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Phenotypic Variation and Modulation in *Bordetella bronchiseptica*

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Most of the isolates of *Bordetella bronchiseptica* obtained by this laboratory possessed a characteristic colonial morphology when grown on Bordet-Gengou agar (BGA) at 37°C. The colonies appeared domed (Dom^+) with a smooth colonial surface (Scs^+) and a clear zone of hemolysis (Hly^+). From these Dom^+ Scs^+ Hly^+ BGA colony types arose flat (Dom^-), smooth colonial surface (Scs^+) and nonhemolytic (Hly^-) variants at frequencies of 10^{-2} to 10^{-3}. Isogenic pairs of Dom^+ Scs^+ Hly^+ and Dom^- Scs^+ Hly^- BGA phenotype variants (BGA-PVs) were picked from 11 strains of *B. bronchiseptica*, and their whole cell lysates were compared with each other by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Characteristic SDS-PAGE profiles were observed for each of the Dom^+ Scs^+ Hly^+ and Dom^- Scs^+ Hly^- BGA-PVs with regard to (i) surface-exposed proteins, based on autoradiographs of ^{125}I-Iodogen-labeled organisms, (ii) polypeptide differences, based on gels stained with Coomassie brilliant blue R-250, and (iii) lipopolysaccharide differences based on gels stained with silver after oxidation with periodic acid. SDS-PAGE profiles were then used to monitor the phenotypes expressed by Dom^+ Scs^+ Hly^+ and Dom^- Scs^+ Hly^- BGA-PVs transferred and grown on brucella agar, Trypticase soy agar, and nutrient agar. When grown on non-BGA media, the Dom^+ Scs^+ Hly^+ BGA-PVs from six of eight strains showed SDS-PAGE profiles identical to those of Dom^- Scs^+ Hly^- BGA-PVs. This phenotypic change was reversible even after 15 subcultures on the non-BGA media, since Dom^+ Scs^+ Hly^+ organisms passed back onto BGA expressed both Dom^+ Scs^+ Hly^+ colonial morphology and Dom^+ Scs^+ Hly^+ SDS-PAGE profiles. The influence of cultural conditions on maintenance of virulence is discussed.

Two nomenclatures currently exist to describe colonial phenotypes of *Bordetella bronchiseptica*. The first nomenclature was described by Nakase in 1957 (9, 10) wherein blood agar was used for discriminating among the phenotype variants (PVs) or phases of *B. bronchiseptica* (Table 1). Nakase found that *B. bronchiseptica* freshly isolated from guinea pigs were in phase I but would rapidly change upon subculture to phases II and III or, more rarely, to "rough phases."

A second nomenclature was instituted by Bemis et al. in 1977 (1) wherein brucella agar (BA) was used for defining colonial morphotypes of *B. bronchiseptica* (Table 2). Most of the isolates studied by Bemis et al. were defined as being in the intermediate phase, regardless of the animal species from which they had been isolated (1).

In addition to the stable phenotypes just described, both Lacy (7) and Nakase (9, 10) have reported that the composition of the medium used to grow *B. bronchiseptica* can affect or modulate the antigenic phenotype expressed. Hence, when Nakase grew phase I organisms on nutrient agar (NA) or on blood agar containing 1% magnesium sulfate, his cultures agglutinated with the specificity of phase III organisms. When returned to regular blood agar or Bordet-Gengou agar (BGA), the organisms regained their phase I-specific antigens. This reversible, medium-dependent phenotypic shift was called antigenic modulation by Lacey (7).

Our interest in phenotypic variation of *B. bronchiseptica* stemmed from work done with phenotypic variation in *Bordetella pertussis* (11). We were impressed with the general similarities in colonial morphologies on BGA of PVs from both species, but we were also confused by the discrepancies in the literature regarding phenotype nomenclature and properties. In the present paper, we have shown that *B. bronchiseptica* colonial PVs could be further defined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with either Coomassie brilliant blue staining, ^{125}I-Iodogen labeling, or periodate-silver staining for lipopolysaccharide (LPS), and that these techniques could be used to monitor the effect of different media on phenotype expression.

MATERIALS AND METHODS

Organisms and media. *B. bronchiseptica* strains 214, 2320, Rab 10, 22067, and 899L were obtained as phase I cultures from John J. Munoz (Rocky Mountain Laboratories). Strain 10540 was obtained from the National Collection of Type Cultures, London, England, also as a phase I culture. Strain 8442 (virulent) and strain 201 (avirulent) were received from Gail H. Cassell, University of Alabama, Birmingham. The following strains were obtained from David A. Bemis, University of Tennessee, Knoxville; the phase designations for these strains are those defined previously by Bemis et al. (1): 110H, 501, and Rat 1 (all phase I); 87, 17640 SAC, and BTS (all intermediate phase); 110NH, Ft. Collins, and Columbus (all rough phase).

