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Physical and Genetic Map of the *Pasteurella multocida* A:1 Chromosome

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A physical and genetic map of the *Pasteurella multocida* A:1 genome was generated by using the restriction enzymes *Apa*I, *Ceu*I, and *Not*I. The positions of 23 restriction sites and 32 genes, including 5 *rrn* operons, were localized on the 2.35-Mbp single circular chromosome. This report presents the first genetic and physical map for this genus.

The gram-negative, facultative bacterium *Pasteurella multocida* is an important veterinary pathogen with worldwide distribution. It is a member of the *Pasteurellaceae* family (16), which includes the genera *Haemophilus*, *Actinobacillus*, and *Lonepinella* (15). Certain serotypes are the etiological agents of a number of severe pasteurelloses, such as fowl cholera in poultry, atrophic rhinitis in swine, and hemorrhagic septicemia in cattle and buffalo. Despite much research into the various diseases caused by *P. multocida*, there has been little characterization of this organism at the molecular level, and few of its genes have been characterized.

Here we describe the construction of a physical and genetic map of *P. multocida* serotype A:1, an Australian fowl cholera isolate, strain PBA100 (7). Restriction enzymes with GC-rich recognition sequences were chosen, as the G+C content of *P. multocida* is between 40 and 43% (14). The intron-encoded endonuclease *Ceu*I, which recognizes a 26-bp sequence found exclusively in the 23S rRNA gene (12, 23), was used for restriction mapping and the analysis of the *P. multocida* rRNA (*rrn*) operons.

Restriction fragment analysis and estimation of genome size. High-molecular-weight DNA was purified by the method of Smith and Cantor (21), except that the detergent Brij 58 was omitted from the lysis solution. The enzymes *Apa*I (Boehringer Mannheim), *Not*I, and *Ceu*I (both from New England Biolabs) were used in physical mapping of the *P. multocida* genome according to the manufacturers' instructions. Double digestions were performed as two consecutive 16-h digests, with *Ceu*I digestion always performed second.

The resulting restriction fragments were separated by pulsed-field gel electrophoresis (PFGE) in 1% agarose gels in 0.5× Tris-borate-EDTA buffer (20) at 14°C with a contour-clamped homogeneous electric field apparatus (CHEF DRII; Bio-Rad). A range of conditions for electrophoresis was used to visualize and size the macrorestriction fragments. Typically, fragments between 50 and 500 kb were separated by using a linear ramp time of 10 to 60 s for 24 h at 200 V. Larger restriction fragments were observed by using longer pulse times of 10 to 150 s and 180 V for 24 h.

PFGE profiles resulting from single and double restriction digestions are shown in Fig. 1A. The sizes of the resulting macrorestriction fragments estimated from a number of pulsed-field gels run under different conditions to optimize the separation of bands are listed in Table 1. When standard agarose gel electrophoresis was used to visualize restriction fragments under 20 kb, only the 9-kb NF band was detected (Fig. 1B). The *Apa*I and *Apa*I/*Ceu*I restriction profiles appeared identical (Fig. 1A, lanes 5 and 6), indicating the close linkage of the five *Ceu*I sites to five *Apa*I sites. No evidence of extrachromosomal elements was found in the strain studied by using either a standard alkaline lysis plasmid preparation or the method of Kado and Liu (8) for the extraction of large plasmids. Based on these data, the *P. multocida* A:1 genome was estimated to be 2.35 Mbp.

Construction of the physical and genetic map. Single and double restriction digestion, partial *Apa*I digestion, Southern hybridization using homologous and heterologous gene probes, the generation of linking clones, and reciprocal hybridization using macrorestriction fragments as probes were used to generate a physical and genetic map of the *P. multocida* A:1 chromosome. All hybridization, probe labeling, and chemiluminescence detection steps were performed according to the Dig System User's Guide (Boehringer Mannheim). Hybridizations with homologous probes were performed under conditions of high stringency at 68°C, whereas those with heterologous probes were performed at 55°C under medium-stringency conditions consisting of two 5-min washes at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) and two 15-min washes at 37°C in 1× SSC–0.1% SDS.

