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Analysis Of High-Frequency Chromosomal Rearrangements In Mycoplasma Pulmonis.

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ANALYSIS OF HIGH-FREQUENCY CHROMOSOMAL REARRANGEMENTS IN *MYCOPLASMA PULMONIS*

by

BINDU BHUGRA

A DISSERTATION

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ABSTRACT OF DISSERTATION
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From an evolutionary viewpoint, one of the most intriguing and enigmatic aspects of mycoplasmas is their high rate of genetic variation. Mycoplasmal diseases of animals and humans usually involve colonization of respiratory or genital tracts and are noted for their chronicity. The basis for the persistence of mycoplasmal infections is poorly understood. However, many mycoplasmal species undergo high-frequency phenotypic switching phenomena that involve changes in surface antigen structures. These variations may contribute to the ability of these organisms to evade host defenses and thereby contribute to disease chronicity. The aim of this study was to examine genetic variation in mycoplasmas. The species we chose for these studies was Mycoplasma pulmonis, a murine pathogen that causes chronic respiratory disease in laboratory rats and mice and a species for which high-frequency phenotypic variation is well documented.

Our results indicate that chromosomal rearrangements occur during growth of M. pulmonis at a high rate of about

10^{-2} to 10^{-3} per CFU per generation, making this one of the most variable genomes known. Some of the rearrangements were found to correlate with changes in the cell's phenotype. Resistance to P1 virus has previously been shown to be mediated at the level of adsorption and to be correlated with changes in the surface antigen V-1. Specific DNA rearrangements that correlate with P1 virus susceptibility and V-1 antigen structure were identified. Further, one of the cloned DNA fragments associated with these rearrangements expressed V-1 epitopes in Escherichia coli.

Another mechanism of chromosomal variability in M. pulmonis that was identified is the transposition of insertion sequence (IS) elements. IS1138, a novel IS element from M. pulmonis, was cloned and characterized. It is a 1288 bp element related to the IS3 family, and upon transposition it generates a 3 bp duplication of target sequences that flank its ends as direct repeats. IS1138 is the first example of a native, mobile mycoplasmal element.

Abstract Approved by: **Committee Chairman** Kevin Dybvig

Program Director Susan Jackson

Date 12/29/92 **Dean of Graduate School** W. C. Dubay

DEDICATION

This thesis is dedicated to my parents, with all my love.

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LIST OF ABBREVIATIONS

A	adenine
bp	base pair
C	cytosine
cm	centimeter
CsCl	cesium chloride
CFU	colony-forming unit
d	day
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
DEAE	diethyl aminoethyl
E.	Escherichia
EtBr	ethidium bromide
Fig.	figure
G	guanine
GCG	Genetics Computer Group
Hd	restriction endonuclease Hind III
Hc	restriction endonuclease Hinc II
h	hour
IS	insertion sequence
kb	kilobase pair
M.	Mycoplasma
min	minutes

LIST OF ABBREVIATIONS (Continued)

ml	milliliter
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PPLO	pleuropneumonia-like organism
R	P1 virus-resistant phenotype
RFP	restriction fragment polymorphism
RBS	ribosome binding site
RNA	ribonucleic acid
RT	room temperature
rRNA	ribosomal ribonucleic acid
S	P1 virus-sensitive phenotype
S	restriction endonuclease Sst I
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylaminde gel electrophoresis
T	thymine
tRNA	transfer ribonucleic acid
U	uracil
Vcm^{-1}	volts per centimeter
^{o}C	degrees Celsius
^{32}P	32-phosphorous
µg	microgram

INTRODUCTION

An intriguing aspect of mycoplasmas is their high rate of evolution. In the absence of a cell wall, the limiting unit membrane of mycoplasmas is faced with a unique challenge of resisting damage by the rapidly changing host environment. High-frequency phenotypic switching has been documented for almost all species of mycoplasmas and involves variation in structures of surface antigens. The genetic mechanisms that produce this remarkable heterogeneity in mycoplasmas are unknown. The goal of this study was to elucidate mechanisms of phenotypic variation and to gain insight into the rapidly evolving world of wall-less pathogens. The results described here indicate that mycoplasmas have one of the most dynamic chromosomes on record. The high levels of genetic recombination that occurred in Mycoplasma pulmonis may provide insight into the unusually high rate of evolution and the extensive phenotypic variability in this organism.

Mycoplasmal diseases

The era of mycoplasmology was initiated in 1898 when the first mycoplasma was isolated from cattle with contagious bovine pleuropneumoniae (CBPP) (Edwards and Freundt, 1967; Nocard and Roux, 1990). At present, more than 100 well-characterized species of mycoplasmas have been isolated from

humans, animals, insects and plants (Tully, 1989). Mycoplasmas, included in the Division Tenericutes, class Mollicutes, are wall-less prokaryotes with a cell size small enough to pass through 0.2 μm bacteriological filters, and have unusually small genomes with low G+C content (Razin, 1985; Razin and Freundt, 1984).