All strains and their PVs were grown on BGA prepared as previously described (11). BGA was chosen so that the zone of hemolysis as well as colonial size and morphology could be assessed and so that analogies could be made with *B. pertussis* grown on the same medium. These phenotypic markers were monitored with a stereomicroscope (either a Stereozoom 4 or a Stereozoom 7; Bausch & Lomb, Inc., Rochester, N.Y.) with both reflected light as described

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TABLE 1. Properties of *B. bronchiseptica* PVs isolated from guinea pigs and observed on BA[a]

Name	Colony morphology	Capsule stain	Flagella	Agglutinated by antiserum to antigen[b]:				
				L	S	H	O1	O5
Phase I	Small, round, transparent, convex, clear zone of hemolysis	++	−	++	++	± or −	−	−
Phase II	Variable combination of phase I and phase III	+ or −	+	+ or −	+ or −	+	+	+
Phase III	Large, smooth, entire, low convex, nonhemolytic	−	+	−	−	++	++[c] or −[d]	−[c] or ++[d]

[a] According to Nakase (9, 10).
[b] Antigens: L, heat-labile (70°C, 1 h) capsular antigen; S, slightly heat-stable (100°C, 1 h) surface antigen; H, heat-labile (70°C, 1 h) flagellar antigen; O1, O5, heat-stable (120°C, 1 h) somatic antigens.
[c] Phase III-1.
[d] Phase III-2.

before (11) and also with substage lighting with a silvered mirror as described by Swanson (13). Single colonies of the desired colonial morphology were passed at 2- to 3-day intervals, and the plates were incubated at 37°C in a humidified incubator.

BA was made by adding 15 g of Bacto agar (Difco Laboratories, Detroit, Mich.) per liter to either commercial brucella broth (Albimi Laboratories, Flushing, N.Y.) or to brucella broth made according to a formula described previously (15). Trypticase soy agar (TSA) was prepared by adding 15 g of Bacto agar to 1 liter of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Nutrient agar was purchased from Difco.

Frequency of variation. Single colonies of a desired morphology were harvested after growth on BGA for 2 days at 37°C and streaked onto fresh BGA. After 2 days of growth, the organisms were harvested and diluted in 50 mM Tris–43 mM sodium glutamate–90 mM sodium chloride (TGS; pH 7.5). Fifty microliters of the dilution was plated onto fresh BGA and allowed to grow for 2 days. Colonial morphology was observed, and the number of variant-type colonies and parent-type colonies was recorded. The number of variant colonies divided by the total number of colonies equals the frequency of variation.

SDS-PAGE and gel staining techniques. SDS-PAGE of whole cell lysates of *B. bronchiseptica* was performed essentially as described for *B. pertussis* (11). Organisms of the desired phenotype were suspended in TGS to an absorbance of 0.12 at 540 nm in tubes (13 by 100 mm). A 1.4-ml portion of this suspension was centifuged in 1.5-ml tubes in a Microfuge B (Beckman Instruments, Palo Alto, Calif.) for 3 min. The pelleted organisms were suspended in 50 μl of SDS-solubilizing solution before heating in a boiling water bath for 5 min. Ten microliters of this suspension was applied per lane. This sample size represented ca. 28 μg of organism protein (based on the Lowry assay with bovine serum albumin as a standard [8]). Total acrylamide concentration in the slab gels (12 by 14 cm) was varied from 12.8 to 16% (wt/vol) to effect optimal separation of putative LPS bands. For all gels, *N,N'*-methylenebisacrylamide was 2.6% (wt/vol), and a 5% (wt/vol) total acrylamide stacking gel was used.

To visualize protein bands after electrophoresis, gels were first fixed overnight in 5% acetic acid–25% isopropanol (vol/vol) and then stained with 0.2% Coomassie brilliant blue R-250 as previously described (11). Putative LPS bands were visualized by the silver staining technique of Tsai and Frasch (14). Fifty microliters of whole cell lysate, prepared as above, was incubated with 10 μg of freshly prepared proteinase K (Boehringer Mannheim Corp., Indianapolis, Ind.) at 56°C for 3 h with vigorous vortex mixing at 30-min intervals (5, 12). Ten microliters of the digested lysate was then applied per lane of a 16% (wt/vol) total acrylamide gel for separation by SDS-PAGE.

^{125}I iodination of whole organisms. Whole cells of *B. bronchiseptica* were suspended in TGS to an absorbence of 0.12 at 540 nm in test tubes (13 by 100 mm). Duplicate 1.5-ml samples were centrifuged for 3 min in a Beckman Microfuge B. One of the duplicate samples was resuspended in SDS-solubilizing solution to serve as an unlabeled control. The other sample was resuspended in 50 μl of TGS, and the entire volume was transferred to a dram vial previously coated with 1 μg of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen; Pierce Chemical Co., Rockford, Ill.) (11). Twenty microcuries of Na^{125}I (high specific activity; New England Nuclear Corp., Boston, Mass.) in 10 μl of TGS was then added, and the mixture was incubated at ambient temperature for 10 min with occasional swirling. The reaction mixture 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 1.0 ml of TGS, then resuspended in 50 μl of SDS-solubilizing solution, and heated as described above. The lysed, ^{125}I-labeled organisms were added to unlabeled whole cell lysates of the same organism to give a final activity of 2×10^3 cpm per microliter. Ten-microliter samples were applied per lane for SDS-PAGE. Gels were stained for protein as above and then dried on a Bio-Rad model 224 slab gel drier. Autoradiographs were made by exposing Kodak X-Omat AR film (Kodak, Rochester, N.Y.) at −70°C for 11 h in the presence of a DuPont Cronex Lightning Plus intensifying screen (E. I. duPont de Nemours & Co., Wilmington, Del.).