A small number of *Not*I and *Apa*I linking clones were constructed by digesting 1 μg of genomic DNA to completion with *Bam*HI, *Eco*RI, or *Hin*dIII. Restriction digestion products were self-ligated and digested with either *Not*I or *Apa*I. The resulting linear fragments were then cloned into *Not*I- or *Apa*I-digested pBluescript II KS (Stratagene) and used as probes in Southern hybridization experiments. The linkages NA-NE1, NB-NC2, and NC1-ND were established by using the isolated *Not*I linking clones. These linkages were found to occur in the AE and CC fragments, the AD and CD fragments, and the AC and CA fragments, respectively.

A number of the *Apa*I linking clones isolated were of limited use in linkage analysis, as they hybridized to multiple *Apa*I fragments, all *Ceu*I fragments, and *Not*I fragments NA, NB, and NC. The remaining *Apa*I linking clone linked AA with AG, and this linkage was found to lie within the CB and NA fragments. This approach thus definitively identified overlap-

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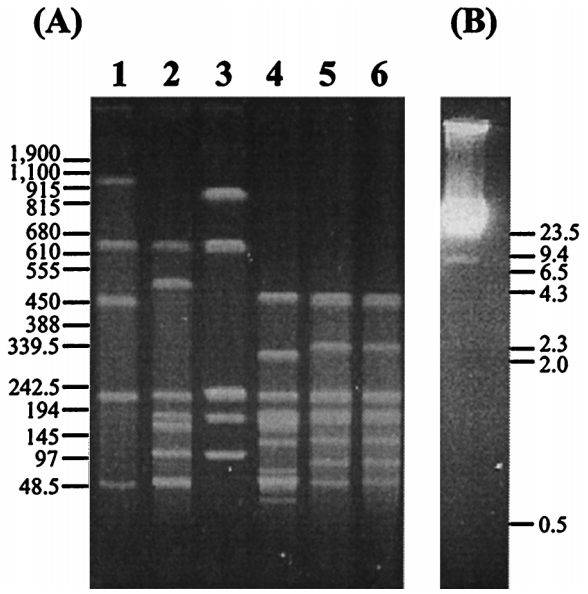

FIG. 1. (A) PFGE of *P. multocida* DNA fragments produced after digestion with *Ceu*I (lane 1), *Not*I/*Ceu*I (lane 2), *Not*I (lane 3), *Not*I/*Apa*I (lane 4), *Apa*I (lane 5), and *Apa*I/*Ceu*I (lane 6). The positions of standard DNA size markers (in kilobases) are shown on the left. Electrophoresis was performed in 1% agarose with a pulse time of 10 to 80 s at 200 V for 24 h. In this figure, a single band represents each of the *Apa*I fragment pairs, AA/AB, AD/AE, and AF/AG (lane 5). (B) Standard agarose gel electrophoresis, showing the migration of *P. multocida* genomic DNA after *Not*I digestion, to visualize the 9-kb NF restriction fragment. The positions of *Hin*dIII-digested lambda DNA size markers (in kilobases) are shown on the right. The originals were scanned with a Hewlett-Packard ScanJet 4cse by using the Adobe Photoshop 2.5.1 LE program and exported into Deneba Canvas 3.5.4 for presentation.

ping groups of restriction fragments between the three restriction enzymes.

Reciprocal hybridization experiments using macrorestriction fragments as probes extended the linkage data and allowed for the placement of fragments into a circular genomic map. However, because AA and AG occurred entirely within NA and CB, their order could not be determined by reciprocal hybridization or linker probe data alone. Partial *Apa*I restriction digestion was used to determine the orientations of these fragments. *Apa*I partial fragments of 790 and 415 kb, which may comprise AA-AC (800 kb) and AG-AE (408 kb), respectively, were observed. If the *Apa*I fragments were present in the order AC-AG-AA-AE, a 688-kb fragment representing the linkage AA-AE and a 520-kb fragment representing AC-AG would be expected. Although a 680-kb fragment was seen, no partial fragment in the vicinity of 520 kb was observed. This suggested that the most likely arrangement of these fragments was AC-AA-AG-AE.

