
	2753	1.8	0.0004	115	113	0.003	114	117	0.09	112	118
D^-H^-	3779	2.3	57.0	71	69	63.0	86	78	71.0	96	73
	Tohama I	2.7	78.0	96	96	89.0	80	98	93.0	86	102
	353/Z	2.8	76.0	94	98	95.0	96	102	95.0	101	104
	2753	2.2	36.0	107	113	60.0	115	98	21.0	100	112

[a] BSA percentages are weight/volume.

different times during the passage of the organisms on BGA.

Efficiency of plating and frequency of variation. SSA was supplemented with various concentrations of Tris and BSA in an attempt to ascertain more quantitatively the conditions for D^-H^- selection and D^+H^+ stabilization. Table 1 summarizes the plating efficiencies of the four strains and their two colony types on the various SSA media. The efficiencies of the D^+H^+ colony types on SSA with 12.5 mM Tris and no BSA varied greatly among the strains from 0.0003% in Tohama I up to 12% in 3779. Increasing just the Tris concentration to 25 mM appeared to enhance the efficiencies of 3779, 353/Z, and 2753 D^+H^+. Further increasing the Tris concentration to 50 mM continued to enhance plating efficiency of 2753 D^+H^+, but had no further effect on 3779 D^+H^+ and was actually less supportive than 25 mM Tris for 353/Z D^+H^+.

The addition of BSA, whether 0.1 or 1.0%, raised all plating efficiencies to near 100%. An exception was Tohama I D^+H^+, where growth on 1.0% BSA was slightly but consistently more efficient than growth on 0.1% BSA. BSA also improved growth on SSA as evidenced by colony diameter (Table 2). In general, individual colony diameters were larger on the plates containing 1.0% BSA than on plates of the same Tris concentration but containing no or 0.1% BSA. The concentration of Tris also affected colony diameter. This was most noticeable in strains Tohama I and 2753, where increasing Tris caused decreased colony diameter. Based on the combined data of efficiency of plating and colony diameter, SSA containing 25 mM Tris and 1.0% BSA appeared to be the most suitable modification of SSA medium for growth of D^+H^+ organisms.

The frequency of D^-H^- colony types within the suspensions of D^+H^+ organisms was determined by replica plating those SSA plates containing 12.5 mM Tris and no BSA and showing growth of the individual colonies at 10 days (cf. above). The net frequencies of D^-H^- colony types for Tohama I and 2753 were 4.5×10^{-6} and 9.7×10^{-5}, respectively. Individual D^-H^- colonies were not discernible in strains 3779 or 353/Z by this technique. However, the confirmed absence of D^-H^- colony types in a known population of D^+H^+ colonies predicts a net frequency of $<1.4 \times 10^{-3}$ for 3779 and $<4.5 \times 10^{-5}$ for 353/Z. SSA made with three different batches of Noble agar (manufacturing dates November 1979, December 1979, and April 1980; personal communication, Richard Bell, Difco)

TABLE 2. Colony diameters of D^+H^+ organisms after 10 days of growth on SSA containing various Tris and BSA concentrations

Strain	Colony diameter (mm) at Tris concn:									
	BGA control	12.5 mM			25 mM			50 mM		
		No BSA	0.1% BSA	1.0% BSA	No BSA	0.1% BSA	1.0% BSA	No BSA	0.1% BSA	1.0% BSA
3779	2.8–3.3	0.9–1.9	2.1–2.3	2.5–2.6	2.2–2.5	2.1–2.2	2.5–2.7	1.4–2.4	2.0–2.1	2.5–2.6
Tohama I	3.3–3.4	2.3–2.5	2.6–2.7	2.4–2.5	1.7–1.8	2.2–2.5	2.7–3.0	0.4–1.2	1.8–2.1	2.0–2.3
353Z	3.4–3.5	2.6–2.7	2.3–2.6	2.6–2.7	2.5–2.7	2.5–2.6	2.9–3.0	1.4–1.5	2.2–2.4	2.4–2.5
2753	2.7–3.0	2.2–3.1	2.1–2.2	3.0–3.2	0.7–1.9	2.1–2.4	2.6–2.9	<0.1–1.5	1.6–1.7	2.4–2.9

gave identical efficiencies of plating with net frequencies of variation between 5×10^{-5} and 5×10^{-6}.