TABLE 2. Properties of *B. bronchiseptica* PVs isolated from dogs and other mammals and observed on BA

Name	Colony morphology	Flagella	Pili
Phase I	Small (1 mm), pulvinate, smooth, opaque, entire, hemolytic on blood agar	−	+
Intermediate	Medium (1–2 mm), convex, smooth, opaque-translucent, entire, nonhemolytic	+	+ or −
Rough phase	Large (2 mm) umbonate, rough, translucent, undulate edge, nonhemolytic	+	+ or −

[a] By Bemis et al. (1).

RESULTS

PVs, their colonial morphology on BGA, and frequency of variation. The majority of the *B. bronchiseptica* strains we observed could be divided into two major PVs based on colonial morphology on BGA: domed, smooth colonial surface, and hemolytic (Dom$^+$ Scs$^+$ Hly$^+$) and flat, smooth colonial surface, and nonhemolytic (Dom$^-$ Scs$^+$ Hly$^-$). Other BGA-PVs of intermediate morphologies, e.g. Dom$^+$ Scs$^+$ Hly$^-$, Dom$^+$ Scs$^-$ Hly$^-$, etc., were also noted. Characteristic colonial morphologies of the major BGA-PVs are illustrated in Fig. 1A. The distribution of the major BGA-PVs among the strains we studied is listed in Table 3, column A. As Table 3 suggests, isogenic Dom$^-$ Scs$^+$ Hly$^-$ could be easily isolated from the Dom$^+$ Scs$^+$ Hly$^+$ colony type of any strain since the frequency of variation from Dom$^+$ Scs$^+$ Hly$^+$ to Dom$^-$ Scs$^+$ Hly$^-$ ranged from 10^{-2} to 10^{-3} for six strains tested (214, 2320, 10540, 22067, Rab 10, and 899L). Once cloned, these two major colony types were stable to multiple passages on BGA as long as single colonies of the desired phenotypes were cloned at each passage. No reversion of Hly$^-$ to Hly$^+$ was noted on BGA up to a frequency approaching 10^{-9}.

SDS-PAGE profiles of BGA-PVs. Whole cells from isogenic pairs of two major colony types were harvested off BGA, labeled with ^{125}I by Iodogen, and subjected to SDS-PAGE. As Fig. 2A illustrates, subtle, but nevertheless characteristic, Coomassie blue-stained polypeptide profiles could be assigned to organisms of either the Dom$^+$ Scs$^+$ Hly$^+$ or Dom$^-$ Scs$^+$ Hly$^-$ colony type (except 899L; see below). Arrowheads denote Coomassie blue-stained bands seen in Dom$^+$ Scs$^+$ Hly$^+$ organisms but absent or in lower concentrations in Dom$^-$ Scs$^+$ Hly$^-$ organisms. Figure 2B shows the autoradiograph of the dried gel in Fig. 2A. Marked differences in ^{125}I labeling are seen, which are characteristic for organisms of each colony type, an exception again being strain 899L, whose intermediate colonial morphologies both have Coomassie (Fig. 2A) and ^{125}I profiles (Fig. 2B) which appear to be mixtures of the Dom$^+$ Scs$^+$ Hly$^+$ and Dom$^-$ Scs$^+$ Hly$^-$ PVs.

Unlabeled samples of the same suspension used in Fig. 2A were digested with proteinase K, subjected to SDS-PAGE in 16% (wt/vol) total acrylamide gels, and stained with silver after periodate oxidation. Figure 2C, therefore, represents the LPS profiles (5, 12) from the two major phenotypes and shows that Dom$^+$ Scs$^+$ Hly$^+$ organisms possess a heavy-staining, slower-migrating band *a* and a lightly stained band *c*, whereas Dom$^-$ Scs$^+$ Hly$^-$ organisms possess a moderately staining band *b* and a lesser amount of a band *d*. An additional band *e*, which migrated faster than band *d*, was also observed in the Dom$^-$ Scs$^+$ Hly$^-$ PVs of strains Rat 1 and 8442 (not shown). Additional broad silver-stained bands (brackets) were seen in all PVs tested. These bands were not stainable by Coomassie brilliant blue R-250 and were evident