Gene probes (Table 2) were hybridized to single and double digests of *P. multocida* genomic DNA. This led to the positioning of genes which covered much of the genome onto the physical map and provided further evidence for the overlapping linkage groups of restriction fragments, hence permitting a more precise alignment of the three restriction maps with respect to each other. The combined physical and genetic map is shown in Fig. 2A.

Location, orientation and operon structure of the *P. multocida* A:1 *rrn* genes. The intron-encoded endonuclease *Ceu*I has been found to cut exclusively in the *rrl* genes of many bacteria (12). Thus, the five restriction fragments generated indicated that PBA100 has five copies of the *rrl* gene. To determine if rRNA genes in *P. multocida* were organized into operons, heterologous *rrn* probes from *Dichelobacter nodosus*, probe 1, probe 3, and probe 4 (11), and a homologous PCR-derived 16S rRNA probe, probe 2, with the primers 5′-AGAGTTTGATC CTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTTC-3′, which bind to conserved regions of the 16S rRNA gene, were used in Southern hybridization analysis (Fig. 2B). With all *rrn* probes used, an invariant *Not*I hybridization profile of NA, NB, and NC was seen, indicating that *rrn* genes resided only on these *Not*I restriction fragments. The linkage of the five *Ceu*I fragments to five *Apa*I fragments was apparent from the similarity of the *Apa*I and *Apa*I/*Ceu*I digests (Fig. 1) and the resulting physical map. Hybridization results, taken in conjunction with the physical mapping data, indicated that the *rrs* genes in *P. multocida* traverse *Apa*I sites. This was confirmed by the isolation of *Apa*I linking clones that hybridized to the same set of *Not*I fragments (NA, NB, and NC), many *Apa*I

TABLE 1. Sizes of restriction fragments observed by PFGE after digestion of *P. multocida* A:1 (PBA100) genomic DNA with restriction enzymes as indicated

*Apa*I		*Ceu*I		*Not*I		*Apa*I-*Ceu*I	*Not*I-*Ceu*I
Fragment	Size (kb)	Fragment	Size (kb)	Fragment	Size (kb)	Size (kb)	Size (kb)
AA	460	CA	980	NA	850	460	630
AB	450	CB	630	NB	630	330	520
AC	340	CC	450	NC1	230	230	230
AD	230	CD	230	NC2	230	185	180
AE	228	CE	55	ND	180	180	175[a]
AF	185			NE1	110	175[a]	110[a]
AG	180			NE2	110	125	55[b]
AH	130			NF	9	75	9
AI	95					55[c]	
AJ	55					53	
						33	
						9	
Total[d]	2,353		2,345		2,349	2,360	2,359

[a] Band contains two comigrating fragments.
[b] Band contains four comigrating fragments.
[c] Band contains six comigrating fragments.
[d] The genome size of 2.35 Mbp was derived from the mean of the five total genomic size estimations from each restriction digestion shown above.

