

A family of phase-variable restriction enzymes with differing specificities generated by high-frequency gene rearrangements

(phase variation/mycoplasmas/DNA inversion/antigenic variation/mycoplasma virus)

KEVIN DYBVIG*†‡, RAMAKRISHNAN SITARAMAN‡, AND C. TODD FRENCH†

Departments of †Comparative Medicine and ‡Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294-0019

Edited by Clyde A. Hutchison, III, University of North Carolina, Chapel Hill, NC, and approved September 15, 1998 (received for review July 27, 1998)

ABSTRACT The *hsd* genes of *Mycoplasma pulmonis* encode restriction and modification enzymes exhibiting a high degree of sequence similarity to the type I enzymes of enteric bacteria. The S subunits of type I systems dictate the DNA sequence specificity of the holoenzyme and are required for both the restriction and the modification reactions. The *M. pulmonis* chromosome has two *hsd* loci, both of which contain two *hsdS* genes each and are complex, site-specific DNA inversion systems. Embedded within the coding region of each *hsdS* gene are a minimum of three sites at which DNA inversions occur to generate extensive amino acid sequence variations in the predicted S subunits. We show that the polymorphic *hsdS* genes produced by gene rearrangement encode a family of functional S subunits with differing DNA sequence specificities. In addition to creating polymorphisms in *hsdS* sequences, DNA inversions regulate the phase-variable production of restriction activity because the other genes required for restriction activity (*hsdR* and *hsdM*) are expressed only from loci that are oriented appropriately in the chromosome relative to the *hsd* promoter. These data cast doubt on the prevailing paradigms that restriction systems are either selfish or function to confer protection from invasion by foreign DNA.

Bacterial restriction and modification (R-M) systems function as a defense mechanism conferring protection from phage infection and other types of DNA invasion. Incoming DNA lacking the appropriate base modifications is cleaved by the restriction endonuclease while host DNA is protected by the presence of sequence-specific base modifications introduced by the activity of the DNA methyltransferase (MTase). These systems usually confer incomplete protection from phage infection because some phage DNA molecules are modified by the MTase before the endonucleolytic cleavage can occur, giving rise to progeny phage containing DNA modifications identical to that of the host and, therefore, resistant to restriction activity.

The type I restriction enzymes are considerably more complex than the more prevalent type II enzymes. Type II restriction activity and MTase activity are performed by two distinct enzymes encoded by gene pairs. The endonuclease reaction is sequence-specific and occurs at or very near to the site modified by the MTase. In the type I systems, the nuclease and MTase activities are performed by the same holoenzyme, which consists of three types of subunits. The S subunit dictates the sequence specificity of the MTase activity. Both the S and M subunits are necessary and sufficient for MTase activity, which occurs at specific adenine residues. All three types of subunits (S, R, and M) are required for the nuclease reaction,

which occurs at essentially random sites up to 7 kilobases from the recognition sequence (1). Translocation of the holoenzyme from the recognition site to the cleavage site occurs through the ATP-dependent helicase activity of the R subunit.

It has been proposed that some restriction systems are selfish because the daughter cells that have lost the R-M genes may be killed if the MTase activity is lost more rapidly than the nuclease activity (2, 3). Therefore, it is possible that some restriction systems persist in bacteria because of selfish behavior and are not maintained for cellular defense. Data supporting the selfish behavior hypothesis are based on type II systems. In contrast, a recent study concluded that type I systems may not be selfish and that cellular defense is a possible function (4).

Mycoplasma pulmonis possesses the most complex *hsd* genes described thus far (5, 6). The *M. pulmonis* chromosome has two *hsd* loci, each encoding S, M, and R subunits highly homologous to type I restriction systems. Both loci are site-specific DNA inversion systems containing two *hsdS* genes flanking *hsdR* and *hsdM*. DNA inversions occur within the coding regions of the *hsdS* genes and result in extensive genetic polymorphisms. We show here that DNA inversions regulate the phase-variable production of restriction activity and that polymorphic *hsdS* genes encode S subunits of differing specificity. The phase-variable nature of the system strongly argues against the selfish behavior hypothesis and raises the issue of how cells with unmodified genomic DNA survive when restriction activity is induced. We conclude that cultures of *M. pulmonis* contain a significant subpopulation of cells in which the *hsdR* and *hsdM* genes are not transcribed. Such cells lack detectable restriction activity and are highly susceptible to phage infection. Therefore, the *hsd* loci in *M. pulmonis* are ineffective as barriers against invasion by foreign DNA and may serve alternative functions.

MATERIALS AND METHODS

Mycoplasmas. *M. pulmonis* was propagated in mycoplasma medium as described (7). Subclones of *M. pulmonis* (Table 1) were derived by using filter clone methodology (8). In brief, cell cultures were passed gently through a 0.2- μ m filter immediately before assaying for colony-forming units. This technique removes cell aggregates, and the resulting colonies are presumed to be derived from single cells. The resulting colo-

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: R-M, restriction and modification; MTase, methyltransferase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF076984–AF076990).