Biological activities of D^+H^+ and D^-H^- colony types from four strains of *B. pertussis*. The D^+H^+ and D^-H^- colony types of each of the four strains tested were assayed for FHA, LPF, agglutinogen 1, and adenylate cyclase activity. Table 3 summarizes the differences in biological activities between the two colony types of single strains as well as the differences among strains. Overall, the D^-H^- colony types of each strain have notably lower activities than their isogenic D^+H^+ counterparts. An exception is the low FHA titer for both colony types from strain 2753.

The occurences of soluble, diffusible antigens were compared for the two colony types of each of the four strains. Replica-plated organisms on SSA-BSA were grown from 2 to 6 days, and antigen production was assessed by immunodiffusion against horse anti-353/Z serum (Fig. 3A). Few precipitin bands were visible until day 5. After 5 days, pronounced precipitin bands were seen which showed identity among the four D^+H^+ colony types. Although the D^-H^- colony types all grew as well as their D^+H^+ counterparts on both SSA-BSA and BGA (Fig. 3B), no major precipitin bands were seen by immunodiffusion for D^-H^- colonies.

Triton X-100 extracts of both D^-H^- and D^+H^+ colony types from each of the four strains were compared by diffusion in agar containing anitserum. Figure 4 shows that all D^+H^+ colony types possessed various heat-labile antigens and FHA, but all of the D^-H^- colony types did not. Likewise, small amounts of LPF were detected only in the D^+H^+ colony types.

SDS-PAGE and ^{125}I surface labeling. Whole-cell lysates of the two colony types of each strain were compared on SDS-PAGE after labeling with ^{125}I as described above. Figure 5A shows the banding profile after staining with Coomassie brilliant blue. Two bands, with apparent molecular weights of 25.5K and 30.5K, appeared in each D^+H^+ pattern, but were missing in the D^-H^- patterns. Other protein bands which could be seen in some but not all D^+H^+ organisms were absent in their D^-H^- counterparts. For example, a large, approximately 200K band was seen only in the D^+H^+ organisms of all strains except 2753. Likewise, only the D^-H^- organisms from 3779 and 2753 appeared to lack 80K bands.

In addition to the protein banding differences between D^+H^+ and D^-H^- colony types, intrastrain similarities could also be seen in the region of 26K to 30K. Specifically, both colony types of strain 3779 had a prominent band at 27K. In strain 353/Z, an intensely staining band was seen at approximately 27.5K, and in strain 2753 a band was easily seen at 29K. The Tohama I colony types resembled those of strains 3779, with a dominant band at 27K. Thus, most strains could be distinguished from one another by differences in banding patterns in the region 26K to 30K.

Additional differences were discernible in SDS-PAGE patterns of ^{125}I-labeled *B. pertussis* by autoradiography (Fig. 5B). Although the concentration of organisms and labeling conditions were kept constant for all the strains and their colony types, differences in the degree of labeling could be seen for bands of similar apparent molecular weight in different strains, e.g., 3779 D^-H^- and 353/Z D^-H^-. Nonetheless, there was

TABLE 3. Biological activities of D^+H^+ and D^-H^- colony types of four *B. pertussis* strains

Strain	Colony type	FHA[a]	Promotion of leukocytosis[b]	Heat-stable agglutinogens[c]	Adenylate cyclase activity (pmol/min per 10^9 CFU)
3779	D^+H^+	4	10,783 ± 756	320	36.3
	D^-H-	<2	4,154 ± 278	20	0.03
Tohama I	D^+H^+	64	8,014 ± 569	320	45.8
	D^-H^-	<2	5,720 ± 498	20	0.03
353/Z	D^+H^+	64	10,094 ± 632	320	137.9
	D^-H^-	<2	6,183 ± 385	<10	ND[d]
2753	D^+H^+	<2	12,137 ± 1,072	640	45.4
	D^-H^-	<2	5,047 ± 551	<10	ND

[a] Reciprocal titer of last positive dilution showing disruption of erythrocyte settling.