Although most mycoplasmas are commensals, a large number of species are pathogens capable of producing a variety of chronic diseases in diverse hosts. In animals, mycoplasmas frequently colonize the epithelial linings of the respiratory, urogenital, and alimentary tracts, and the eyes, mammary glands and joints (Razin and Barile, 1985). Mycoplasma-associated diseases in humans can present a wide spectrum of symptoms ranging from acute respiratory infections, with extrapulmonary manifestations such as the involvement of central nervous system, to infection of the genitourinary tract and joints (Razin and Barile, 1985). Mycoplasma pneumoniae, the causative agent of primary atypical pneumonia in man, is responsible for over 15 million respiratory infections in the United States alone per year (Clyde, 1979). Ureaplasma urealyticum is the organism most often associated with chorioamnionitis, pneumonia and meningitis in very low birth weight infants (Cassell et al., 1986a; Cassell and Waites, 1989). Mycoplasma hominis is a cause of pyelonephritis and pelvic inflammatory disease in adults and of pneumonia and meningitis in newborns (Mardh et al., 1983). Recently, a previously unrecognized pathogenic

mycoplasma, Mycoplasma fermentans, was detected in tissues and peripheral blood of patients with AIDS, suggesting a potential role of this mycoplasma in the development of AIDS-related opportunistic infections (Lo, 1986; Lo et al., 1989). Enhancement of HIV-associated cytopathogenicity observed in vitro in the presence of mycoplasmas, including M. fermentans, has led to the hypothesis that mycoplasmas may act as cofactors in the development of AIDS (Lemaitre et al., 1992; Lo et al., 1991). Avian, swine, and ruminant mycoplasmas have a major economic impact on farm animals in Europe, the United States, and in particular, the third world countries (Tully and Whitcomb, 1979). Mycoplasma pulmonis is a cause of chronic respiratory and genital disease in laboratory rodents, and is responsible for millions of lost research funds (Cassell et al., 1986b). The cost of mycoplasmal diseases is phenomenal considering that over 10 million rats and 30 million mice are used yearly for research in the United States alone. In addition to being a diagnostic problem, the murine mycoplasmal diseases also interact with other host factors to induce extremely subtle changes in animal physiology and behaviour, and thereby affect the outcome of work in many different fields that utilize these animal species. The impact of plant diseases, caused mostly by spiroplasmas, is fairly extensive and involves economically important diseases of vegetable, ornamental, forest, and plantation crops. Several of these diseases are of such epidemic potential that they threaten to eliminate

production of their host crops in which they are endemic or have been introduced (McCoy, 1979; Tully, 1989).

Chronicity: A property of mycoplasmal diseases

Many pathogenic mycoplasmas are known to cause slowly progressive, chronic diseases of the respiratory tract, genital tract and the joints which are often difficult to eradicate (Cassell, 1982; Cassell et al., 1978; Razin and Barile, 1985). The persistence of mycoplasmal infections implies that these organisms have the potential to successfully adapt to a wide range of environments, since they have the ability to survive in rapidly changing conditions encountered in the animal hosts. Although the basis for the chronicity of mycoplasmal diseases is not understood, it is known that several mycoplasmal species undergo high-frequency phenotypic switching phenomena involving variable surface antigens (Christiansen et al., 1987; Dybvig et al., 1988; 1989; 1990; Krause et al., 1982; Liss and Heiland, 1983; Rosengarten and Wise, 1990; 1991; Watson et al., 1990a). Such variability may contribute to the ability of these organisms to adapt successfully and evade host defenses.

Basic Biology of Mycoplasmas

Ultrastructure and morphology

The name Mollicutes (mollis, soft; cutes, skin) denotes the lack of a cell wall and is a characteristic feature for the members of this class (Edward et al., 1967). In ultra thin sections, mycoplasmas are seen to lack a traditional form of

the prokaryotic cell wall and are confined by a 'unit' membrane. The width of the limiting membrane, which has a typical trilaminar structure, varies from 7.5 to 10.0 nm (Domermuth et al., 1964) and it may or may not show asymmetry. A number of common traits such as resistance to penicillin, sensitivity to osmotic shock and extremely variable shapes are the result of this single distinguishing property (Razin and Freundt, 1984).

Gliding motility has been described for several species of mycoplasma, e.g., M. pneumoniae, M. pulmonis, M. mobile, M. genitalium and M. gallisepticum. Some of these possess specialized tip-like structures, which also render the function of attachment organelles and have some bearing upon locomotion (Boatman, 1979). In M. pneumoniae, the role of the 170-kd surface protein, P1, located at the tip-like structure of virulent organisms in mediating cytadherence is well established (Baseman et al., 1982). Spiroplasmas, on the other hand, possess a rapid rotatory motion and flexional movement in keeping with their helical morphology (Cole et al., 1973).