*To whom reprint requests should be addressed at: Department of Comparative Medicine, Volker Hall, Room 418A, University of Alabama at Birmingham, Birmingham, AL 35294-0019. e-mail: dybvig@uab.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1998 by The National Academy of Sciences 0027-8424/98/9513923-6\$2.00/0 PNAS is available online at www.pnas.org.

Table 1. *M. pulmonis* strains used in this study

Strain	Parent strain	Restriction group, enzymes	Reference
KD735-15	KD735	I, no <i>MpuU</i> enzymes	6
KD735-16K	KD735	II, <i>MpuUI</i> and <i>MpuUII</i>	5
KD735-16H	KD735	VIII, <i>MpuUI</i>	This study
KD117	KD735-16H	II, <i>MpuUI</i> and <i>MpuUII</i>	This study
KD129	KD735-16H	III, <i>MpuUI</i> and <i>MpuUIV</i>	This study
KD131	KD735-16H	IV, <i>MpuUIII</i> and <i>MpuUIII</i>	This study
KD136	KD735-16H	II, <i>MpuUI</i> and <i>MpuUII</i>	This study
KD208	KD735-16H	V, <i>MpuUV</i> and <i>MpuUVI</i>	This study
KD231	KD735-16H	VI, <i>MpuUV</i> and <i>MpuUIV</i>	This study
KD255	KD735-16H	VIII, <i>MpuUI</i>	This study
KD262	KD735-16H	VII, <i>MpuUI</i> and <i>MpuUVIII</i>	This study
KD297	KD735-16H	IV, <i>MpuUIII</i> and <i>MpuUII</i>	This study
T3	KD736-16K	V, <i>MpuUV</i> and <i>MpuUVI</i>	This study
KD117-18	KD117	VIII, <i>MpuUI</i>	This study

nies (filter clones) were selected randomly for propagation and analysis for R-M activity.

R-M Assays. R-M properties of *M. pulmonis* strains were assessed by determining the plaquing efficiency of mycoplasma virus P1 on lawns of host cells as described (6, 9).

Gene Analysis. Genomic DNA was isolated as described (5). For Southern blot analysis, *Hind*III-digested chromosomal DNA fragments were subjected to agarose gel electrophoresis, were transferred to MSI MagnaCharge nylon membrane (Fisher), and were hybridized by using normal (high) stringency conditions (8). The probe was a 362-bp PCR product derived from sequences located within the conserved *hsdS* domain illustrated in Fig. 1 by black shading and was obtained by PCR amplifying DNA from strain KD735-15 using primers 5'-CAATTCATATGACTTTAATGGTG-3' and 5'-TGTTGAAGACTTTTTTGCTCAC-3'. The probe was radiolabeled with ³²P by the random primer method by using Ready-To-Go DNA Labeling Beads (Pharmacia).

The *hsdS* genes were isolated from subclones of KD735-16H by PCR (Fig. 1). Primer o.4 (5'-CATCAAGACTAGTGTTAAATTTTGTAAC-3') specifically binds to a 65-bp sequence located in the promoter region of *hsd2* but absent altogether from *hsd1*. Therefore, primers o.4 and o.2 (5'-GTGCTTGAAAAGAAAAAGAGAAGAGG-3') were used to amplify specifically the transcribed *hsdS* gene of *hsd2* from subclones having *hsd2* oriented as shown in Fig. 1, and primers

o.4 and o.3 (5'-GCGAATCAAATCTTTTACCC-3') were used to amplify the transcribed *hsdS* gene from subclones having the locus in the inverted orientation. No primers were available for the specific PCR amplification of the expressed *hsdS* gene from *hsd1* because *hsd1* and *hsd2* have identical promoter regions other than the 65-bp sequence that is unique to *hsd2*. Therefore, the *hsd1*-specific primer o.1 (5'-ATTTT-TGAACAAATACCAGAGAC-3') and primer o.3 were used to amplify the silent *hsdS* gene of *hsd1* from subclones having *hsd1* oriented as shown in Fig. 1. Primers o.1 and o.2 were used for subclones having *hsd1* in the inverted orientation. For some experiments, the *hsd2*-specific primer o.5 (5'-ATCAAGGA-ATTTATTCTGTTATATC-3') was paired with o.3 or o.2, depending on the orientation of the locus, to amplify the silent *hsdS* gene from *hsd2*.

PCR cycling conditions were as described (5), but the number of cycles was reduced to 25. PCR products were gel-purified and cloned by using the pGEM-T Easy vector (Promega). DNA sequencing was performed by automated fluorescent dye terminator methods at the Sequencing Core Facility, University of Alabama at Birmingham. Primers were obtained from the Oligonucleotide Synthesis Core Facility at the University of Alabama at Birmingham.

