Novel Genetic and Phenotypic Heterogeneity in *Bordetella bronchiseptica* Pertactin

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The *Bordetella bronchiseptica* outer membrane protein pertactin is believed to function as an adhesin and is an important protective immunogen. Previous sequence analysis of the pertactin gene identified two regions predicted to encode amino acid repeat motifs. Recent studies have documented DNA sequence heterogeneity in both regions. The present study describes additional variants in these regions, which form the basis for six novel pertactin types. Immunoblotting demonstrated phenotypic heterogeneity in pertactin consistent with the predicted combined sizes of the repeat regions. A revised system for classifying *B. bronchiseptica* pertactin variants is proposed.

Bordetella bronchiseptica causes respiratory disease in a wide range of domesticated and wild animals, including atrophic rhinitis and pneumonia in pigs, bronchopneumonia in dogs, and rhinitis in rabbits. Pertactin is an outer membrane protein proposed to function as an adhesin for *B. bronchiseptica*, although this has yet to be proven. More clearly defined is its role as a protective immunogen. Several studies have demonstrated that pertactin-specific active or passive immunization protects against mortality and disease in mice and pigs (6, 10, 14).

Previous sequence analysis of the *B. bronchiseptica* gene encoding pertactin, *prn*, revealed two regions predicted to encode either GGXXP_n (region 1) or PQP_n (region 2) amino acid repeats (8). A recent study first described heterogeneity in both repeat regions of the *B. bronchiseptica prn* gene from a limited number of swine field and vaccine isolates (K. B. Register, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. B187, 2000). Additional *prn* variants in both regions have subsequently been reported based on an analysis of isolates of animal and human origin (1). Since the sources of those isolates were not further defined, the number of host species they represent is not clear. Corresponding phenotypic evidence of pertactin variants identified in these studies was not provided. The purpose of the present study was to examine the *prn* repeat regions of *B. bronchiseptica* isolates obtained from a variety of host species to ascertain whether additional variants exist. Furthermore, the pertactin proteins synthesized by strains possessing variations in the *prn* sequence were evaluated by Western blotting to determine whether accompanying phenotypic heterogeneity could be detected.

Genetic analysis of the repeat regions. The 14 *B. bronchiseptica* isolates included in this study were chosen to provide a diverse representation of host species, geographic origins, and ribotypes (Table 1). All but one have been previously described (13, 16, 17). Isolate SO3287-99 was acquired during 1999 and has only recently been characterized and ribotyped (C. Stave-

ley, K. B. Register, S. Yang, S. L. Brockmeier, and M. Chelchowitz, submitted for publication).

Detection of *prn* heterogeneity was initially based on a comparison of the mobility in agarose gels of PCR amplicons encompassing either region 1 or region 2, corresponding to bp 651 to 1211 or bp 1211 to 2063, respectively, relative to the published sequence of strain CN7531 (8). Multiple variants differing in mobility for each region were detected, some of which are shown in Fig. 1. To more specifically define regions of heterogeneity, fragments resulting from digestion of PCR amplicons with restriction enzymes were evaluated. The previously reported sequence predicts that digestion of region 1 amplicons with *Sau3A* should generate three detectable fragments, with the GGXXP_n repeat contained in a fragment of 153 bp. For some strains, the expected fragments were present and slight differences in mobility could be detected in the GGXXP_n-containing fragment (Fig. 2A, lanes 1, 2, and 5). In other strains, some *Sau3A* sites appeared to be absent or were located such that fragments of an unexpected size were apparent (Fig. 2A, lanes 3 and 4). In these cases, the identity of the repeat-containing fragment is not immediately obvious. Similar results were acquired when region 2 amplicons were digested with *BstXI*, which is predicted to generate two detectable fragments under the conditions used here. Mobility variants of the 360-bp PQP_n-encoding fragment were obvious for some strains (Fig. 2B, lanes 1 to 4). Other strains appeared to lack the *BstXI* site responsible for generating these two fragments (Fig. 2B, lane 5). In some cases, restriction digestion revealed heterogeneity in regions 1 or 2 that was not apparent from the comparison of full-length amplicons.