Nutrition and growth characteristics

Mycoplasmas have limited biosynthetic capabilities in keeping with the small size of their genome. The sizes range from the smallest genome size observed for Mycoplasma genitalium (600 kb) (Colman et al., 1990) to a larger genome size for Acholeplasma laidlawii (1580 kb) (Robertson et al., 1990). Accordingly, they require a wide array of precursor

molecules, like vitamins, nucleic acid precursors, amino acids, and lipids, often in a complex yet membrane-permeable form for macromolecular synthesis (Razin, 1985; Rodwell and Mitchell, 1979). Apart from Acholeplasmas and Asteroleplasmas all of the Mollicutes require exogenous sterol. Mycoplasmas must consume large amounts of substrate to supply sufficient energy for macromolecular synthesis as they generally possess an inefficient energy-yielding mechanism. On the basis of acid production from carbohydrate, the genus Mycoplasma can be divided into fermentative or non-fermentative species, utilizing either glucose (fermentative) or arginine (non-fermentative) as the source of energy.

Most mycoplasmas have the property to produce on solid medium colonies of a typical "fried egg" appearance (Freundt and Edward, 1979). Colonial appearance depends, to a wide extent, on the composition of the growth medium. Because mycoplasma colonies grow into the agar, they are minute in size and replica plating techniques have not been successful so far (except in A. laidlawii).

Genetics

The mycoplasma genome is an appealing model for genetic studies because of its small size (600 to 1500 kb). However, genetic mapping requires mutants. Auxotrophic markers have been particularly powerful tools for studying bacterial genetics, but the fastidious nature of mycoplasmas has prevented the development of such markers because of the lack of defined media (Dybvig, 1990; Razin, 1985). Despite these

inherent difficulties in working with mycoplasmas, promising approaches for the development of mycoplasmal genetic systems are becoming available.

Phylogeny and classification of mycoplasmas

The unusual and unique properties of mycoplasmas have led to their being assigned into a taxonomic class separate from other eubaceria. The class Mollicutes contains six genera comprising of Mycoplasma, Acholeplasma, Spiroplasma, Ureaplasma, Anaeroplasma, and Asteroleplasma. The distinctions between these are based primarily on differences in morphology, genome size, and nutritional requirements (Robinson and Freundt, 1987; Weisburg et al., 1989). The species of Mycoplasmas, Acholeplasma, Spiroplasma and Ureaplasma are facultative anaerobes. Anaeroplasmas and Asteroleplasmas are obligate anaerobes, the only difference being Anaeroplasmas require sterols for growth. In the last decade or so, considerable progress has been made in the understanding of mollicute phylogeny and evolution (Fox et al., 1980; Maniloff, 1983; Rogers et al., 1985). Mycoplasmas have been placed into five groups on the basis of phylogenetic analysis done using the 16S rRNA sequences from over 40 mycoplasmal species (Weisburg et al., 1989). These analyses have also indicated that mollicutes are related to gram-positive bacteria with low G+C DNA compositions; and within this group they appear to be more closely related to the bacillus-lactobacillus cluster.

The evolution of mycoplasmas is noteworthy in that multiple reductions in genome size have occurred and the usual bacterial genetic code has been modified (Dybvig, 1990; Weisburg et al., 1989; Woese, 1987). It is suggested that an ancestral gram-positive bacterium underwent significant reductions in genome size and lost its ability to synthesize a cell wall, resulting in the formation of an ancestral mycoplasma with a genome size similar to the modern day A. laidlawii (1500 kb). Further diminishing of the genome size during evolution would have created additional, divergent branches containing mycoplasmas, which have a smaller genome size.

A+T-biased mutation pressure and an altered genetic code

Mycoplasmas are thought to be undergoing selective mutation pressure to replace G+C base pairs with A+T base pairs (Muto and Osawa, 1987). The G+C content of some mycoplasmal DNAs is as low as 25%, with M. pneumoniae and some anaerobic mycoplasmas being exceptional in having about 40% G+C content (Muto et al., 1987). This pressure may have played a vital role in the evolution of the mycoplasmal genetic code. Unlike other eubacteria that use UGA as a termination codon and UGG to code for tryptophan, some mycoplasmas use both UGA and UGG to code for tryptophan (Yamao et al., 1985). A. laidlawii, the only known exception, still uses UGA as a termination codon (Tanaka et al., 1989).

A rapid rate of evolution

The uniquely different phenotype and properties of

mycoplasmas may be a result of a rapid evolutionary rate as compared to other eubacteria (Woese, 1987; Weisburg et al, 1989). The evidence for a quickened evolutionary pace comes from the analysis of rRNA genes of mycoplasmas which shows that sequence positions that tend to be invariant in most bacteria are relatively variable in mycoplasmas (Maniloff, 1983; Weisburg et al., 1989; Woese, 1987). It has been suggested that mycoplasmas may be able to withstand a high mutation rate because of their small genome size (Woese, 1987). In fact, a high mutation rate is expected because the mycoplasmal DNA polymerase may lack a 3' to 5' exonuclease (proofreading) activity (Boxer and Korn, 1979; Mills et al., 1977).