[b] Expressed as total leukocytes per microliter of blood, mean ± SE of the mean of five mice; 20 μl was bled from each mouse 24 h after intravenous inoculation with 0.2 ml of phosphate-buffered saline containing 10^9 organisms. Negative control = 5,199 ± 831. The *P* values from the Student *t* test were <0.001 for comparisons of D^+H^+ with D^-H^- from all strains, except Tohama I, where the *P* value was 0.016.

[c] Reciprocal agglutination titer of organisms versus horse anti-353/Z adsorbed with autoclaved 353/Z.

[d] ND, Not detectable; <0.03 pmol/min per 10^9 CFU.

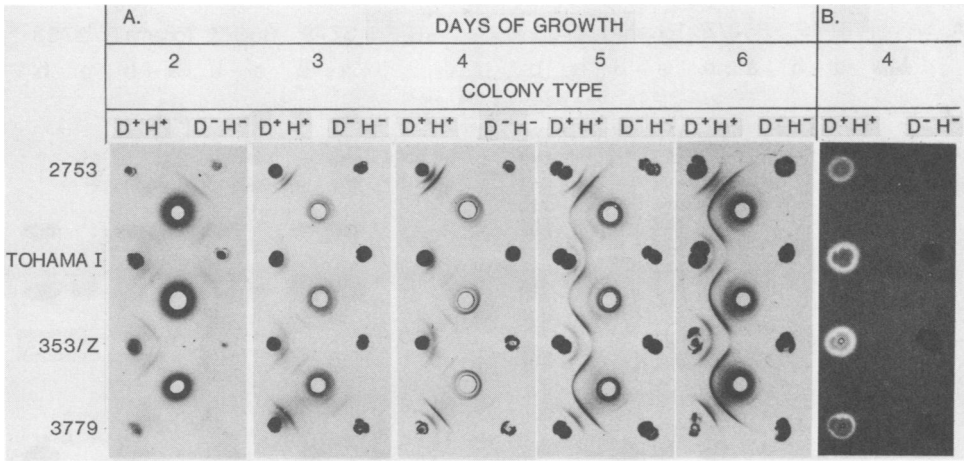

FIG. 3. A, Immunodiffusion plates showing antigens released during growth of D^+H^+ and D^-H^- colony types of *B. pertussis* strains 3779, 353/Z, Tohama I, and 2753. The medium was SSA supplemented with 1% BSA, and the organisms were replica plated onto 10 plates which were then incubated at 37°C for the times indicated. The three 2.5-mm center wells were then cut under sterile conditions, 5 μl of horse anti-353/Z (D^+H^+) serum adsorbed with autoclaved 353/Z (D^+H^+) organisms was added to each well, and the plates were incubated at ambient temperature for 3 days. The colonies were then washed off the plates, excess agar was trimmed away, and the remaining agar, which contained the precipitin bands, was soaked overnight in 0.85% saline–0.02% azide. The next day the agar strips were further washed with distilled water, dried onto microscope slides, and stained with Coomassie brilliant blue R-250. B, The same organisms in A, replica plated onto BGA and grown for 4 days. Back lighting was used to demonstrate hemolysis in the D^+H^+ colony types.