A formal definition of the sequence encompassed by regions 1 and 2 has not been previously proposed but would facilitate comparisons among different studies. In the only published report describing *B. bronchiseptica* region 1 and 2 variants (1), they are presented as including amino acids 254 to 299 and 559 to 610, respectively, relative to the published sequence of pertactin (8). I propose maintaining these boundaries, even though they include short stretches of sequence both upstream and downstream of the repeats themselves. Substitutions, insertions, or deletions immediately adjacent to the repeats may

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TABLE 1. *B. bronchiseptica* strains included in this study

Isolate	Host	Ribotype	Country of origin
KM22	Pig	3	Hungary
PV6	Pig	18	Hungary
MBORD676	Pig	2	Australia
MBORD606	Pig	3	Canada
MBORD847	Pig	2	Switzerland
MBORD831	Rabbit	9	Switzerland
5107	Rabbit	17	Hungary
ATCC 19395 (type strain)	Dog	ND ^a	United States
MBORD591	Dog	13	United States
MBORD731	Horse	8	Denmark
St. Louis	Human	15	United States
MBORD901	Turkey	15	Germany
MBORD626	Leopard	6	United States
SO3287-99	Sea otter	4	United States

^a ND, not determined.

lead to local changes in conformation, charge, or other properties that could directly affect functions dependent upon GGXXP_n or PQP_n sequences. Therefore, in accordance with previous practice (1), novel variants in this study were defined as those having any predicted amino acid substitution, inser-

FIG. 1. PCR amplicon mobility variants. Region 1 (A) or region 2 (B) PCR amplicons were resolved by agarose gel electrophoresis and visualized by staining with ethidium bromide. (A) Lanes: 1, MBORD847; 2, PV6; 3, MBORD731; 4, MBORD831; 5, St. Louis. (B) Lanes: 1, MBORD901; 2, ATCC 19395; 3, MBORD591; 4, St. Louis; 5, MBORD731. Lanes marked M contain molecular size markers, with their sizes indicated on the left.

FIG. 2. PCR amplicon restriction fragment analysis. Fragments resulting from digestion of region 1 amplicons with *Sau3A* (A) or digestion of region 2 amplicons with *BstXI* (B) were resolved by agarose gel electrophoresis and visualized by staining with ethidium bromide. (A) Lanes: 1, MBORD731; 2, MBORD831; 3, MBORD901; 4, St. Louis; 5, 5107. (B) Lanes: 1, MBORD676; 2, MBORD606; 3, ATCC 19395; 4, MBORD626; 5, MBORD591. The locations of molecular size markers are indicated on the left.

tion, or deletion in the areas specified as regions 1 and 2, as compared to variants already identified.

The five region 1 amplicons exhibiting unique patterns in agarose gels were purified and sequenced directly at the Iowa State University DNA Sequencing and Synthesis Facility, Ames. The predicted amino acid sequences of all five region 1 amplicons were found to constitute unique variants of the GGXXP_n region, based on differences in the number of repeats as well as amino acid substitutions. Substitutions occurred both in the degenerate amino acid positions and in sequence immediately adjacent to the repeats (Fig. 3A). Variant I-2 has been previously described (1). None of the unique region 1 variants identified here is identical to the six previously identified *Bordetella pertussis* region 1 variants (9, 11, 12; H. F. L. M. van Oirschot, unpublished data [GenBank accession no. AJ132095]).

Region 2 amplicons from the five strains with novel region 1 sequences, as well as additional amplicons representing all unique patterns observed in agarose gels, were analyzed similarly. Eight region 2 variants could be distinguished, based on the presence of six, seven, or eight PQP repeats, as well as amino acid substitutions and/or deletions in adjacent sequence (Fig. 3B). The predicted amino acid sequences for four variants, II-1, II-2, II-4, and II-5, have been previously reported (1). However, it is important to note the sequences presented in that publication are not consistent with the DNA sequence deposited in GenBank for the representative strains indicated. Since the authors of that study did not include DNA sequences in their report, it is not possible to determine the reason for this discrepancy. Therefore, it is presently unclear whether

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I-1  QRATIRRGDAPAGGAVPGGAVPG-----GAVPGGFGPLLDGWYGVDVSDSTVDLAQ
I-2  QRATIRRGDAPAGGAVPG-----GAVPGGFGPLLDGWYGVDVSDSTVDLAQ
I-4  QRATIRRGDAPAGGGVPGGAVPG-----GFGPGGFGPVLDDGWYGVDVSGSSVELAQ
I-5  QRATIRRGDAPAGGGVPGGAVPGGFDPGGFGPGGFGPGGFGPGGFGPVLDDGWYGVDVSGSTVELAQ
I-6  QRATIRRGDAPAGGGVPGGAVPGGFDPGGFGPGGFGPGGFGPGGFGPVLDDGWYGVDVSGSTVELAQ
I-7  QRATIRRGDAPAGGGVPGGAVPG-----GFGPGGFGPGGFGPVLDDGWYGVDVSGSTVELAQ
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B