Other enzyme activities associated with DNA repair may also cause the biased mutation pressure (Muto and Osawa, 1987; Williams and Pollack, 1988). Uracil-DNA glycosylase, an enzyme that specifically removes uracil residues from DNA, was shown to have a decreased activity in mollicutes (Williams and Pollack, 1990). Uracil residues in DNA may arise either by spontaneous deamination of deoxycytidine monophosphate residues or the misincorporation of dUTP into DNA by DNA polymerase. Since deamination of cytosine residues in DNA occurs under physiological conditions (Maniloff, 1983), the inability to remove uracil residues from DNA would result in A.T transition mutations and ultimately in a decrease in the G+C content of DNA. Thus, a decreased capacity of the glycosylase suggests that an inefficiency in

removing uracil residues may have a vital role in the low G+C content and the A+T-biased mutation pressure (Williams and Pollack, 1990).

Repetitive sequences in mycoplasmas

Repetitive DNA sequences have been identified in the genome of many organisms, where they carry out a variety of different functions. Repetitive regions of DNA are considered as hotspots for recombination and may play a role in modulating gene expression (Stern et al., 1984). Numerous repeated elements have been described in mycoplasmas. Several repetitive sequences have been identified in M. pneumoniae, particularly within the P1 gene that codes for the major adhesin protein. Also, sequences with homology to segments of the P1 gene have been found at several sites within the M. pneumoniae chromosome (Su et al., 1988; Wenzel et al., 1990). To add to this diversity, sequences unrelated to the P1 gene were also found scattered throughout the genome (Colman et al., 1990; Ruland et al., 1990; Wenzel and Hermann, 1988).

Recombination events mediated by repetitive sequences may increase the potential for mutation and phenotypic changes in these organisms. A 1.5 kb repetitive sequence, that has structural characteristics of a prokaryotic insertion sequence, has been identified in M. hyorhinis (Ferrell et al., 1989). This IS-like sequence is present in about 16 copies in all M. hyorhinis strains examined and in fewer copies in M. hyopneumoniae and M. floculare, suggesting possible interspecies transfer of genetic material among

organisms inhabiting a common (porcine) host (Taylor et al., 1988). These observations indicate that the repetitive sequence may be a transposable element, though transposition events mediated by this element have not been observed. Repetitive sequences dispersed throughout spiroplasmas have been generated as a result of the insertion of extrachromosomal elements (plasmids and viral DNA) into the chromosome. For example, sequences homologous to plasmid pRA1 and plasmid pMH1 are present in several copies in the chromosome of several S. citri strains as well as other unrelated spiroplasma species (Mouches et al., 1983; Ranhand et al., 1987). Another repetitive element with structural features similar to prokaryotic IS elements is present in M. fermentans (Hu et al., 1990).

Alteration of Surface Properties

Structural variation of surface antigens

Organisms in the genus Mycoplasma have successfully adapted to very diverse environments, probably by using mechanisms which enable them to survive in rapidly changing conditions encountered in their animal hosts. The major surface antigen of M. pulmonis, designated V-1, has a complex and heterogenous, laddered pattern on immunoblots of one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), showing multiple components with shared, as well as distincitve, epitopes (Watson et al., 1988; 1989). The V-1 protein varies both among different strains and within subclones of the same strain (Dybvig et

al., 1988; 1989; Watson et al., 1988). Analysis of the proteins from M. pulmonis using SDS-PAGE revealed that individual subclones of a single strain may have any of the numerous, structurally distinct forms of V-1 (Watson et al., 1988). A high rate of variation in V-1, about 2 x 10^{-3} per cell per generation, occurred in any given subclone. This potential for variability in a major surface antigen could have important implications as to how the organism interacts with its host.

V-1 is a prominent antigen recognized during natural infection (Watson et al., 1987), and populations with different structural variations of the V-1 antigen differ in the type of disease produced in mice (Watson et al., unpublished). The in vivo analyses showed that mice infected with the nonhemadsorbing phenotype developed an acute, fatal disease whereas infection with the hemadsorbing phenotype resulted in a chronic airway disease with no significant mortality. Thus, the variabilty in the surface antigen of the infecting organism has the potential to produce altered phenotypes, and therefore affect the outcome of the disease.

