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)

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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-μm filter immediately before assay 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).

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subclones having
hsd2
sequence located in the promoter region of
hsdS
...ATTTATTCTGTTATATC-3

9
ATTCATATGACTTTAATGGTG-3

promoter driving
hsd
hrs1
a
labeled
hsdS
Homologous regions within
hsdM
(AMPLIFYING DNA FROM STRAIN KD735–15 USING PRIMERS 5 ...S CHARACTERIZED BY BLACK SHADING AND WAS OBTAINED BY PCR FROM SEQUENCES LOCATED WITHIN THE CONSERVED...)

...TCTTTTGCTC-3’. THE PROBE WAS RADIOLABELED WITH 33P BY THE RANDOM PRIMING METHOD BY USING READY-TO-GO DNA LABELING BEADS (PHARMACIA).

The
hsdS
genomes were isolated from subclones of KD735–16H by PCR (Fig. 1). Primer o.4 (5’-CATCAAGACTAGTGTT- AAAATTTTGGTTAC-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’-GTTGAGTTAGCTTTTGGTAGCT-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’-GCGAATCAAATC TTTTACCC-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’-ATTTTTGTAACAAATACAGAGAC-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’-ATCAAGGAAATTATTCTGGTTATAC-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
MpuU1 (10). Southern hybridization analysis using probes specific for the
hsdS
genomes 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
MpuU1. To this end, 147 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
MpuU1 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 subpopulations were analyzed by using the probes illustrated in Fig. 1. The
hsd1
loci of KD735–15 are shown in Fig. 1, and the
hsd2
loci of KD735–16H are shown in Fig. 2. The
hsd1
loci of KD735–16H are shown in Fig. 2. The
hsd2
loci of KD735–16H are shown in Fig. 2.

**Table 1.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent strain</th>
<th>Restriction group, enzymes</th>
<th>Reference</th>
</tr>
</thead>
</table>
| KD735-15     | KD735         | I, no
MpuU enzymes            | 6         |
| KD735-16K    | KD735         | II, MpuU1 and MpuU1I       | 5         |
| KD735-16H    | KD735         | VIII, MpuU1                | This study|
| KD117        | KD735-16H     | II, MpuU1 and MpuU1I       | This study|
| KD129        | KD735-16H     | III, MpuU1 and MpuU1IV     | This study|
| KD131        | KD735-16H     | IV, MpuUIII and MpuU1I     | This study|
| KD136        | KD735-16H     | II, MpuU1 and MpuU1I       | This study|
| KD208        | KD735-16H     | V, MpuU1V and MpuU1VI      | This study|
| KD231        | KD735-16H     | VI, MpuUV and MpuU1UV      | This study|
| KD255        | KD735-16H     | VIII, MpuU1                | This study|
| KD262        | KD735-16H     | VII, MpuU1 and MpuU1VIII   | This study|
| KD297        | KD735-16H     | IV, MpuUIII and MpuU1I     | This study|
| T3           | KD736-16K     | V, MpuUV and MpuU1VI       | This study|
| KD117-18     | KD117         | VIII, MpuU1                | This study|

**Fig. 1.** Organization of the
M. pulmonis
hsd1 (Upper) and
hsd2 (Lower) loci. To better illustrate features within the
hsdS
genomes, the
hsdM
and
hsdR
genomes were shortened and were not drawn to scale. Homologous regions within
hsdS
are shown by black shading. The sites labeled α, β, γ, and δ refer to yip 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.
subclones were identical to KD735–15. Because hasd1 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 (KD225) was chosen for further study and was found to be indistinguishable (in terms of R-M properties and configurations of the hasd 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 MpuUI. Stocks of P1 virus propagated on each of these subclones were prepared and used to assess R-M properties as summarized in Table 2. P1 virus propagated on each of these subclones were prepared and used to assess R-M properties as summarized in Table 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 MpuUI. Stocks of P1 virus propagated on each of these subclones were prepared and used to assess R-M properties as summarized in Table 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 MpuUI. Stocks of P1 virus propagated on each of these subclones were prepared and used to assess R-M properties as summarized in Table 2.