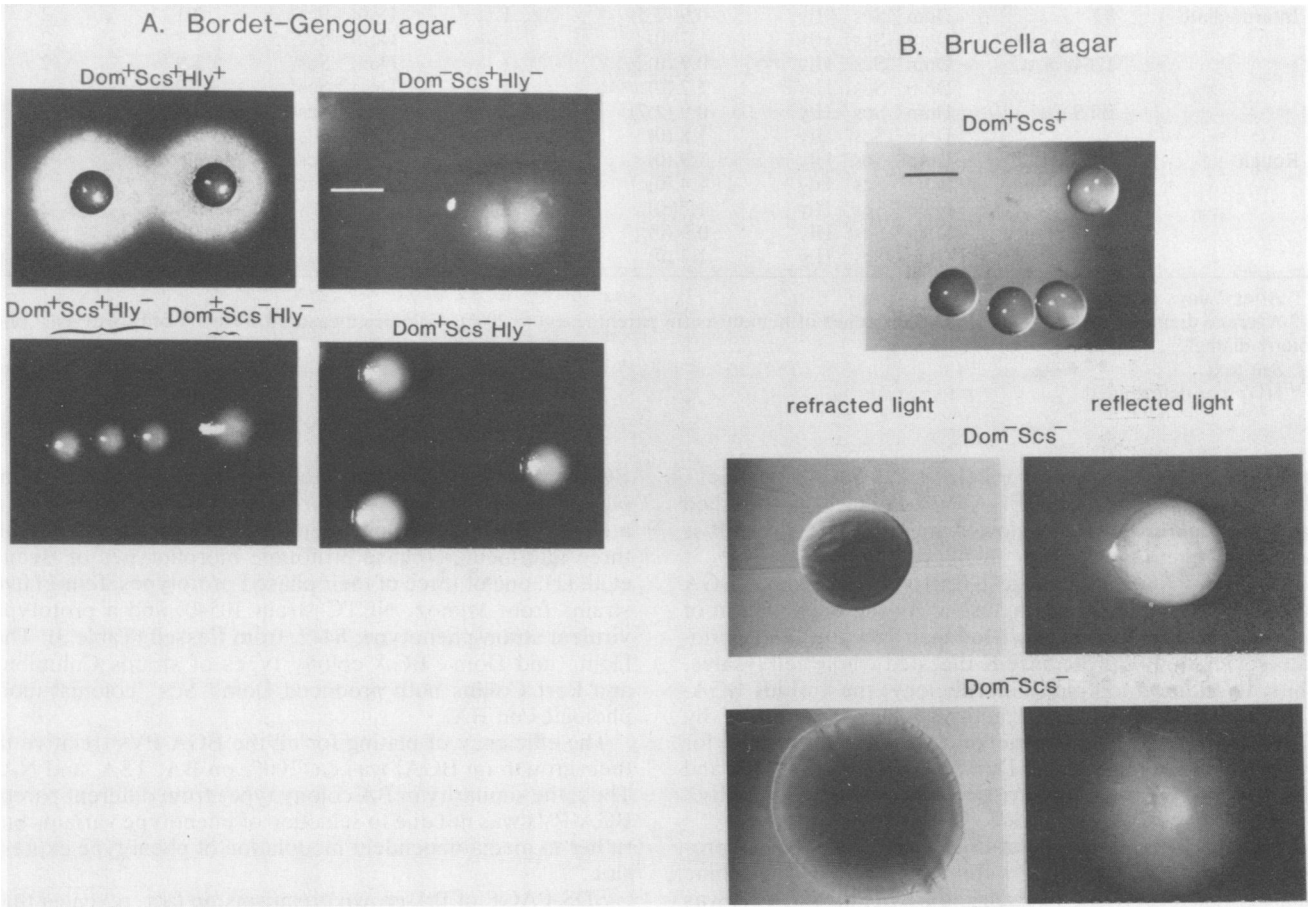

FIG. 1. Representative colonial morphologies of *B. bronchiseptica* after 2 days of growth at 37°C on (A) BGA and (B) BA. Colonies were viewed and photographed through a Bausch & Lomb Stereozoom 7. Bar = 1.0 mm.

TABLE 3. Colonial and SDS-PAGE phenotypes of *B. bronchiseptica* grown at 37°C on BGA or BA