This potential can be extended to other mycoplasmas which have been shown to have variable antigens with structural characteristics similar to V-1 (Cassell et al., 1988; Rosengarten and Wise, 1991; Sasaki et al., 1989; Washburn and Hirsch, 1990). Immunoblots of proteins from U. urealyticum, A. laidlawii, Mycoplasma arthritidis, and Mycoplasma muris (Statdlander and Watson, 1992; Watson,

1990b) also display a ladder pattern similar to that of V-1 antigen. Also, the L-1 surface antigen from U. urealyticum undergoes a high rate of variation when passaged in broth (Watson et al., 1990b). The variant, amphiphilic, surface-exposed lipoproteins (VlpA, VlpB and VlpC) of M. hyorhinis are known to undergo high-frequency changes in size and expression (Rosengarten and Wise, 1990; 1991). On SDS-PAGE immunoblots, isogenic variants generate spontaneous ladders, similar to the V-1 molecule of M. pulmonis (Watson et al., 1988). Additionally, the Vlp antigens were found to be expressed and immunogenic in M. hyorhinis-induced disease (Rosengarten and Wise, 1991). What is noteworthy about this system is that Vlp size variation is superimposed on Vlp phase variation, and also that the changes in the size of each Vlp can occur either independently or concomitantly with phase variation.

Phenotypic Switching

Many mycoplasma species are known to undergo high-frequency phenotypic switching phenomena that involve variable surface antigens. These variations might contribute to the ability of the mycoplasmal cells to evade host defenses, thereby playing a role in chronicity of mycoplasmal infections. A large number of studies have indicated that these variations have a genetic basis. Several examples of high-frequency genetic variation have been described in M. pulmonis. Dybvig et al. (1988) have described the generation of spontaneous mutants that are resistant to mycoplasma virus P1, a virus that

infects some strains of M. pulmonis (Dybvig et al., 1987). Mutants resistant to the virus were isolated at a frequency of 10^{-3} per CFU and resistance in some of these mutants resulted from the failure of P1 virus to attach to these cells (Dybvig et al., 1988). It was implied that V-1 antigen could be a possible factor involved in the binding of P1 virus to host cells because there was a correlation between the electrophoretic properties of V-1 and the adsorption of P1 virus to host cells.

Watson et al. (1990a) have shown that subcloning of a highly virulent strain of M. pulmonis results in the isolation of two phenotypic variants. One variant had the ability to adhere to polystyrene during growth and to hemadsorb to red blood cells while the other exhibited neither property. In vitro, both variants showed a high rate of switching between the two phenotypes, with the rate from adherent to non-adherent (10^{-3} per cell per generation) being tenfold higher than the reverse switch. These phenotypic differences were correlated with the high frequency of structural variation in the V-1 antigen.

The size of M. pulmonis colonies is another property that varies at a high frequency, and data from subcloning experiments indicate that colony size variation resulted from genetic changes (Dybvig et al., 1989). About 3% of the colonies from a given subclone were variants, with as much as a fourfold change in colony diameter. This variation in colony size was shown to correlate with variation in V-1

antigen, with each of the rare colonies (with a relatively large or a small diameter) having a structure different from the parent strain. In addition, subclones with varied colony size (and V-1 antigens) also displayed different growth rates in broth, suggesting that V-1 can affect growth of the organism on agar and in broth. There was no correlation noted between variation in colony size and P1 virus susceptibility. Variation in growth properties, colony morphology, adsorption to red blood cells and polystyrene and resistance to virus infection are some of the phenotypic characteristics affected by the variation in V-1.

Genetic variation affecting colony size has also been described for Mycoplasma mycoides subsp. mycoides (Valdivieso-Garcia and Rosendal, 1982). A related phenomenon may occur in M. hyorhinis which is known to undergo high-frequency changes in colony morphology as well as in the expression of diverse lipid-modified, cell surface protein antigens (Boyer and Wise, 1989; Rosengarten and Wise, 1990). Cultures of M. hyorhinis were shown to contain a stable proportion of three morphotypes, small (S), medium (M) and large (L), when plated on standard agar medium. Oscillating morphotypic switches of S, M, and L morphotypes occur at a frequency of 0.2×10^{-4} to 14.4×10^{-4} (Rosengarten and Wise, 1990). An independent, reversible high-frequency switching in colony opacity was also observed. An additional phenotypic instability identified in subcloned M. hyorhinis populations was phase variation in the surface epitope expression of

distinct sets of lipoprotein antigens (Rosengarten and Wise, 1990; 1991).

A high frequency of isolation of mutants in M. pneumoniae suggests that a phenomenon may be occurring that is similar to phase variation in Neisseria gonorrhoea (Sparling et al., 1986). The spontaneous appearance of non-hemadsorbing mutants of M. pneumoniae occurs at a high frequency of 7 X 10^{-3} mutants per CFU (Hansen et al., 1979), and similar mutants which fail to hemadsorb were also isolated using treatment with nitrosoguanidine (Krause et al., 1983). These mutants have been grouped into four classes (Krause et al., 1983). Specifically, class I consists of mutants lacking five high molecular-mass proteins designated HMW1 to HMW5, and Class III mutants lack proteins designated A, B and C. In both cases, re-acquisition of the missing proteins resulted in the hemadsorption-positive phenotype in the revertants. These data suggest that the expression of proteins involved with hemadsorption may be coordinately regulated.