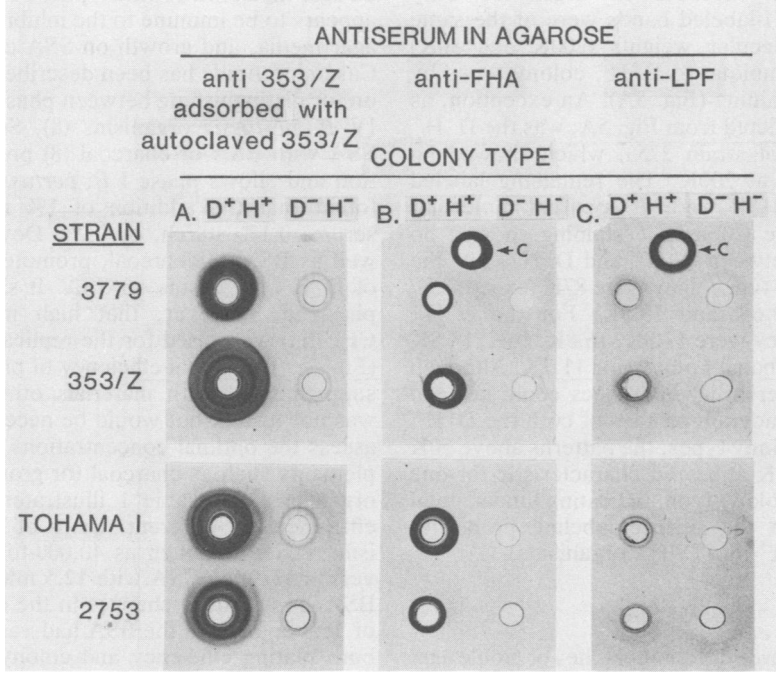

FIG. 4. Radial immunodiffusion plates to detect the presence of antigens in Triton X-100 extracts of D^+H^+ and D^-H^- colony types of *B. pertussis* strains 353/Z, 3779, Tohama I, and 2753. Antisera in the plates were (final concentration) (A) 1% horse anti-353/Z (D^+H^+) adsorbed with autoclaved 353/Z (D^+H^+), (B) 1% rabbit anti-FHA, and (C) 2% rabbit anti-LPF. The well (2.5 mm in original gel) labeled "←C" in B contained 1.5 μg of reference FHA and in C contained 0.3 μg of reference LPF.

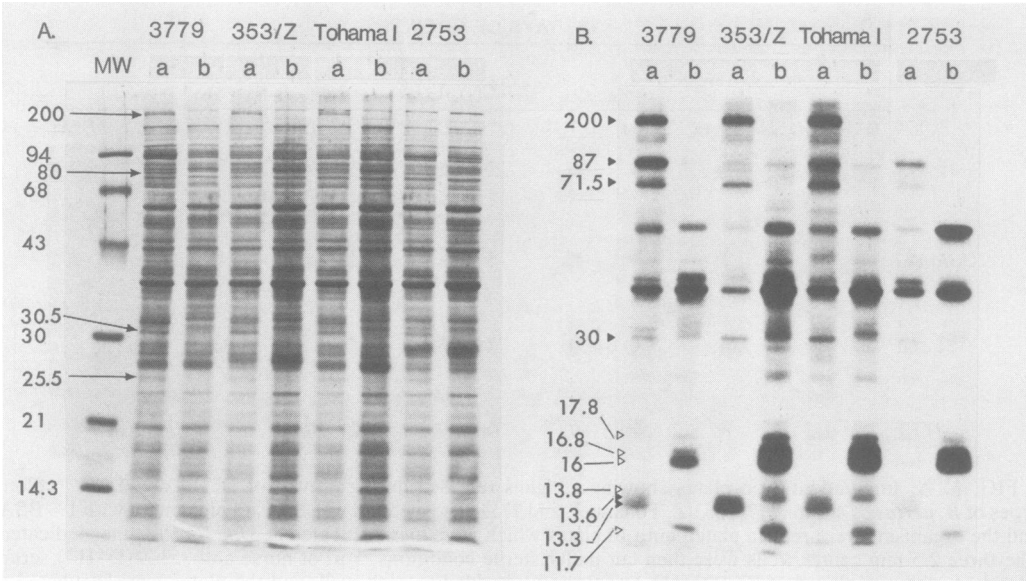

FIG. 5. SDS-PAGE of whole *B. pertussis* cells from strains 3779, 353/Z, Tohama I, and 2753. Organisms were grown for 3 days on BGA and ^{125}I surface labeled by Iodo-gen as described in the text. A, Gel stained with Coomassie brilliant blue R-250 and dried onto filter paper. B, Autoradiogram of the dried gel in A. Lanes a contained D^+H^+ colony types, and lanes b contained D^-H^- colony types from the indicated strains.