Source	Strain	BGA			BA		
		Colony phenotype[a]	Colony diam (hemolysis diam) (mm)[b]	SDS-PAGE profile type	Colony phenotype[a]	Colony diam (mm)[b]	SDS-PAGE profile type[c]
Munoz	214	$Dom^+ Scs^+ Hly^+$	0.7 (3.2)	1	$Dom^+ Scs^+$	1.2	2
		$Dom^- Scs^+ Hly^-$	1.8 (0)	2	$Dom^+ Scs^+$	1.5	2
	2320	$Dom^+ Scs^+ Hly^+$	1.0 (2.5)	1	$Dom^+ Scs^+$	1.7	2
		$Dom^- Scs^+ Hly^-$	1.9 (0)	2	$Dom^+ Scs^+$	1.5	2
	899L	$Dom^+ Scs^+ Hly^\pm$	0.9 (1.2)	1	ND[d]	ND	ND
		$Dom^- Scs^+ Hly^\pm$	1.6 (1.8)	1			
	Rab 10	$Dom^\pm Scs^+ Hly^+$	1.1 (2.2)	1	ND	ND	ND
		$Dom^- Scs^+ Hly^-$	1.5 (0)	2			
	22067	$Dom^+ Scs^+ Hly^+$	0.7 (2.4)	1	ND	ND	ND
		$Dom^- Scs^+ Hly^-$	1.5 (0)	2			
NCTC	10540	$Dom^+ Scs^+ Hly^+$	1.0 (2.6)	1	$Dom^+ Scs^+$	1.5	2
		$Dom^- Scs^+ Hly^-$	2.0 (0)	2	$Dom^+ Scs^+$	1.7	2
Cassell	8442	$Dom^+ Scs^+ Hly^+$	0.9 (2.6)	1	$Dom^+ Scs^+$	1.8	ND
		$Dom^- Scs^+ Hly^-$	1.7 (0)	2	$Dom^+ Scs^+$	2.0	
	201	$Dom^- Scs^\pm Hly^-$	1.7 (0)	2	$Dom^\pm Scs^-$	3.0	ND
Bemis et al.							
Phase I	110H	$Dom^+ Scs^+ Hly^+$	1.0 (3.0)	1	$Dom^+ Scs^+$	1.2	1
		$Dom^- Scs^+ Hly^-$	1.9 (0)	2	$Dom^- Scs^-$	2.5	2
	501	$Dom^+ Scs^+ Hly^+$	0.9 (3.4)	1	$Dom^+ Scs^+$	1.4	1
		$Dom^- Scs^+ Hly^-$	1.9 (0)	2	$Dom^- Scs^-$	2.4	2
	Rat 1	$Dom^+ Scs^+ Hly^+$	0.7 (2.5)	1	$Dom^+ Scs^+$	2.0	1–2
		$Dom^- Scs^+ Hly^-$	1.8 (0)	2	$Dom^+ Scs^+$	1.8	2
Intermediate	87	$Dom^+ Scs^+ Hly^+$	0.9 (2.5)	1	$Dom^+ Scs^+$	2.1	2
		$Dom^- Scs^\pm Hly^-$	1.7 (0)	1	$Dom^+ Scs^+$	1.7	2
	17640 SAC	$Dom^+ Scs^+ Hly^-$	0.7 (0)	1	$Dom^+ Scs^+$	1.9	2
		$Dom^- Scs^- Hly^-$	1.7 (0)	2	$Dom^\pm Scs^+$	1.9	2
	BTS	$Dom^+ Scs^+ Hly^+$	0.9 (2.7)	1	$Dom^+ Scs^+$	2.0	2
		$Dom^- Scs^+ Hly^-$	1.8 (0)	2	$Dom^\pm Scs^+$	1.9	2
Rough	110 NH	$Dom^- Scs^+ Hly^-$	1.9 (0)	2	$Dom^- Scs^-$	2.5	2
	Ft. Collins	$Dom^+ Scs^- Hly^-$	1.4 (0)	2	$Dom^- Scs^-$	2.3	2
		$Dom^- Scs^+ Hly^-$	1.7 (0)	2	$Dom^- Scs^-$	2.4	2
	Columbus	$Dom^+ Scs^+ Hly^-$	0.9 (0)	1	$Dom^- Scs^-$	4.5	2
		$Dom^- Scs^+ Hly^-$	1.7 (0)	ND	$Dom^- Scs^-$	5.0	ND

[a] After 2 days of growth.
[b] Average diameter of 15 colonies and their zones of hemolysis (in parentheses) (±20%). Colonies measured were spaced by at least one colony diameter.
[c] See text.
[d] ND, Not determined.

in phenol–water-extracted LPS (16) from both $Dom^+ Scs^+ Hly^+$ and $Dom^- Scs^+ Hly^-$ BGA-PVs (Peppler, unpublished data). The nature of these slower-migrating broad bands is currently being investigated.

The SDS-PAGE data suggested that the two major BGA colony types could be distinguished by the combination of Coomassie blue staining, ^{125}I-Iodogen labeling, and periodate-silver stain of proteinase K-digested whole cell lysates. Thus, in addition to colony morphology, the various BGA-PVs described in Table 3 could also be characterized by SDS-PAGE profiles into one of two classes: type 1 for profiles characteristic of the $Dom^+ Scs^+ Hly^+$ BGA-PV and type 2 for profiles characteristic of the $Dom^- Scs^+ Hly^-$ BGA-PV (Table 3).

Phenotype expression on non-BGA media. SDS-PAGE profiles of whole cell lysates were then used to determine phenotype expression on various non-BGA media. This was particularly useful since the distinct colonial morphologies seen on BGA were often lost when cloned colonies of each BGA-PV were plated onto other media. On BA, for example, the two major BGA-PVs from most strains both showed a $Dom^+ Scs^+$ morphology (Fig. 1B). This occurred with all three intermediate-phase prototype morphotypes of Bemis et al. (1), one of three of their phase I prototypes, four of five strains from Munoz, NCTC strain 10540, and a prototype virulent strain-phenotype, 8442, from Cassell (Table 3). The Dom^+ and Dom^- BGA colony types of strains Columbus and Fort Collins both produced $Dom^- Scs^-$ colonial morphologies on BA.

The efficiency of plating for all the BGA-PVs (relative to their growth on BGA) was ca. 100% on BA, TSA, and NA. Thus, the similarity in BA colony types from different parent BGA-PVs was not due to selection of phenotype variants but rather to media-dependent modulation of phenotype expression.