The Proposal

M. pulmonis: A model system for studying genetic variation

There are several factors that make M. pulmonis an attractive system for evaluating mechanisms of genetic variation in mycoplasmas. Murine respiratory mycoplasmosis (MRM), caused by M. pulmonis, is one of the most common and important naturally ocurring diseases of laboratory rats and mice (Cassell et al., 1985). MRM is easily reproducible, and

experimental M. pulmonis infections provide an excellent model system to evaluate potential mechanisms of other mycoplasmal diseases of humans and animals, and also for the study of the pathogenesis of chronic infectious respiratory disease (Cassell, 1982; Cassell et al., 1985). Therefore, M. pulmonis infections in rodents provide an excellent natural animal model.

M. pulmonis is one of the better characterized systems for phenotypic switching among mycoplasmas. The known examples of phenotypic variation in M. pulmonis affect hemadsorption, surface antigen structure, growth properties, and virus susceptibility of the host cell (Dybvig et al., 1988; 1989; Watson et al., 1987; 1990a). As discussed previously, the surface antigens of M. pulmonis also have analogous molecules on other mycoplasmas. Therefore, the progress made in understanding these mechanisms in M. pulmonis will provide an impetus in elucidating mechanisms in many mycoplasma species. Since it is a pathogen of rats and mice, it is also feasible to compare virulence of different phenotypic variants and examine the outcome of the disease.

M. pulmonis is also relatively easy to work with. It is easy to grow, and gives fairly good yields. The gene transfer systems for M. pulmonis have been well studied and developed (Dybvig and Cassell, 1987; Dybvig and Alderete, 1988). Moreover, recently Dybvig et al. (1992) reported the isolation and cloning of the recA gene from M. pulmonis. Thus, together with the genetic tools available, it is

possible to test whether the mechanisms generating variability are recA mediated. As discussed above, P1 virus, which infects some strains of M. pulmonis, provides another useful tool for genetic studies of this organism.

Why study genetic recombination ?

It can be predicted from the evolutionary scheme for mycoplasmas that they have an inclination for undergoing chromosomal deletions. A high rate of genetic recombination is likely given the diminutive size and a high rate of evolution in these cells. Therefore a study of DNA recombination and chromosomal rearrangements will provide information on mechanisms of evolution. Moreover, phenotypic switching in many bacterial systems is associated with DNA rearrangements. Similar mechanisms might be operating in mycoplasmas, or mycoplasmas may employ novel mechanisms to generate variation.

Experimental design

The overall aim of my proposal was to elucidate the mechanisms of mycoplasmal genetic variation and to examine the association between chromosomal and phenotypic variation. The present studies demonstrate that the chromosome of M. pulmonis undergoes rearrangements at a high frequency, suggesting DNA recombination may be an important participant in generating variability.

Additionally, the small mycoplasmal genome offers distinct advantages over other bacterial systems to examine chromosomal rearrangements. Restriction endonuclease

digestion of the small mycoplasmal genome results in few fragments relative to most bacterial genomes. Therefore, it is possible to detect novel DNA fragments on routine agarose gels stained with ethidium bromide. Using such an approach, rearrangements in the chromosome can be easily identified and these variant fragments can be isolated and characterized.

The first paper presented here deals with the investigation of genetic variation within a single strain of M. pulmonis, by comparing restriction endonuclease digested DNA banding patterns of parental strain with several of its subclones. Restriction fragment polymorphisms (RFPs) were identified and some of the variant bands were cloned and used as hybridization probes. The data indicate that the variant bands arose via recombination. Some of the rearrangements correlated with changes in the susceptibility of the cells to mycoplasma virus P1, an example of phenotypic switching involving changes in surface antigen structure. This paper was published in the May 1992 issue of Molecular Microbiology.

Other DNA rearrangements, unrelated to phenotypic switching, resulted from the insertion of a copy of a repetitive element. The nucleotide sequence of this element, designated IS1138, indicates that it is an insertion sequence element related to the IS3 family. Although recent reports have described the isolation of IS-like elements from mycoplasmas (Ferrell et al.,1989; Hu et al., 1990), IS1138 is the first element indigenous to mycoplasmas that has been

shown to actively transpose within the mycoplasmal chromosome. These results have been written in the form of a manuscript that has been accepted for publication in the journal of Molecular Microbiology. The manuscript is presented as the second paper in the Results section.