RESULTS

R-M Properties of *M. pulmonis* Strains. Strain KD735-16 has been maintained in the laboratory for several years and has been shown both to restrict and modify mycoplasma virus P1 (6). The restriction enzyme produced in KD735-16 has been designated in the REBASE database as *MpuUI* (10). Southern hybridization analysis using probes specific for the *hsdS* genes demonstrated that KD735-16 is considerably heterogeneous, possessing subpopulations in which the *hsd* loci have undergone gene rearrangements (Fig. 2). Because stocks of KD735-16 that had been maintained in the laboratory by separate individuals may contain different cell subpopulations, the stocks were assigned new strain designations (e.g., KD735-16H and KD735-16K). Experiments were undertaken to determine whether some subpopulations possessed R-M enzymes with specificities different from *MpuUI*. To this end, 147 filter clones of KD735-16H were isolated.

R-M properties were examined by assaying plaque-forming units of mycoplasma virus P1. KD735-15 is a strain of *M. pulmonis* that lacks detectable R-M activity and has *hsd1* inverted in the chromosome relative to KD735-16 (6). Stocks of P1 virus prepared by infection of KD735-15 (designated P1•15) are susceptible to restriction by the *MpuUI* enzyme when assayed on lawns of KD735-16H. Of the 147 subclones of KD735-16H that were analyzed, 17 (12%) failed to restrict P1•15. The phenotype and the *hsd1* orientation of these 17

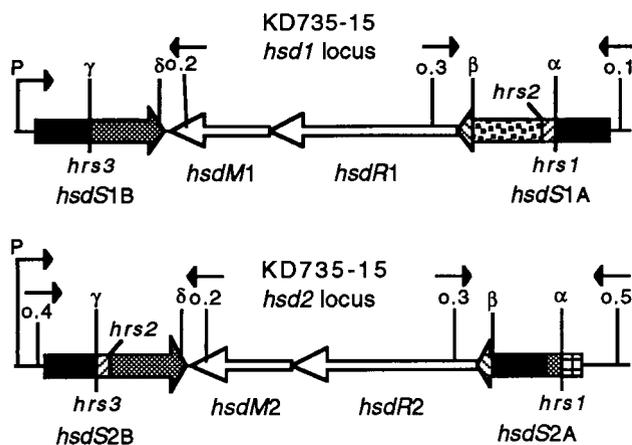


FIG. 1. Organization of the *M. pulmonis* *hsd1* (Upper) and *hsd2* (Lower) loci. To better illustrate features within the *hsdS* genes, the *hsdM* and *hsdR* genes were shortened and were not drawn to scale. Homologous regions within *hsdS* are shown by like shading. The sites labeled α , β , γ , and δ refer to *vip* DNA inversion sites. The sites labeled *hrs1*, *hrs2*, and *hrs3* are additional sites for DNA inversion. The single promoter driving *hsd* transcription is labeled by P. The orientations of primer binding sites for PCR analysis are indicated by arrows.

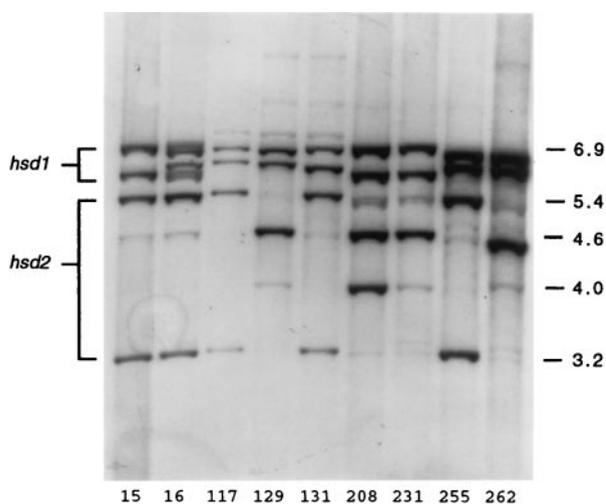


FIG. 2. Southern blot analysis of *Hind*III-digested *M. pulmonis* chromosomal DNA hybridized with the *hsdS*-specific probe described in Materials and Methods. Strain abbreviations: 15, KD735-15; 16, KD735-16H; 17, KD117; 129, KD129; etc.

subclones were identical to KD735-15. Because *hsd1* inversions leading to the KD735-15 phenotype have been described, the current study focused on the 130 subclones of KD735-16H that retained R-M activity. Of these 130 subclones, 96 (65%) did not restrict the P1 virus that had been propagated on KD735-16H (P1•16). One of these subclones (KD255) was chosen for further study and was found to be indistinguishable (in terms of R-M properties and configurations of the *hsd* loci) from the majority cell population in KD735-16H. Southern hybridization analysis confirmed that KD255 is more homogeneous than KD735-16H (Fig. 2).