SDS-PAGE of BA-grown organisms, in fact, revealed that most of the $Dom^+ Scs^+ Hly^+$ BGA-PVs expressed type 2 profiles when grown on BA. Table 3 summarizes these

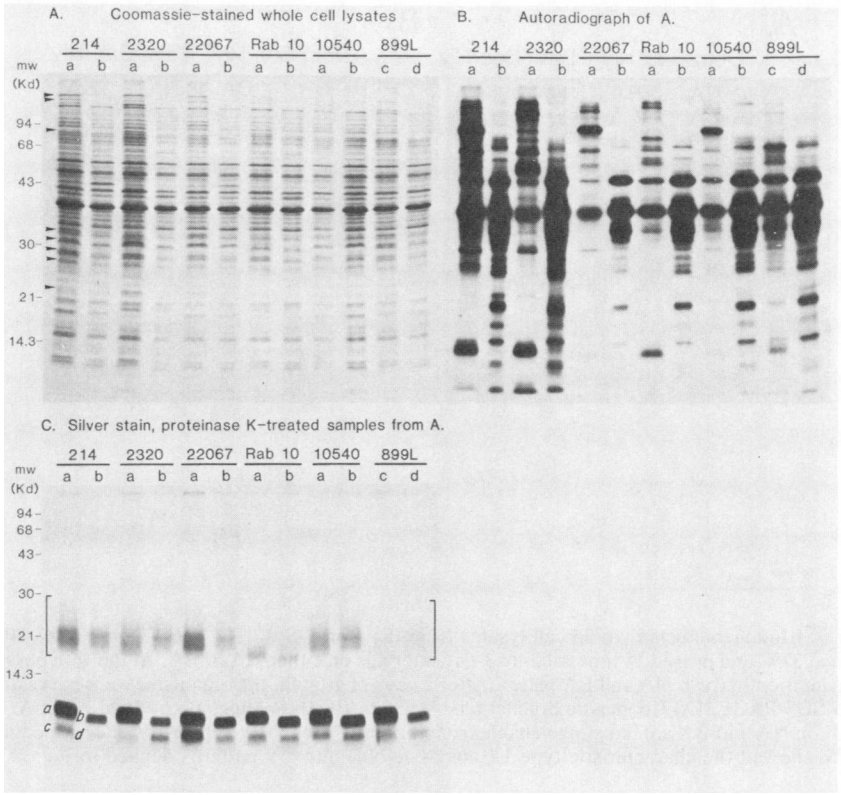

FIG. 2. SDS-PAGE of ^{125}I-Iodogen-labeled whole cell lysates of isogenic pairs of (a) Dom$^+$ Scs$^+$ Hly$^+$ and (b) Dom$^-$ Scs$^+$ Hly$^-$ PVs from strains 214, 2320, 22067, Rab 10, and 10540, and isogenic (c) Dom$^+$ Scs$^+$ Hly$^+$ and (d) Dom$^-$ Scs$^+$ Hly$^+$ PVs of strain 899L. (A) Gels stained with Coomassie brilliant blue. Arrows denote protein bands characteristic of the Dom$^+$ Scs$^+$ Hly$^+$ PVs and absent (or in reduced amounts) in the Dom$^-$ Scs$^+$ Hly$^-$ PVs. (B) Autoradiograph of the dried gel in A. Note the tendency of higher-molecular-weight bands to be labeled in the Dom$^+$ Scs$^+$ Hly$^+$ PVs, whereas lower-molecular-weight bands tend to be labeled in the Dom$^-$ Scs$^+$ Hly$^-$ PVs, irrespective of their staining densities with Coomassie blue. These grossly different ^{125}I-labeled patterns have been termed type 1 for the Dom$^+$ Scs$^+$ Hly$^+$ type profile and type 2 for the Dom$^-$ Scs$^+$ Hly$^-$ type profile. (C) Same sample preparations as in A, only unlabeled and digested with proteinase K for 3 h at 56°C and then electrophoresed. After electrophoresis, the gel was treated with periodate and stained with silver. Bands *a* and *c* are characteristic of the Dom$^+$ Scs$^+$ Hly$^+$ LPS profile. Bands *b* and *d* are characteristic of the Dom$^-$ Scs$^+$ Hly$^-$ LPS profile. In addition, broad bands of silver-stained material are seen in each PV preparation (brackets).

observations. These phenotypic changes were reversible, however, as exemplified by strain 214 in Fig. 3. After 15 serial subcultures on either BA or NA, a single passage onto BGA of the originally Dom$^+$ Scs$^+$ Hly$^+$ BGA-PV showed characteristic type 1 Coomassie blue and ^{125}I profiles. Continued passage on BA or NA resulted in type 2 profiles. The Dom$^-$ Scs$^+$ Hly$^-$ BGA-PV was little affected by growth medium as judged by the same SDS-PAGE criteria (Fig. 3).

Modulation of SDS-PAGE profile on non-BGA media extended to LPS expression and included TSA as well as NA and BA. Figure 4 shows that growth of the Dom$^+$ Scs$^+$ Hly$^+$ PV of strain 214 on BA (lane 2), TSA (lane 3), and NA (lane 4) affects its SDS-PAGE Coomassie blue (Fig. 4A) and LPS (Fig. 4B) profiles when compared to its profiles after growth on BGA (lane 1). In contrast, the SDS-PAGE Coomassie and LPS profiles from the Dom$^-$ Scs$^+$ Hly$^-$ PV of strain 214 were influenced little by the non-BGA media.