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

<table>
<thead>
<tr>
<th>Infecting virus Stock†</th>
<th>Relative plaque-forming units on M. pulmonis strain*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I</strong></td>
<td></td>
</tr>
<tr>
<td>KD735–15</td>
<td>KD117</td>
</tr>
<tr>
<td>P1•15</td>
<td>2 x 10^-5</td>
</tr>
<tr>
<td>P1•117/P1•136</td>
<td>1</td>
</tr>
<tr>
<td>P1•129</td>
<td>2 x 10^-3</td>
</tr>
<tr>
<td>P1•131/P1•297</td>
<td>&lt;10^-4</td>
</tr>
<tr>
<td>P1•208/P1•T3</td>
<td>2 x 10^-5</td>
</tr>
<tr>
<td>P1•231</td>
<td>1 x 10^-6</td>
</tr>
<tr>
<td>P1•262</td>
<td>1 x 10^-2</td>
</tr>
<tr>
<td>P1•255</td>
<td>4 x 10^-2</td>
</tr>
</tbody>
</table>

*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.
<table>
<thead>
<tr>
<th>Group</th>
<th>hsdS gene from hsd1</th>
<th>hsdS gene from hsd2</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KD117</td>
<td>α-γ or hrs1-hrs3 inversion of KD735-15 (MpuUI S subunit, GenBank accession no. AF076984)</td>
<td>same as in KD735-15 (MpuUII S subunit, GenBank accession no. AF076985)</td>
</tr>
<tr>
<td>KD735-16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KD129</td>
<td>MpuUI S subunit (see Group II)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KD131</td>
<td>β-δ inversion of KD735-15 (MpuUV S subunit, GenBank accession no. AF076986)</td>
<td></td>
</tr>
<tr>
<td>KD297</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KD208</td>
<td>same as in KD735-15 (MpuUV S subunit, GenBank accession no. AF076986)</td>
<td>β-δ inversion of KD735-15 (MpuUVI S subunit, GenBank accession no. AF076989)</td>
</tr>
<tr>
<td>VI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KD231</td>
<td>MpuUV S subunit (see Group V)</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KD262</td>
<td>MpuUI S subunit (see Group II)</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KD255</td>
<td>MpuUI S subunit (see Group II)</td>
<td>MpuUI S subunit (see Group II)</td>
</tr>
</tbody>
</table>

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.

hrsI (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 hsdI 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 MpuUV. 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 MpuUV, 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 MpuUV. 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 MpuUV is functional and distinct from MpuUI and MpuUV. A comparison of groups V and VI indicates that MpuUV is functional and distinct from MpuUI and MpuUV. A comparison of group VII to groups II and III indicates that MpuUVIII is functional and distinct from MpuUI, MpuUII, and MpuUV. This latter comparison is of interest because the sequences of MpuUV and MpuUVIII are identical but for the presence of the 33-aa region in MpuUV that is encoded by the 99-nt region bounded by hrs2
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 MpuUUI. The S subunits of these enzyme pairs differ only at their carboxyl-terminal regions with undetermined functional consequence.

**Limited R-M Activity in Group I Subclones.** Group I subclones are devoid of detectable R-M activity but should produce the S subunits of MpuUUV and MpuUUI (compare the expressed hsdS genes illustrated in Fig. 1 (left end of the hsd loci) with the MpuUUV and MpuUUI S subunits shown in Fig. 3). Because MpuUUV and MpuUUI 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 MpuUUI and MpuUVI. The subclones that modify P1 virus, rendering it resistant to restriction by group VIII subclones, all produce MpuUUI whereas subclones lacking MpuUUI 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 MpuUUI but not MpuUUI (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 MpuUUI 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, MpuUUI 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 MpuUUI is undetermined. A 65-bp sequence present in the 5′ untranslated region of the hsdS mRNA from hsd2 but is absent in hsdI (5). Therefore, hsd2 gene expression may be regulated differently from hsdI, and an undetermined factor may repress MpuUUI 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 *hsdRI* 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 *vs* (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 *vs* genes are transcriptionally silent and lack a promoter, ribosome binding site, and the first 700 nucleotides of the *vs* coding region. Only a single *vs* 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 *vs* inversions and phase transitions (16, 20). Perhaps inversions at these loci are regulated coordinately. Alternatively, the induction of R-M activity associated with *hsd* inversion may stimulate *vs* inversions. Stimulation of *vs* 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 *vs* inversions. Regardless of the explanation for the association between DNA inversions at *hsd* and *vs*, it seems likely that the *hsd* loci have some role beyond phage restriction, possibly in the pathogenic process.

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