a marked difference in the overall labeling patterns of D^+H^+ versus D^-H^- organisms. Only two of the ^{125}I-labeled bands were of the same apparent molecular weights (200K and 30K) shown to be unique to D^+H^+ colony types by Coomassie staining (Fig. 5A). An exception, as might be predicted from Fig. 5A, was the D^+H^+ colony type of strain 2753, which showed no labeled band at 200K. The remaining labeled bands in Fig. 5B were at apparent molecular weights where Coomassie staining showed no differences between D^+H^+ and D^-H^-. For the D^+H^+ colony types, they were 87K (except 353/Z), 71.5K 13.6K and 13.3K. For the D^-H^- organisms, they were 17.8K, 16.8K, 16K, 13.8K (353/Z and Tohama I only), and 11.7K. Although numerous interstrain differences could be seen in the autoradiogram profiles of both the D^+H^+ and D^-H^- colony types, the patterns above 50K and below 20K appeared characteristic for one or the other colony type, indicating fundamental differences in the surface labeling properties between D^+H^+ and D^-H^- organisms.

DISCUSSION

Although three different batches of Noble agar gave identical results, the mechanism by which D^-H^- colony types are selected on SSA is not known. However, several substances, including fatty acids, organic peroxides, colloidal sulfur (31), and Mn^{2+} (26), have been implicated as growth inhibitors of phase I *B. pertussis* plated on agar media. In contrast, phase IV *B. pertussis* appears to be immune to the inhibitory action of agar media, and growth on SSA containing 1% Casamino Acids has been described as a criterion for distinguishing between phase I and phase IV *B. pertussis* organisms (8). Supplementing SSA with BSA or charcoal (8) prevents inhibition and allows phase I *B. pertussis* to grow. I found that the addition of 1% normal horse serum, 0.1% starch, or 0.2% Dowex 1-×8, as well as BSA or charcoal, promoted the growth of D^+H^+ organisms on SSA. It should be emphasized, however, that high inocula ($>10^8$ CFU/ml) were used for the replica plating tests (Fig. 2). The actual efficiency of plating on SSA supplemented with materials other than BSA was not tested, but would be necessary to fully assess the optimal concentrations of other supplements such as charcoal for growth of D^+H^+ organisms. As Table 1 illustrates, the plating efficiencies of different strains of D^+H^+ organisms varied as much as 40,000-fold (Tohama I versus 3779) on SSA with 12.5 mM Tris and no BSA. In addition, changes in the concentration of Tris or BSA in the SSA had varied effects in both plating efficiency and colony size for the different strains (Tables 1 and 2). Thus generalizations about growth or nongrowth of D^+H^+ organisms on unsupplemented SSA must be tempered by the differences in plating efficiency among strains. For example, Dobrogosz et al.

(8) reported two unclassified strains of *B. pertussis* which were hemolytic on BGA and possessed numerous phase I characteristics but nevertheless grew on unsupplemented SSA.

Surprisingly, plating efficiencies of the D^-H^- colony types also varied among strains. In addition D^-H^- types from most strains also showed improved efficiencies on SSA supplemented with BSA. This indicates that not all D^-H^- organisms possess the ability to grow on SSA with no additions. This could explain why isolate 15 grew on SSA containing 1% horse serum (Fig. 2B), but did not grow in 9 days on unsupplemented SSA (Fig. 2A). Mechanisms which might explain the differences in plating efficiencies among various isolates or strains were not investigated further.