DISCUSSION

The Dom$^+$ Scs$^+$ Hly$^+$ BGA-PV described here is analogous to the phase I of Nakase (9, 10). Likewise the Dom$^-$ Scs$^+$ Hly$^-$ BGA-PV is analogous to the phase III of Nakase. Phase I has been considered the virulent phase of *B. bronchiseptica* based on its production of capsule (9), its adherence to cells (17), and its possession of adenylate cyclase (2). Phase III, in contrast, has none of these properties. The colonial morphologies on BGA and the ^{125}I-Iodogen-labeled whole-cell profiles shown here are reminiscent of those obtained with the domed, hemolytic and flat, nonhemolytic phenotype variants of *B. pertussis* (11) and support the idea that phase I *B. bronchiseptica* is the virulent PV. If this is so, then the methods we've used to monitor the PVs of *B. bronchiseptica* are of particular value.

First, we have shown that the medium on which *B. bronchiseptica* is grown can influence the phenotype it expresses. More specifically, the majority of our clearly distinguishable BGA-PVs had similar colonial morphologies on BA, and, as judged by Coomassie blue staining, ^{125}I labeling, and periodate-silver staining, the Dom$^+$ Scs$^+$ Hly$^+$ BGA-PV from most strains could modulate from a type 1 SDS-PAGE profile to a type 2 SDS-PAGE profile on TSA and NA in addition to BA. Nakase (10) has also shown, by serological methods, that phase I *B. bronchiseptica* modulates to produce phase III antigens (including heat-stable O antigens) on NA.

Thus, we caution the use of media other than BGA or blood agar to monitor colonial phenotypes unless it is

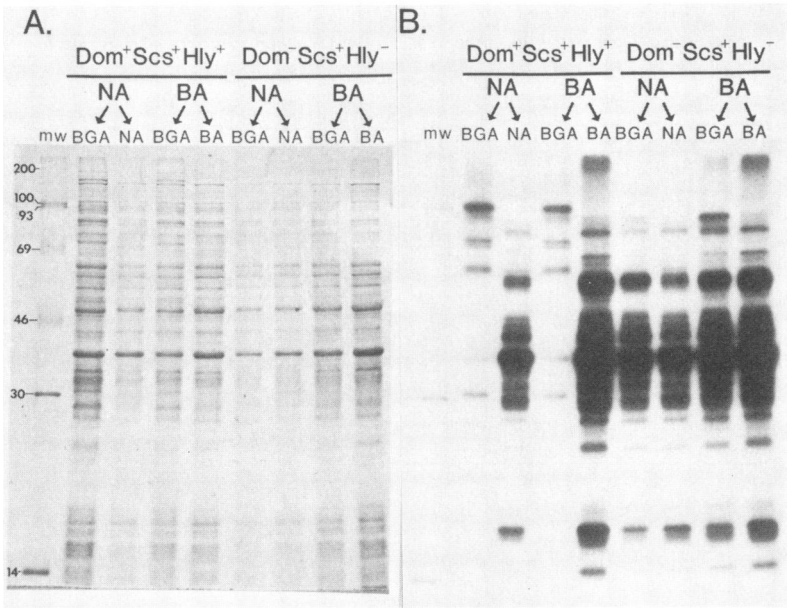

FIG. 3. SDS-PAGE of ^{125}I-Iodogen-labeled whole cell lysates from the Dom$^+$ Scs$^+$ Hly$^+$ and Dom$^-$ Scs$^+$ Hly$^-$ PVs of strain 214. The organisms had been grown at 37°C and passed 15 times at 2- to 3-day intervals on either NA or BA. At the 16th passage, organisms were transferred (arrows) to BGA in addition to fresh NA and BA plates. After 2 days of growth, the organisms were harvested off each medium labeled with ^{125}I, and subjected to SDS-PAGE. (A) Coomassie brilliant blue stained. (B) Autoradiograph of dried gel in A. SDS-PAGE patterns of the Dom$^+$ Scs$^+$ Hly$^+$ BGA-PV on NA and BA are indistinguishable from the Dom$^-$ Scs$^+$ Hly$^-$ BGA-PV on any medium. On BGA, however, the Dom$^+$ Scs$^+$ Hly$^+$ BGA-PV showed the characteristic type 1 Coomassie blue and ^{125}I patterns defined in Fig. 2.

determined, e.g., by the SDS-PAGE techniques described here, that no modulation of phenotype has occurred on the non-BGA medium being tested. In this regard we question the usefulness of the BA morphotype system of Bemis et al. (1). As Table 3 summarizes, the three prototype morphotypes of Bemis et al. were a heterogeneous mixture of BGA-PVs, many of which modulated to type 2 SDS-PAGE profiles when transferred onto BA, despite their being Dom$^+$ Scs$^+$ Hly$^+$ BGA-PVs. This leads to an erroneous classification of potentially virulent Dom$^+$ Scs$^+$ Hly$^+$ BGA-PVs as avirulent BA morphotypes in the system of Bemis et al. (1). The use of media or growth conditions in which modulation occurs has its obvious drawbacks, especially in the growth of organisms for isolation of virulence-associated components.

Second, the SDS-PAGE profiles of whole cell lysates can reveal polypeptides, surface-exposed proteins, and LPS unique to virulent PVs and aid in defining the molecular basis of virulence in *B. bronchiseptica*.