TABLE 2. Gene probes used for physical and genetic mapping

Probe	Gene	Function or description	Source[b]
Homologous			
pMEC100	*aroA*	Aromatic amino acid biosynthesis	6
pPBA822	*gidA*[a]	Glucose-inhibited cell division protein	A
pPBA826	*atpG*[a]	ATP synthetase gamma subunit	A
pPBA838	*hemN*[a]	Heme biosynthesis	A
pPBA1039	*rpsF*[a]	Ribosomal protein	A
pPBA1137	*yaeL*[a]	Hypothetical *E. coli* protein	18
pPBA1137	*oma87*	87-kDa outer membrane protein	19
pPBA1137	*skp*[a]	Chaperone protein	18
pPBA1155	*clpP*[a]	Protease	A
pPBA1166	*murCG*[a]	Peptidoglycan synthesis	A
pPBA1169	*groEL*[a]	Heat shock protein	A
pPBA1211	*adh*[a]	Alcohol dehydrogenase	A
pPBA1211	*mesA*	Esterase	A
pPBA1211	*HI0568*[a]	Orthologue of HI0568, *H. influenzae* Rd	A
pPBA1211	*greB*[a]	Transcriptional elongation factor	A
pPBA1217	*recA*	Homologous recombination	A
pPBA1245	*lapB*[a]	Membrane protein	A
pPBA1248	*aspS*[a]	Aspartyl-tRNA synthetase	A
pPBA1267	*tonB*[a]	Iron uptake	A
pPBA1268	*tbpI*[a]	Transferrin binding protein	A
pPBA1271	*ampD*[a]	β-Lactamase regulation	18
pPBA1271	*ptfA*[a]	Type IV fimbrial subunit gene	18
pPBA1285	*HI1252*[a]	Orthologue of HI1252, *H. influenzae* Rd	A
pPBA1458	*pzfA*[a]	Putative zinc finger; regulation	A
pPBAYZ1	*rpoB*[a]	RNA polymerase beta subunit	A
PCR amplicon	*ompH*	Major outer membrane porin	B
PCR amplicon (probe 2)	*rrs*	16S rRNA gene 1.5-kb PCR product	A
Heterologous			
pJSK3	*bexAB*	Polysaccharide export; *H. influenzae* type b	10
pJIR632 (probe 1)	*rrs*	5′ region of *D. nodosus rrs* gene	11
pJIR697 (probe 3)	*rrs-rrl*	3′ region of *rrs* and 5′ region of *rrl* gene of *D. nodosus*	11
pJIR873 (probe 4)	*rrl-rrf*	3′ region of *rrl* and complete *rrf* gene of *D. nodosus*	11

[a] Gene names assigned on the basis of similarity to GenBank sequence entries.
[b] Source or reference of each gene probe. A, our laboratory; B, 943-bp product generated by using primers 5′-AAA AAG ACA ATC GTA GC-3′ and 5′-TAA CCA AAA CCT ACA GCG-3′, designed from GenBank sequence U50907 (13).

restriction fragments, and all *Ceu*I fragments. One such clone, a 5.5-kb *Eco*RI-generated *Apa*I linking clone, was partially sequenced, revealing the presence of an *rrs* gene containing a single *Apa*I site and both *rrl* and *rrf* genes. Considering the sequencing data and the *rrn* hybridization profiles, *P. multocida* rRNA genes appear to be organized into five operons, with the gene order of these operons, like that of many other eubacteria, being *rrs-rrl-rrf*. *Ceu*I and *Apa*I/*Ceu*I digestion of these linking clones indicated the presence of a unique *Ceu*I site and demonstrated an *Apa*I/*Ceu*I fragment of either 2.9 or 3.1 kb, thus indicating the presence of two classes of *rrn* loci with different spacing of the *Apa*I and *Ceu*I restriction sites. The direction of transcription of each operon, designated *rrnA* through *rrnE*, was ascertained from hybridization analysis (Fig. 2).

Genetic organization. This study, which positions 23 restriction sites and 32 genetic markers onto a 2.35-Mbp circular chromosome, details the first physical and genetic map for the species *P. multocida* and the genus *Pasteurella*. Key genetic markers such as *gidA*, the five *rrn* operons, *recA*, and *groEL* have been positioned onto the chromosome. The genome size derived in this study places *P. multocida* in bacterial genome group 2 as defined by Cole and Saint Girons (2), which includes genomes between 1.5 and 3 Mbp. This group includes a range of human and animal pathogens, such as the gram-positive bacteria *Streptococcus pneumoniae* and *Staphylococcus aureus* and the gram-negative bacteria *Campylobacter jejuni*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* (2).

Several features of genome organization common among many bacteria appear to be conserved in *P. multocida*. The *gidA* gene, encoding the glucose-inhibited division protein, is adjacent to the origin of replication, *oriC*, in *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, and the closely related strain *H. influenzae* Rd (3, 22). If *gidA* is also linked to the origin of replication in *P. multocida*, then genes involved in transcription, as in many other bacteria, map close to the origin: *greB* and *atpG* are both located on the same fragment as *gidA*, and *rpoB* and *aspS* occur within approximately 285 kb of this site (Fig. 2A).