Biological activities or antigens present in D^+H^+ were markedly reduced or absent in D^-H^- organisms, strongly suggesting a parallel loss of several characteristics. An exception to this was the apparent absence of detectable FHA in the D^+H^+ colony type of strain 2753 (Table 3), an observation which is discussed further below. In certain strains of *B. pertussis*, all of the agglutinogens present on the organisms may not be exposed on the surface to allow for agglutination by antibody (6, 14). The immunoprecipitation patterns of the naturally released and Triton X-100-extracted organisms shown in Fig. 3 and 4 confirmed the differences seen in agglutination patterns in Table 3; i.e., D^-H^- colonies exhibited few, if any, agglutinogens and no FHA. These data support the concept of loss of antigen production rather than a mere masking of these antigens from agglutinating antibody. Interestingly, the D^+H^+ colony type (but not the D^-H^- type) of strain 2753 showed a positive FHA reaction in the radial immunodiffusion plate (Fig. 4B) despite its inability to agglutinate chicken erythrocytes (Table 3). Either the D^+H^+ colony of 2753 had a variant FHA that was still recognized by anti-FHA, but did not agglutinate chicken erythrocytes, or FHA was not expressed on its surface.

The apparent loss in parallel of the above-mentioned antigens and biological activities suggests that they may be linked genetically or are perhaps controlled by a common gene product(s). In D^+H^+ organisms, the gene(s) would be switched on, whereas in D^-H^- organisms the gene(s) would be switched off. Presently there is no genetic evidence to support this hypothesis.

SDS-PAGE has been used to analyze the differences in the peptide composition of phase I and phase IV *B. pertussis* cell envelopes (8, 11, 27, 36, 37). The Coomassie-stained whole-cell patterns in Fig. 5A are in general agreement with the observations of Wardlaw and Parton (36) and Dobrogosz et al. (8). These authors showed the presence of two proteins with molecular weights of approximately 30K and 28K which were present only in the envelope preparations of phase I. In addition, Dobrogosz et al. (8) have described two other phase I envelope proteins with molecular weights of approximately 98K and 88K. The differences between D^+H^+ and D^-H^- colony types shown in Fig. 5A reflect a similar pattern: D^+H^+ colony types alone possessed a 30.5K and a 25.5K band, and some, but not all, of the D^+H^+ colony types also had an 80K and a 200K band, absent in the D^-H^- colony types. The discrepancies between the apparent molecular weights reported here and the molecular weights reported by the authors cited above (8, 36) are probably trivial and may reflect differences in SDS-PAGE technique, growth conditions of the bacteria, or the inherent inaccuracy of assigning molecular weights to bands well out of the linear range of the gel. Regardless of the exact molecular weight, the absence of both of these smaller bands in phase IV organisms and in C-mode (18) cells (27, 36, 37) parallels the absence of LPF, histamine-sensitizing factor, protective antigen, adenylate cyclase, and agglutinogen production. Several authors (8, 36) have speculated that the biologically active molecules are somehow associated with these smaller envelope protein bands. As likely as this connection may be, no published evidence exists to support the contention.

One advantage of comparing whole-cell lysates was that differences among strains were not limited to observations of proteins associated with outer membranes. Thus, protein patterns conserved in whole-cell lysates of the D^-H^- and D^+H^+ colony types from one strain were shown to differ in other strains (Fig. 5A). Any relationship of these protein changes with serotype differences was not examined.

A second advantage of using whole cells for SDS-PAGE was that proteins exposed on the surface of intact organisms could be labeled in situ by ^{125}I and observed by autoradiography (Fig. 5B). Gow et al. (11) reported difficulty in labeling *B. pertussis* cells with lactoperoxidase-catalyzed ^{125}I iodination. Using Iodo-gen, as reported here, I have had no difficulty in obtaining repeatable labeling of *B. pertussis* whole cells. The striking differences seen in the ^{125}I labeling patterns of D^+H^+ and D^-H^- colony types were not predictable by the basis of Coomassie-stained banding patterns (Fig. 5A). Gow et al. (11) noticed a similar phenomenon in ^{125}I-labeled cell envelopes of X-mode and C-mode *B. pertussis* wherein the predominant 28K and 30K X-mode-specific bands seen by Coomassie staining were radiolabeled poorly, relative to their abundance in the membranes.