The subclones of KD735-16H that restricted both P1•15 and P1•16 were studied in detail because they likely possessed R-M activity with specificity different from *Mpu*UI. Stocks of P1 virus propagated on each of these subclones were prepared and used to assess R-M properties as summarized in Table 2. Each subclone of KD735-16H was assigned to one of eight distinct restriction groups. Group I consisted of the 17 subclones that failed to restrict P1•15 and had restriction properties identical to KD735-15. Group II consisted of 26 subclones (20%) typified by KD117, having R-M properties similar to a stock of KD735-16 that had been maintained in the laboratory for several years and had been assigned the strain designation KD735-16K. Groups III through VII consisted of subclones that possess previously unobserved R-M activities. Members of each of these groups restricted stocks of P1 virus propagated on any of the other groups. Group VIII consisted of the 96 subclones typified by KD255 that restricted P1•15 but

not P1•16. Group VIII subclones were unusual in that they restricted virus propagated on some subclones (groups I, IV, V, and VI) but not others. Southern hybridization analysis indicated that most restriction groups had a distinct hybridization profile, indicating that differences in R-M activity resulted from gene rearrangements (Fig. 2).

Sequence Analysis of *hsdS* Genes. The *hsd* gene rearrangements identified in previous studies were site-specific DNA inversions that occurred at sites designated *vip*. Each *hsd* locus has four *vip* sites (two per *hsdS* gene) referred to as *vip* α , *vip* β , *vip* δ , and *vip* γ (Fig. 1). Previously, four types of DNA inversions were identified, resulting from recombination between *vip* α and *vip* γ (α - γ inversion), *vip* α and *vip* δ (α - δ inversion), *vip* β and *vip* γ (β - γ inversion), and *vip* β and *vip* δ (β - δ inversion) (5). For representative members of each restriction group, the nucleotide sequence of one of the *hsdS* genes from each *hsd* locus was determined and used to deduce the particular type of DNA inversion that had occurred, if any, along with the sequence of the complementary *hsdS* gene. For each *hsd* locus, one *hsdS* gene is expressed (Fig. 1, left end) and the other is transcriptionally silent (Fig. 1, right end) (5). The expressed gene from *hsd2* and the silent gene from *hsd1* were PCR amplified as described in Materials and Methods. The nucleotide sequences of the PCR products derived from these two genes permitted the identification of the type of DNA inversion (α - γ , α - δ , β - γ , or β - δ) that had occurred at each locus. By using this strategy, the particular DNA inversions giving rise to subclones within restriction groups II, IV, V, VII, and VIII were identified as summarized in Fig. 3. These subclones had all undergone predictable DNA inversions at *vip* sites.

Identification of New Site-Specific Gene Rearrangements in *hsdS*. The PCR products derived from the *hsd2* locus of group III (KD129) and group VI (KD208) subclones had unexpected sequences requiring further study. The *hsd2* locus from these subclones was oriented such that the transcribed *hsdS* gene was PCR amplified by using primers o.4 and o.3. To document the configuration of the *hsd2* locus in these subclones, the silent *hsdS* gene was also PCR amplified by using the *hsd2*-specific primer o.5 paired with primer o.2 (Fig. 1). Sequence comparison of the silent and expressed *hsdS* genes of KD129 and KD208 indicated that the *hsd2* locus of both subclones had undergone the same gene rearrangement, which apparently consisted of a DNA inversion at a site other than *vip*. The inversion occurred between sites designated in Fig. 3 as *hrs1* (*hsd* recombination site) and *hrs2*. There are three *hrs* sites per *hsd* locus, and these sites had been recognized as 20-bp direct repeats present at each end of a 99-bp region that is present only in the genes labeled in Fig. 1 as *hsdS1A* and *hsdS2B* (5, 6). Although the nucleotide sequences of the *hrs* sites appear distinct from the sequences of *vip*, the *vip* γ and *hrs3* sites are adjacent in the chromosome with some overlap, as are *vip* α and

Table 2. Summary of restriction and modification properties of *M. pulmonis* subclones

Infecting virus Stock [†]	Relative plaque-forming units on <i>M. pulmonis</i> strain*							
	Group I KD735-15	Group II KD117 KD136	Group III KD129	Group IV KD131 KD297	Group V KD208 T3	Group VI KD231	Group VII KD262	Group VIII KD255 KD117-18
P1•15	1	2 × 10 ⁻⁵	1 × 10 ⁻⁴	3 × 10 ⁻³	1 × 10 ⁻³	2 × 10 ⁻³	1 × 10 ⁻⁴	2 × 10 ⁻³
P1•117/P1•136	1	1	4 × 10 ⁻³	6 × 10 ⁻⁴	7 × 10 ⁻³	1 × 10 ⁻³	3 × 10 ⁻⁴	1
P1•129	1	2 × 10 ⁻³	1	8 × 10 ⁻⁴	2 × 10 ⁻²	2 × 10 ⁻¹	6 × 10 ⁻³	1
P1•131/P1•297	1	<10 ⁻⁴	<10 ⁻⁴	1	9 × 10 ⁻³	2 × 10 ⁻³	<10 ⁻⁵	1 × 10 ⁻⁴
P1•208/P1•T3	1	2 × 10 ⁻⁵	8 × 10 ⁻⁶	1 × 10 ⁻³	1	8 × 10 ⁻⁴	2 × 10 ⁻⁵	2 × 10 ⁻³
P1•231	1	1 × 10 ⁻⁶	3 × 10 ⁻³	2 × 10 ⁻³	3 × 10 ⁻²	1	2 × 10 ⁻⁵	2 × 10 ⁻³
P1•262	1	2 × 10 ⁻³	2 × 10 ⁻⁴	3 × 10 ⁻³	1 × 10 ⁻²	5 × 10 ⁻³	1	1
P1•255	1	4 × 10 ⁻²	5 × 10 ⁻³	3 × 10 ⁻³	1 × 10 ⁻³	5 × 10 ⁻³	5 × 10 ⁻³	1