The *rrn* operons of *P. multocida* A:1 strain PBA100 occur in two unequal groups, transcribed divergently from the putative site of *oriC*, which may itself be linked to the *rrnA* operon. This is a common feature of many bacterial chromosomes, in which the *rrn* genes are often located within one-third to one-half of the chromosome (17). The operons *rrnA*, *rrnB*, *rrnC*, and *rrnD*, which are transcribed in the same direction away from the presumed location of *oriC*, lie within a 735-kb region (31% of the chromosome). In many bacteria, a single *rrn* operon is transcribed divergently from the other *rrn* loci. However, in PBA100 the single divergently transcribed *rrnE* operon is located at an unusually large distance of almost 980 kb away from the putative origin of replication, with the result that the five *rrn* operons of *P. multocida* are spread over a much greater proportion of the chromosome than is common in other bacteria.

Varying degrees of genome structure conservation are seen within and between bacterial genera and species (4). A com-

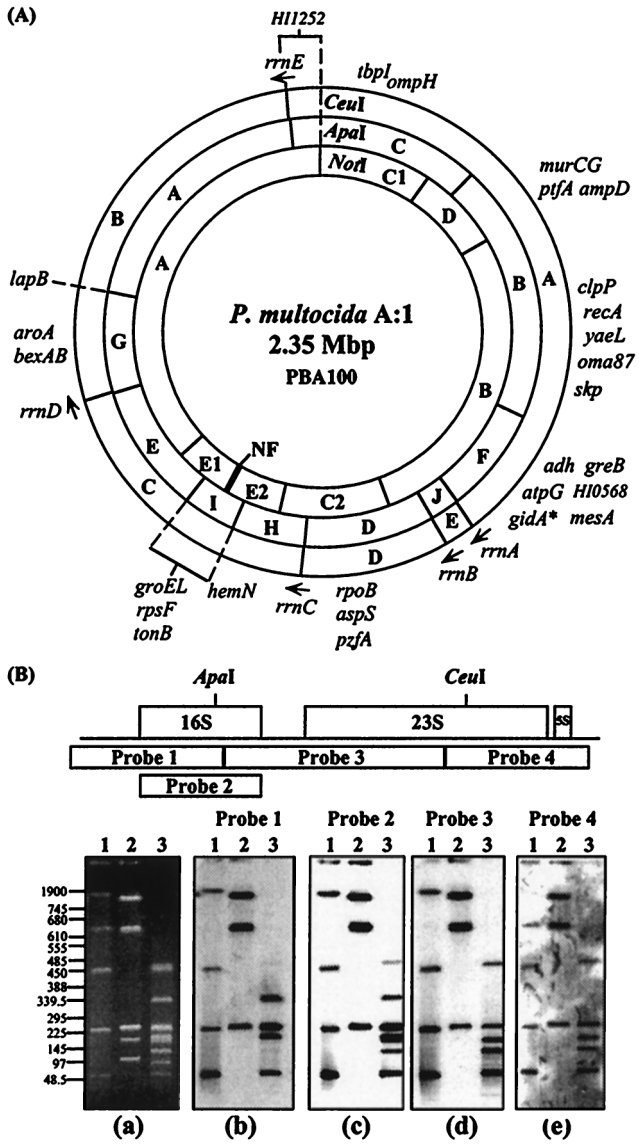

FIG. 2. (A) Physical and genetic map of the 2.35-Mbp circular chromosome of *P. multocida* A:1 PBA100. The positions of the *Apa*I, *Ceu*I, and *Not*I restriction sites are shown, and fragment names are indicated. The genes are positioned on the map to the minimum region localized by hybridization. The order of genes given in a particular region is arbitrary and does not necessarily represent the actual order of genes in that area. The genes *groEL*, *rpsF*, and *tonB* can be localized only to the 95-kb AI fragment, as it is not known to which NE fragment these genes belong. Asterisk, putative location of the origin of replication, linked to *gidA*. The arrows indicate the presumed 5′→3′ direction of transcription of the *rrn* operons given the gene order *rrs-rrl-rrf*. (B) Determination of *rrn* gene position and orientation on the *P. multocida* chromosome. (Top) Diagrammatic representation of a ribosomal operon and the positions of heterologous *D. nodosus* probes 1, 3, and 4 and homologous *rrs* gene probe 2. The approximate positions of *Apa*I and *Ceu*I sites in the *P. multocida rrn* operons are indicated. (a) PFGE-resolved restriction fragments of *P. multocida* DNA obtained by using running conditions of 20 to 60 s at 200 V for 24 h. The positions of standard DNA size markers (in kilobases) are shown on the left. (b through e) Resulting Southern hybridization profiles obtained by using the probes indicated above. In all panels, lanes 1, 2, and 3 represent *Ceu*I, *Not*I, and *Apa*I restriction digestion fragments, respectively. The originals were scanned with a Hewlett-Packard ScanJet 4cse by using the Adobe Photoshop 2.5.1 LE program and exported into Deneba Canvas 3.5.4 for presentation.