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II-1  GAKAPPAPKPAPQPGPQPGPQP--PQPPQPPQ-----RQPEAPAPQPPAGRELSAA
II-2  GAKAPPAPKPAPQPGPQPGPQP--PQPPQPPQPPQ---RQPEAPAPQPPAGRELSAA
II-4  GAKAPPAPKPAPQPGPQPGPQP--PQPPQPPQPPQ---RQPEAPAPQPPAGRELSAA
II-5  GAKAPPAPKPAPQPGPQPGPQP--PQPPQPPQPPQPPQ---RQPEAPAPQPPAGRELSAA
II-10 GAKAPPAPKPAPQPGPQPPQPPQ--PQPQPQPQP-----QPEAPAPQPPAGRELSAA
II-11 GAKVPPAPKPAPQPGPQPPQPPQ--PQPQPQPQP-----PEAPAPQPPAGRELSAA
II-12 GAKVPPAPKPAPQPGPQPPQPPQPPQPQPQP-----QPEAPAPQPPAGRELSAA
II-13 GAKVPPAPKPAPQPGPQPPQPPQPPQPQPQP-----QPEAPAPQPPAGRELSAA
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FIG. 3. Multiple alignments of the predicted amino acid sequences for *pm* variants in region 1 (A) or region 2 (B). Dots and dashes indicate identical amino acids and gaps in the sequence, respectively. Unmarked positions indicate the location of amino acid substitutions.

variants II-1, II-2, II-4, and II-5, as described here, have been previously identified.

Proposed modifications to nomenclature. The results presented identify four novel repeat variants in region 1 of the *pm* gene of *B. bronchiseptica*, as well as four in region 2. Together with data already reported (1), a total of seven region 1 variants and 13 region 2 variants have been described. New variants identified here were initially assigned names consistent with the classification system previously used (1). However, given the large number of variants now identified and the existence of additional variants (K. B. Register, unpublished data), I propose a modified nomenclature that may be more informative and less cumbersome than the existing one. This system, unlike the current one, will clearly convey the number of GGXXP or PQP repeats present without having to refer to some other source and will also indicate which variants differ only in amino acids that do not affect the total number of repeats.

All studies describing pertactin variants (9, 11, 12), except for the most recent one (1), designate the use of Arabic numerals in referring to the repeat regions. Therefore, it is suggested that the originally established designations for regions 1 and 2 not be modified. The remainder of the proposed epithet consists of an additional Arabic numeral, coinciding with the predicted number of amino acid repeats, followed by a lower-case letter to distinguish variants sharing the same number of amino acid repeats (e.g., 1-1a). The first variant reported having a particular number of repeats would be assigned the letter "a," and subsequently identified variants would assume the next available letter, proceeding alphabetically. Region 1 variants differing only in the degenerate positions of the repeats would be assigned the numeral indicative of the total number of repeats, followed by the next available lowercase letter. To provide an example, the region 1 variants in this report previously referred to as I-5 and I-6 each contain eight GGXXP

repeats and are distinguished only by a single amino acid substitution in the second X position of repeat 4 (Fig. 3A). Under the proposed system, these variants would be designated 1-8a and 1-8b, respectively. As another example, the previously described GGXXP₃ variant I-2 would be renamed 1-3a. The designation 1-3b would be reserved for the next GGXXP₃ variant to be identified. Table 2 lists the previous and proposed designations for each of the currently known GGXXP and PQP variants. For the sake of clarity, the proposed nomenclature will be adopted for the remainder of this report.

TABLE 2. Current and proposed nomenclature for *B. bronchiseptica* pertactin variants

Repeat	Current designation	No. of repeats	Proposed designation
GGXXP	I-2	3	1-3a
	I-1	4	1-4a
	I-4	4	1-4b
	I-3	5	1-5a
	I-7	5	1-5b
	I-5	8	1-8a
PQP	I-6	8	1-8b
	II-1	6	2-6a
	II-7	6	2-6b
	II-2	7	2-7a
	II-4	7	2-7b
	II-10	7	2-7c
	II-11	7	2-7d
	II-5	8	2-8a
	II-8	8	2-8b
	II-12	8	2-8c
	II-13	8	2-8d
	II-3	9	2-9a
	II-6	9	2-9b
	II-9	9	2-9c

TABLE 3. *B. bronchiseptica* pertactin types identified in this study

Pertactin type ^a (region 1/region 2)	Strain	Accession no.	
		Region 1	Region 2
1-3a/2-7a	KM22	AY007270	AY007271
1-3a/2-7b ^b	SO3287-99	AY007262	AY007263
1-3a/2-8a ^b	ATCC 19395	AY007264	AY007265
1-4a/2-6a	MBORD831	AF298589	AF298590
1-4b/2-7c	MBORD591	AY007266	AY007267
1-5b/2-8d	PV6	AY007276	AY007277
1-8a/2-7d	St. Louis	AY007274	AY007275
1-8b/2-8c	MBORD901	AY007272	AY007273

^a Indicated using the proposed nomenclature.^b Reported previously (1).