*Similar results were obtained in four independent experiments.

[†]Stocks of P1 virus are abbreviated as follows: P1•15, P1 virus grown on strain KD735-15; P1•117, P1 virus grown on strain KD117; etc.

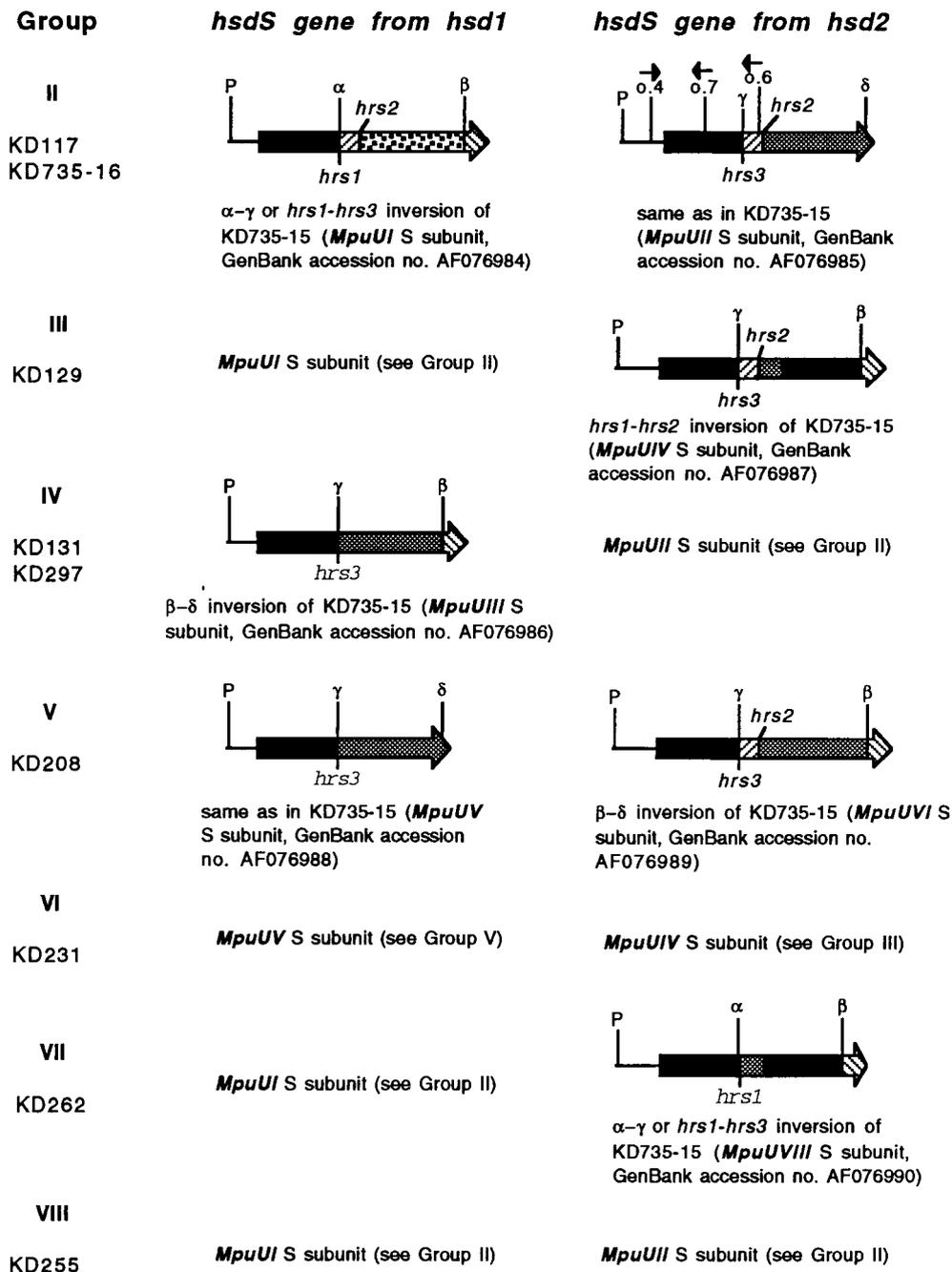


FIG. 3. Schematic diagrams of the *hsdS* genes associated with each restriction group. Shaded segments refer to regions of homology as shown in Fig. 1. Recombination sites (*vip* and *hrs*) and promoter (P) sites are as in Fig. 1.

hrs1 (Fig. 1). An alignment of the *hsd2 vip* and *hrs* sites is shown in Fig. 4.