For example, further investigation may reveal that the Coomassie blue-stained bands unique to the Dom$^+$ Scs$^+$ Hly$^+$ PVs in Fig. 2A represent toxins, pili, or other polypeptides with virulence-associated characteristics.

The differences in SDS-PAGE autoradiographic profiles of ^{125}I-labeled Dom$^+$ Scs$^+$ Hly$^+$ and Dom$^-$ Scs$^+$ Hly$^-$ PVs (Fig. 2B) are also potentially revealing. They suggest that polypeptides judged to be similar by migration and Coomassie blue staining (Fig. 2A) are exposed differently in these two PVs. The expression of a polypeptide but its lack of surface exposure may represent a form of masking, or protection of that polypeptide from its external environment. If the external environment is a host animal, such protection may be envisioned as the inability of masked polypeptides on intact organisms to bind antibody or even to elicit an antibody response. Surface exposure of polypeptides may also help define the molecules responsible for some of the antigenic differences described for *B. bronchiseptica* PVs by agglutination reactions (9).

And finally, the differences seen in the LPS profiles of the Dom$^+$ Scs$^+$ Hly$^+$ and Dom$^-$ Scs$^+$ Hly$^-$ PVs (Fig. 2C) may help define the physical and chemical basis for the antigenic heterogeneity of the heat-stable (120°C, 1 h) somatic antigens of *B. bronchiseptica* PVs (9). In addition to antigenic differences, variation in LPS composition may confer other environment-related advantages to *B. bronchiseptica* such as antibiotic resistance or optimal membrane fluidity at various temperatures.

Further characterization of the polypeptides and LPS from the PVs of *B. bronchiseptica* may reveal structural similarities to PV-associated antigens in *B. pertussis*. Expression of structurally similar antigens by these two organisms would support the concept of Kloos et al., that *B. pertussis* and *B. bronchiseptica* are actually biotypes of the same species (6).

For the above reasons, we feel that SDS-PAGE of whole cell lysates is not only a simpler procedure for monitoring phenotypic variation in *B. bronchiseptica*, but also yields more information than the SDS-PAGE profiles of either cell-associated proteins (3) or supernatants of cell sonicates (4) alone.

In summary, *B. bronchiseptica* is capable of expressing multiple combinations of colonial phenotypes as well as other phenotypic markers such as SDS-PAGE polypeptide profiles (3, 4), piliation (1), flagellation (1, 9, 10), and O antigen expression (10). More BGA colonial phenotypes were reported here than just the Nakase phase I and phase III equivalents. Whether these other colonial phenotypes can all be classified as phase II is questionable. Further-

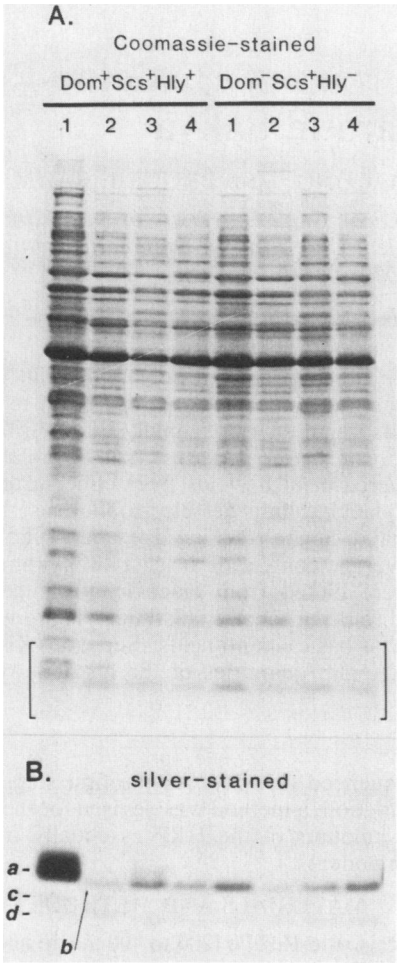

FIG. 4. SDS-PAGE of whole cell lysates from the Dom$^+$ Scs$^+$ Hly$^+$ and Dom$^-$ Scs$^+$ Hly$^-$ BGA-PVs of strain 214. Organisms were passed 15 times on either BGA (lane 1), BA (lane 2), TSA (lane 3), or NA (lane 4) and then subjected to SDS-PAGE. (A) Coomassie brilliant blue stained. (B) Proteinase K-treated and silver-stained as in Fig. 3B. The Dom$^+$ Scs$^+$ Hly$^+$ grown on BGA gives its characteristic *a-c* LPS banding pattern. On BA, TSA, or NA, however, a predominantly *b-d* pattern is seen. The Dom$^-$ Scs$^+$ Hly$^-$ BGA-PV of 214 gives its characteristic *b-d* pattern on all media, although a faint band *a* can be seen on BA in this preparation.

more, do they really represent natural diversity or are they laboratory artifacts caused by indiscriminant passage? We believe that an epidemiological study of BGA colonial phenotypes and their SDS-PAGE profiles as obtained from fresh *B. bronchiseptica* animal infection isolates would help to address this diversity. Experimental infection studies would further elucidate the role of phenotypic variation in the context of virulence determinants.

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