parison of the genetic maps of the *Pasteurellaceae* members *P. multocida* and *H. influenzae* Rd revealed both similarities and differences, but no long-range colinearity of gene order was found. The conservation of gene order among some bacteria is often most marked close to the origin (9). Local gene order and similar genome locations were apparent for the orthologous genes *greB* and *HI0568*. An order and spacing of these two genes in *P. multocida* similar to those in *H. influenzae* Rd were determined from sequence analysis of pPBA1211 (Table 2). These genes mapped to the macrorestriction fragment AF, which contained the putative location of *oriC*.

The *yaeL-oma87-skp-firA* region of *P. multocida* (18, 19) was identified as another region of local conservation of gene order, aligning with the *H. influenzae* Rd genes *yaeL-D15-skp-firA* (3). However, these orthologues have different chromosomal locations. Differences in gene positions are also apparent when the clustering together of *murCG* and *ptfA* within a 125-kb region, the clustering of *aroA* and *lapB* within a 180-kb region, and the close proximity of *tonB* to *groEL* and *rpsF* in the *P. multocida* genome are taken into consideration. In *H. influenzae* Rd, these groups of genes are separated by much greater distances. Despite the fact that the genome of *P. multocida* is 520 kb larger, there was no evidence for the occurrence of multiple *lapB* and *tbpI* loci, such as are found in *H. influenzae* Rd. Also, *P. multocida* has only five *rrn* operons, which are arranged differently from the six *rrn* operons of *H. influenzae* Rd.

Differences between these *Pasteurellaceae* members are not unexpected when the different growth requirements of these two pathogens and the different diseases they cause are considered. Additionally, different *Not*I profiles have been reported previously for other *P. multocida* isolates (24). We have also observed differing *Ceu*I profiles in other serotype A:1 isolates and some isolates of heterologous serotypes (data not shown), indicating that *P. multocida* exhibits significant intraspecies heterogeneity. The marked differences between restriction fragment profiles may be the result of a heterogeneous group of organisms being grouped together as a single species. Alternatively, while no extrachromosomal elements were found in PBA100, a variety of bacteriophages and plasmids have been characterized in other *P. multocida* isolates (1, 5). Hence, the tools for genome plasticity are present in the *P. multocida* species.

This genomic map provides a basis for the study of genomic organization in *P. multocida*. Comparative analysis of the *Apa*I and *Ceu*I profiles of *P. multocida* isolates will be useful in tracking the number and arrangement of the *rrn* loci. The placement on the map of more virulence-associated genes and the future use of this map in comparative studies with other *P. multocida* isolates of the same or different serotypes will provide greater insights into the genomic architecture of *P. multocida* and may begin to answer questions about the molecular basis for the differences in host predilection and pathogenicity in this diverse species.

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