MpuU Enzymes and Nomenclature. Nearly all of the *hsdS* genes expressed by subclones within each restriction group encode functional S subunits. The S subunits encoded by *hsd1* are identified with the odd-numbered restriction enzymes *MpuUI*, *MpuUIII*, etc., and the subunits encoded by *hsd2* are identified with the even-numbered enzymes *MpuUII*, *MpuUIV*, etc. (Fig. 3). The predicted HsdS proteins of group II subclones (e.g., KD117) are the S subunits of *MpuUI* and *MpuUII*. Group III subclones produce the enzymes *MpuUI* and *MpuUIV*. Groups II and III both produce *MpuUI*. Yet, each of these groups restricts P1 virus propagated on the other group. The different restriction properties exhibited by groups II and III are attributed to *MpuUII* and *MpuUIV*, indicating that both of these enzymes are functional but with DNA

recognition sequence specificities distinct from one another and from *MpuUI*. Group IV subclones produce *MpuUIII* and *MpuUII*. Because group IV and group II subclones both produce *MpuUII*, the different restriction properties exhibited by these groups indicate that *MpuUI* and *MpuUIII* are functionally distinct from one another and from *MpuUII*. A comparison of groups V and VI, both of which produce *MpuUV*, indicates that *MpuUVI* is functional and distinct from *MpuUV* and *MpuUIV*. A comparison of groups III and VI indicates that *MpuUV* is functional and distinct from *MpuUI* and *MpuUIV*. A comparison of group VII to groups II and III indicates that *MpuUVIII* is functional and distinct from *MpuUI*, *MpuUII*, and *MpuUIV*. This latter comparison is of interest because the sequences of *MpuUIV* and *MpuUVIII* are identical but for the presence of the 33-aa region in *MpuUIV* that is encoded by the 99-nt region bounded by *hrs2*

hrs sites

```

hrs2 (15)      ATTTAAGTATATAATTAAGATTATTGAACCTTT
hrs2 (129)    ATTTAAGTATATAATTAAGATTATTGAACCTCT
vipα/hrs1 (15) CTCAAAGTGCAATTAATTAAGATTATTGAACCTCT
vipα/hrs1 (129) CTCAAAGTGCAATTAATTAAGATTATTGAACCTTT
vipβ/hrs3 (15) CTCAAAGTGCAATTAATTAAGATTATTGAACCTAA
vipδ          TCCAAAGTGCAATTTAGGTATTAATGAACACTT
vipβ         TTCCAAAGTGCAATTTAGGTATTAATGAACACTT

```

vip sites

FIG. 4. Alignment of the *vip* and *hrs* DNA inversion sites of the *hsd2* locus. Sequences are from strains KD735-15 or KD129 with the abbreviated strain designation in parenthesis. Boxes are drawn around the 12-nt *vip* sequences and the 20-nt *hrs* sequences.

and *hrs3*. From the available data, it is not possible to conclude that all *MpuU* enzymes are functionally distinct. It is possible that *MpuUIII* and *MpuUV* are functionally equivalent, and *MpuUVI* may be equivalent to *MpuUII*. The S subunits of these enzyme pairs differ only at their carboxyl-terminal regions with undetermined functional consequence.

Lack of R-M Activity in Group I Subclones. Group I subclones are devoid of detectable R-M activity but should produce the S subunits of *MpuUV* and *MpuUII* [compare the expressed *hsdS* genes illustrated in Fig. 1 (left end of the *hsd* loci) with the *MpuUV* and *MpuUII* S subunits shown in Fig. 3]. Because *MpuUV* and *MpuUII* are functional, as discussed above, one might predict that KD735-15 should exhibit R-M activity. However, only one promoter (see Fig. 1) has been identified in each *hsd* locus (5). The orientations of *hsdI* and *hsd2* in the KD735-15 chromosome are such that *hsdR* and *hsdM* would not be transcribed from this promoter. Therefore, it is plausible that KD735-15 lacks R-M activity because R and M subunits are not produced.

Limited R-M Activity of Group VIII Subclones. Group VIII subclones (Table 2) are unusual in that they restrict viruses propagated on groups I, IV, V, and VI but not on groups II, III, and VII. The *hsdS* nucleotide sequences of group VIII and group II subclones were identical, and both groups are predicted by sequence analysis to produce *MpuUI* and *MpuUII*. The subclones that modify P1 virus, rendering it resistant to restriction by group VIII subclones, all produce *MpuUI* whereas subclones lacking *MpuUI* produce P1 virus that is restricted by group VIII. This implies that group VIII subclones produce functional *MpuUI*. Also, P1 virus propagated on subclones producing *MpuUII* but not *MpuUI* (group IV) are restricted by group VIII subclones, further indicating that group VIII subclones produce functional *MpuUI*. P1 virus propagated on KD255 (P1•255) is not modified as predicted from sequence analysis because P1•255 was restricted by group II subclones. Because group VIII subclones evidently produce *MpuUI*, we conclude that group VIII subclones fail to produce *MpuUII* even though the *hsdS* sequence data predict otherwise.