Changes in the susceptibility of M. pulmonis to P1 virus is affected by the changes in the structure of the V-1 surface antigen (Dybvig et al., 1988). One of the RFPs analyzed in this study (the 9.5 kb fragment from strain KD735-16) correlated to changes in P1 virus susceptibility and by implication to changes in the V-1 antigen. The next paper describes the initial analysis of a related, cloned fragment that expresses epitopes of the V-1 antigen in E. coli. This work has been written in the form of a small note, and, after additional work, will be submitted to a peer reviewed journal for publication.

HIGH-FREQUENCY REARRANGEMENTS IN THE CHROMOSOME OF *MYCOPLASMA PULMONIS* CORRELATE WITH PHENOTYPIC SWITCHING

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Summary

Mycoplasma pulmonis is a murine pathogen that causes chronic respiratory disease in laboratory rats and mice. Several examples of high frequency phenotypic switching have been reported for M. pulmonis, the molecular basis for which is unknown. We report here that during growth, the M. pulmonis chromosome undergoes DNA rearrangements at a high frequency. Some of the rearrangements we examined correlated with changes in the susceptibility of the cells to mycoplasma virus P1, an example of phenotypic switching involving changes in surface antigen structure. Other rearrangements, unrelated to phenotypic switching, involved a DNA element present in the chromosome in multiple copies. The high level of DNA recombination that occurred in M. pulmonis indicates that this may be one of the most variable genomes studied to date. High levels of DNA recombination may contribute to the unusually high rate of evolution that mycoplasmas are thought to be undergoing. Understanding the molecular basis for this phenomenon may give insight into the chronic nature of many mycoplasmal infections.

Introduction

The genus Mycoplasma contains about 100 characterized species, many of which are pathogens producing significant diseases in man, animals, insects and plants. Mycoplasmal diseases of humans and animals often involve colonization of respiratory or genital tracts and are noted for their chronicity (Tully and Whitcomb, 1979). Mycoplasma pulmonis

causes chronic respiratory and reproductive tract diseases in laboratory rats and mice, providing a model system to study mechanisms of mycoplasmal disease chronicity (Cassell et al., 1978; Cassell, 1982).

The chronicity of mycoplasmal diseases may be related to high frequency phenotypic and antigenic changes that occur during growth of some species, particularly with M. pulmonis. By examining subclones derived from a single strain of M. pulmonis, it has been established that high frequency phenotypic switching affects growth of the organism on agar (Dybvig et al., 1989), susceptibility of the organism to infection with mycoplasma virus P1 (Dybvig et al., 1988), adherence of the organism to plastic surfaces and hemadsorption of red blood cells (Watson et al., 1990a). All of these phenomena correlate with changes in V-1, a major surface antigen of M. pulmonis. V-1 has numerous, structurally distinct forms that vary at a rate of about 2 X 10^{-3} variants per CFU per generation (Watson et al., 1988). Variation in V-1 occurs in vivo and in vitro, and in vivo variation correlates with disease severity and chronicity (Talkington et al., 1989).

The present studies demonstrate that the chromosome of M. pulmonis undergoes rearrangements at a high frequency. Some rearrangements correlated with changes in phenotype, and therefore DNA recombination may be the principal mechanism by which mycoplasmas vary their surface structures. Other DNA rearrangements, apparently unrelated to phenotypic switching,

involved an element present in the chromosome in multiple copies.

Results

Restriction fragment polymorphisms (RFPs) in subclones of M. pulmonis strain KD735

These studies began as an attempt to obtain genetically homogeneous populations of M. pulmonis. To investigate the degree of genetic variation that occurs within a single strain of M. pulmonis strain KD735 was subcloned as depicted in Fig. 1. DNA from each of these subclones was digested with restriction endonuclease HindIII and analyzed by agarose gel electrophoresis.

The ethidium bromide-stained, DNA banding pattern of most first generation subclones was similar to that of the parent strain, but notable differences were sometimes observed. Examples of some of these banding patterns are shown in Fig. 2. The banding pattern from strain KD735-15 (lane 1) is representative of the parental pattern of KD735. Strains KD735-16 (lane 3) and KD735-26 (lane 7) are subclones of KD735 that have DNA fragments of about 9.5 kb and 8.5 kb, respectively, that are absent in the parent DNA. The variant genomes present in these cells are referred to in this paper as RFPs, all of which were identified by digestion with HindIII.

RFPs were also present in second and third generation subclones. Strain KD735-15-53 (Fig. 2, lane 2) lacked a 5.9 kb fragment found in its parent strain KD735-15 (Fig. 2, lane

1). Strain KD735-16-52 (Fig. 2, lane 4) lacked two fragments of about 6.3 and 5.9 kb that were present in its parent strain KD735-16 (Fig. 2, lane 3). Strain KD735-16-12 (Fig. 2, lane 6) is a subclone of KD735-16 that lacked the 9.5 kb fragment characteristic of its parent and instead had a new 8.2 kb fragment. Strain KD735-16-12 was still heterogeneous; for example, one of its derivatives, strain KD735-16-12-1 (Fig. 2, lane 5), was missing the 6.3 and 5.9 kb fragments.