Pertactin types predicted by DNA sequence. Based on the combined region 1 and region 2 sequences observed in this study, eight pertactin types could be distinguished (Table 3). Two have been previously described (1), assuming the region 2 sequences in that report are not in error. However, it should also be noted that the *prn* gene from strain CN7531, previously sequenced by Li et al. (8), codes for pertactin type 1-3a/2-7a, not type 1-3a/2-7b (or I-2/II-4 as it originally appears) as reported (1). That study, together with the present one, identifies 16 pertactin types from 56 isolates of *B. bronchiseptica*. This degree of heterogeneity is quite remarkable, considering that only six pertactin types have been identified from several hundred strains of *B. pertussis* (9, 11, 12; van Oirschot, unpublished data [GenBank accession no. AJ132095]). Recent evidence suggests that the emergence of *B. pertussis* *prn* variants may have been driven by immune selection in response to vaccination (9, 11, 12). Studies addressing this possibility with respect to *B. bronchiseptica* have not been undertaken. While many factors undoubtedly contribute to the multiplicity of *B. bronchiseptica* *prn* variants, the extensive host range of this species and the use of many different vaccine strains (which likely represent multiple pertactin variants) may have led to the simultaneous, but independent, evolution of pertactin in multiple host species.

Boursaux-Eude and Guiso (1) indicated that host specificity was not observed with respect to the pertactin types described, although information was not provided concerning the number or exact identity of hosts represented. While nearly all novel pertactin types reported here were found in isolates from different hosts, only one or a few isolates were examined from each species represented. Furthermore, isolates chosen for sequencing were those from which region 1 or 2 PCR amplicons, or restriction fragments derived from them, displayed unique patterns on agarose gels. Consequently, the pertactin types identified may not be representative of those most frequently occurring in a given host. Analysis of additional strains is necessary before conclusions can be reached regarding the distribution and frequency of pertactin types with respect to host species, geographic location, or other criteria.

Phenotypic heterogeneity detected by immunoblotting. Variation in the number of predicted amino acids in regions 1 and 2 for each of the pertactin types identified here suggests that differences in mobility should be detectable by Western blotting for some strains, provided that compensatory insertions or deletions do not occur in other regions. The monoclonal anti-

body BPE3 (2) was used for detection of pertactin in immunoblots prepared with bacterial extracts enriched in outer membrane proteins, as described (15). Alterations in the size of pertactin that are at least partially consistent with the total number of amino acids predicted in regions 1 and 2 were detectable in three strains. Pertactin from strain MBORD901, predicted to contain a total of 122 amino acids in regions 1 and 2, displayed the highest apparent molecular mass of all strains assessed. Pertactin from strains St. Louis and PV6, predicted to contain a total of 119 and 106 amino acids in the repeat regions, respectively, also demonstrated an increased molecular mass compared to the remaining strains. Other pertactin types are predicted to contain a total of 98 to 102 amino acids in regions 1 and 2. Reproducible differences in the mobility of pertactin from these strains were not observed under the conditions of this study. However, pertactin size heterogeneity may also be partially due to DNA polymorphisms outside regions 1 and/or 2. Compared to strain PV6, pertactin from the St. Louis strain is predicted to contain an additional 13 amino acids, yet the mobility of pertactin from these isolates is indistinguishable. Comparison of the sequences for the entire *prn* gene is required to determine the basis for this observation.

A vital question not addressed by this study is the functional significance of alterations in pertactin amino acid sequence. Investigations with *B. pertussis* suggest that region 1 amino acids may play a role in adherence (5, 7). Although analogous studies have not been carried out with *B. bronchiseptica*, it could be hypothesized that alterations in this region affect the specificity of binding to host cells, with certain pertactin types preferentially adherent to the tissues of one, or a related group, of host species. At least some *B. bronchiseptica* isolates do show reduced ability to infect heterologous hosts (4, 18). The presence of an immunodominant protective epitope in region 2 (3) suggests that polymorphisms in this region may permit escape from immune surveillance through the emergence of novel antigenic epitopes. Future studies are clearly needed to clarify the implications of pertactin heterogeneity in *B. bronchiseptica*.

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