Analysis of Additional Subclones Derived from Groups II and VIII. KD735-16H was not the only stock of *M. pulmonis* possessing subpopulations with differing R-M properties. For example, strain T3 is a group V subclone isolated from KD735-16K (group II). Strain KD117-18 (group VIII) was 1 of 20 subclones of KD117 (group II) that were analyzed. Therefore, cultures of group II subclones probably contain a significant group VIII subpopulation, implying that group VIII subclones do not arise by rare mutational events.

DISCUSSION

Phase-variable R-M enzymes probably are not limited to *M. pulmonis* and may be widespread. *Helicobacter pylori*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* possess genes that

are predicted to encode phase-variable R-M enzymes (11, 12). In these Gram-negative bacterial systems, phase variation would result from frameshift errors occurring during DNA replication by slipped strand mispairing within a reiterated domain. The genes encoding the phase-variable R-M enzymes of *M. pulmonis* and other bacteria are clearly not selfish.

What protects the chromosome from endonucleolytic attack when restriction activity is induced? The transfer of *hsd* genes from one strain of *Escherichia coli* to another is possible because R-M activity is regulated such that the chromosome of recipient cells is modified before restriction activity is produced (13). The *hsdR* gene of *E. coli* is transcribed from a separate promoter than are *hsdM* and *hsdS*. In contrast, *hsdR* precedes *hsdM* in the *hsd* loci of *M. pulmonis*, suggesting that MTase activity is not induced before restriction activity. For bacterial species possessing natural transformation systems, the induction of phase-variable restriction activity may be part of an autolysis process that releases DNA into the environment for uptake by other cells. This is plausible but not generally applicable to mycoplasmas that lack natural transformation (14). It has been noted that KD735-15 (group I, lacking R-M activity) is more stable with respect to *hsd* inversions than is KD735-16 (6). DNA from KD735-16 is significantly more heterogeneous than DNA from KD735-15 (Fig. 2), supporting this observation. This is in spite of KD735-16 having been propagated less frequently in our laboratory than has KD735-15. Perhaps the apparent stability of KD735-15 results from the frequent death of progeny that have undergone induction of restriction activity via *hsd* inversion. Such a self-destructive system would not be maintained in a cell population unless phase-variable R-M activity had an essential function.

R-M activity in *M. pulmonis* is complex and may be modulated by other factors in addition to *hsd* inversions. As described above, *MpuUII* is produced in group II but not in group VIII subclones. The nucleotide sequences of the coding regions and promoter regions of the *hsdS* genes were identical for groups II and VIII, and the reason for the failure of group VIII to produce *MpuUII* is undetermined. A 65-bp sequence is present in the 5' untranslated region of the *hsdS* mRNA from *hsd2* but is absent in *hsd1* (5). Therefore, *hsd2* gene expression may be regulated differently from *hsd1*, and an undetermined factor may repress *MpuUII* production in group VIII subclones.

The heterogeneity of *M. pulmonis* cultures in regards to R-M activity must result in virus stocks with heterogeneity in base modifications. One result of the heterogeneity is that the size of plaques that are obtained varies with the restriction conditions. Uniformly large plaques are obtained when plaque-forming units are assayed on lawns of group I subclones. In contrast, when plaque-forming units are assayed on restrictive strains, a mixture of large and small plaques is obtained. We interpret small plaques as arising from the initial infection of a cell that was a member of a different restriction group than the cell majority within the lawn. This initially infected cell would release progeny viruses that are restricted by neighboring cells as the plaque develops, limiting its size. The percentage of group I cells on the lawn particularly would influence the ratio of large to small plaques because these cells are infected efficiently but release unmodified progeny viruses that are restricted by cells from all other restriction groups.

What is the function of the phase-variable R-M systems of *M. pulmonis*? Mycoplasmas are thought not to possess natural transformation and conjugation systems, and R-M systems therefore would not be required as barriers to these types of gene transfer. The *hsd* loci may be an exotic system for protection against phage infection, but the presence of a significant cell subpopulation lacking R-M systems suggests that the Hsd enzymes of *M. pulmonis* may be an ineffectual barrier. Infection of the restriction-negative subpopulation would release high levels of progeny phage, numerically over-

whelming the restriction enzymes produced in other cells in the population. The potential for the loss of R-M activity on DNA inversion suggests that *M. pulmonis* may have evolved mechanisms to maintain R-M-negative subpopulations. For example, immediately upstream of the *hsdS1A* gene illustrated in Fig. 1 is a transcription terminator signaling the end of the adjacent *polC* gene (6, 15). Were this transcription terminator deleted during the evolution of the *hsd* loci, transcription could proceed through *polC* and into *hsdI*. This would permit transcription of *hsdR1* and *hsdM1* regardless of the orientation of *hsdI* in the chromosome. Thus, all cells in the population would possess R-M activity, and phage protection would be maximal. The *polC* transcription terminator might have been maintained during evolution because it is sometimes advantageous to have subpopulations lacking R-M activity.