Only two of the stained bands unique to

D^+H^+ colony bands (200K and 30.5K) (Fig. 5A) were also labeled by ^{125}I (Fig. 5B). The other differences in ^{125}I-labeled bands between D^+H^+ and D^-H^- colony types were not visible by Coomassie staining. Thus, some of the protein bands adjudged identical in both D^+H^+ and D^-H^- colony types by Coomassie appear to be exposed differently on the surface of the organisms labeled by ^{125}I. One interpretation of these results is that the radioemitting bands in extrinsically ^{125}I-labeled D^+H^+ colony types represent surface proteins which mask underlying structures and prevent them from labeling with ^{125}I. In D^-H^- colony types these D^+H^+-specific labeled bands would not be processed to the surface, allowing for the labeling of the formerly masked proteins. This hypothesis is attractive in light of previous observations which describe the absence of capsule and lower isoelectric point (i.e., altered surface charge) of phase IV organisms (1, 16, 32) compared with phase I organisms.

Most of the differences in biological activities I have described here for D^+H^+ and D^-H^- colony types have been reported for phase I and phase IV *B. pertussis*, respectively (e.g., reference 8). These observed correlations suggest that D^-H^- colony types are phase IV organisms. Are they?

It is certain the D^-H^- colony type is not the rough-phase colony type of Kasuga et al. (15, 16), since D^-H^- colony morphology on BGA is entire, with a smooth surface, and D^-H^- organisms do not autoagglutinate spontaneously in saline. However, the properties of D^-H^- just mentioned are also found in the phase III colony type of Kasuga et al. (15). In fact, the prototype phase III strain, Sakairi (29), is similar to the D^-H^- phenotypes reported here in colonial morphology on BGA and in SDS-PAGE protein and ^{125}I-surface-labeled profiles (unpublished observations). If the D^-H^- phenotype is analogous to the phase III of Kasuga et al. (15), what constitutes a phase IV?

The distinction between phase III and phase IV may have been lost (27), and it is uncertain how many investigators who claim to use phase IV of Leslie and Gardner (20) are actually using phase III of Kasuga et al. (15, 16). Few authors describe the phenotypes of their cultures on BGA, and there are no readily available reference antisera to the phase III, phase IV, or rough colony types (specifically to antigens ϕ_1, ϕ_2, and ϕ_3 of Kasuga et al. [15]) to differentiate among these variants.

It is certain, however, that ambiguous terms such as smooth, rough, or phase have been interpreted differently, which has caused confusion. Alternative nomenclature has been offered by Parker (26), but the terms fresh isolate, laboratory passaged, and degraded are no less ambiguous than the existing terminology.

Toward improved nomenclature, I have described in this paper two major, stable phenotypes of *B. pertussis*: D^+H^+ (wild type) and D^-H^- (variant). These two types are isogenic within a strain, and the frequency of D^-H^- has been calculated. The two phenotypes are defined by their readily observable colonial morphology and degree of hemolysis on BGA, the recommended medium for cultivation of *B. pertussis*. Classification as D^+H^+ prevails over serological differences among strains of wild-type organisms. Likewise, classification as D^-H^- takes precedence to variations in SDS-PAGE profiles of SSA-derived variants from different strains. A nomenclature based on phenotypes like these would be a superior alternative to the ambiguous phase designations. This does not presume the absence of other phenotypes of *B. pertussis* on BGA. Indeed, the rough colony types described by Kasuga et al. (15) can be anticipated as being basically D^-H^- with rough (nonsmooth, S^-) surface and irregular (nonentire, E^-) outline ($D^-H^-S^-E^-$). As better methods are developed for selecting other stable (on BGA) phenotypic variants, we will also be able to describe their frequency within a cloned population of their parent phenotype and quantitate the dynamics of phenotypic variations within *B. pertussis*. In addition, we will have the isogenic sets of organisms in which we can explore the genetic loci involved in these changes.

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