In *M. pulmonis*, there is an apparent association between phase variation in R-M activity and antigenic variation. The *vsa* (variable surface antigen) locus is a large, DNA inversion system that encodes a family of highly reiterated, phase-variable surface proteins known as the V-1 antigens (16, 17). Variation in the V-1 proteins has been associated with disease pathogenesis, and V-1 may facilitate the attachment of *M. pulmonis* to host tissue (18, 19). Most *vsa* genes are transcriptionally silent and lack a promoter, ribosome binding site, and the first 700 nucleotides of the *vsa* coding region. Only a single *vsa* expression site has been identified thus far, and site-specific DNA inversions regulate phase-variable gene expression by recombining silent genes with this expression site. Pedigree analysis of *M. pulmonis* subclones has indicated a correlation between *vsa* inversions and *hsd* inversions (16, 20). Perhaps inversions at these loci are regulated coordinately. Alternatively, the induction of R-M activity associated with *hsd* inversion may stimulate *vsa* inversions. Stimulation of *vsa* inversions could occur as a result of the introduction of double-stranded DNA breaks in the chromosome by the induced restriction activity or, alternatively, the induced modification activity may serve to regulate expression of the gene encoding the site-specific recombinase responsible for catalyzing *vsa* inversions. Regardless of the explanation for the association between DNA inversions at *hsd* and *vsa*, it seems

likely that the *hsd* loci have some role beyond phage restriction, possibly in the pathogenic process.

This work was supported by U.S. Public Health Service grant GM1126 from the National Institutes of Health. The University of Alabama at Birmingham Sequencing Core Facility is a service provided by the Center for AIDS Research (National Institutes of Health Grant 5 P30 AI27767). The University of Alabama at Birmingham oligonucleotide synthesis core facility is supported by National Institutes of Health Grant 5 P50 CA13148.

1. Bickle, T. A. & Kruger, D. H. (1993) *Microbiol. Rev.* **57**, 434–450.
2. Naito, T., Kusano, K. & Kobayashi, I. (1995) *Science* **267**, 897–899.
3. Nakayama, Y. & Kobayashi, I. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 6442–6447.
4. O'Neill, M., Chen, A. & Murray, N. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 14596–14601.
5. Sitaraman, R. & Dybvig, K. (1997) *Mol. Microbiol.* **26**, 109–120.
6. Dybvig, K. & Yu, H. (1994) *Mol. Microbiol.* **12**, 547–560.
7. Dybvig, K. & Cassell, G. H. (1987) *Science* **235**, 1392–1394.
8. Bhugra, B. & Dybvig, K. (1992) *Mol. Microbiol.* **6**, 1149–1154.
9. Dybvig, K., Liss, A., Alderete, J., Cole, R. M. & Cassell, G. H. (1987) *Isr. J. Med. Sci.* **23**, 418–422.
10. Roberts, R. J. & Macelis, D. (1994) *Nucleic Acids Res.* **22**, 3628–3639.
11. Saunders, N. J., Peden, J. F., Hood, D. W. & Moxon, E. R. (1998) *Mol. Microbiol.* **27**, 1091–1098.
12. Hood, D. W., Deadman, M. E., Jennings, M. P., Bisercic, M., Fleischmann, R. D., Venter, J. C. & Moxon, E. R. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 11121–11125.
13. Kulik, E. M. & Bickle, T. A. (1996) *J. Mol. Biol.* **264**, 891–906.
14. Dybvig, K. & Voelker, L. L. (1996) *Annu. Rev. Microbiol.* **50**, 25–57.
15. Barnes, M. H., Tarantino, P. M., Jr, Spacclapoli, P., Brown, N. C., Yu, H. & Dybvig, K. (1994) *Mol. Microbiol.* **13**, 843–854.
16. Bhugra, B., Voelker, L. L., Zou, N., Yu, H. & Dybvig, K. (1995) *Mol. Microbiol.* **18**, 703–714.
17. Simmons, W. L., Zuhua, C., Glass, J. I., Simecka, J. W., Cassell, G. H. & Watson, H. L. (1996) *Infect. Immun.* **64**, 472–479.
18. Talkington, D. F., Fallon, M. T., Watson, H. L., Thorp, R. K. & Cassell, G. H. (1989) *Microb. Pathog.* **7**, 429–436.
19. Watson, H. L., Zheng, X. & Cassell, G. H. (1993) *Clin. Infect. Dis.* **17**, Suppl. 1, S183–S186.
20. Dybvig, K. (1993) *Mol. Microbiol.* **10**, 465–471.