Frequency of isolation of subclones with RFPs

Cultures of M. pulmonis were sufficiently heterogeneous that subclones containing RFPs were routinely isolated. All strains (subclones) were derived by filter cloning methods (see Experimental Procedures). Some RFP-containing subclones were isolated by immediate subcloning of filter cloned strains. However, most of the RFP-containing subclones were isolated from cultures that had been passaged twice prior to the next round of subcloning. Cells were passaged by daily transfer (1:250 dilution) from an actively growing culture into fresh mycoplasma medium followed by overnight incubation. Five out of 12 subclones (about 40%) isolated from the passaged cultures had RFPs.

RFPs and a repetitive DNA element

One of the RFPs was associated with a repetitive DNA element. Plasmid pBK85 contains the cloned 8.5 kb HindIII fragment from strain KD735-26 (see Fig. 2, lane 7). When used as a probe to analyze Southern blots of M. pulmonis DNA, pBK85 hybridized to several HindIII fragments, ranging in size from

1.6 to 4.5 kb, in addition to the 8.5 kb fragment from KD735-26 (Fig. 3, lane 1). The hybridization profile of DNA from strain KD735-15 (representative of the parent strain) was similar to that of KD735-26, except that KD735-15 had a strongly hybridizing 7.2 kb fragment instead of the 8.5 kb fragment (Fig. 3, lane 3). These data suggest that the 8.5 kb variant fragment of KD735-26 arose from a 7.2 kb precursor fragment.

The RFP from strain KD735-16

M. pulmonis strain KD735-16 had an RFP unrelated to the repetitive element identified on pBK85. Plasmid pIR95 contains the cloned 9.5 kb variant fragment from this strain. On Southern blots, a pIR95 probe hybridized to the 9.5 kb fragment from KD735-16 (Fig. 4A, lane 4) and to two fragments of 5.7 kb and 4.9 kb from the parent strain KD735 (Fig. 4A, lane 1). The probe also hybridized to a 2.7 kb fragment that is present in all of the strains that have been examined to date, except for strain KD735-16-12-2, which has this fragment shifted to a lower molecular weight (Fig. 4A, lane 6).

One hypothesis is that the 9.5 kb variable fragment was generated in KD735-16 by the joining of two adjacent, parental fragments of 4.9 and 5.7 kb through the loss of about 1.1 kb of sequences containing the intervening HindIII site. This putative deletion was not a copy of the repetitive element discussed above; as shown in Fig. 3, DNA's from KD735-15 and KD735-16 had the same hybridization profiles

when probed with pBK85. Furthermore, plasmid pIR49, which contained the cloned 4.9 kb parental fragment from KD735-15, did not hybridize to the repetitive element. pIR49 hybridized to the 9.5 and 4.9 kb fragments from KD735-16 and KD735-15, respectively, as well as to the 2.7 kb fragment that is common to these strains (Fig. 4B). In addition, pIR49 hybridized weakly to the 5.7 kb fragment from KD735-15.

Correlation of RFPs with phenotypic switching

A previous report from our laboratory described the isolation of mutants of M. pulmonis that were resistant to infection by mycoplasma virus P1 (Dybvig et al., 1988). Resistance was due to failure of the virus to adsorb to the cells, and the mutants contained an altered form of the variable surface antigen V-1. Studies were undertaken to determine whether chromosomal DNA rearrangements correlated with changes in P1 virus susceptibility. All of the strains examined to date that contained the 5.7 and 4.9 kb fragments, identified by probing with pIR95, were sensitive to infection with P1 virus. However, all of the virus-resistant mutants, isolated in our previous study, to which P1 virus did not adsorb, lacked the 4.9 and 5.7 kb fragments. Replacing these fragments was a higher molecular weight fragment similar in size to the 9.5 kb fragment of KD735-16 (data not shown).

Variation in DNA fragments of 6.3 and 5.9 kb also correlated with changes in P1 virus susceptibility, as documented in Table 1. Strain KD735-16 was virus resistant. In addition to the 9.5 kb fragment, KD735-16 had a 6.3 kb

fragment that was not present in the parent strain. This fragment did not hybridize with the probes described above; its presence was directly detected as a variant band on ethidium bromide-stained gels (see Fig. 2). Other strains that contained the 6.3 kb fragment were KD735-16-12 and 6a14; both of these strains were also P1 virus resistant. We have isolated derivatives of each of these strains, denoted as KD735-16-52, KD735-16-12-1, and 6a14/4 (see Fig. 1), that no longer had the 6.3 kb DNA fragment. Each of these derivatives had also reverted to a P1 virus-sensitive phenotype. In addition to the 6.3 kb fragment, these strains were also missing a 5.9 kb fragment. These strains, however, retained their high molecular weight fragment that hybridized with pIR95 and still lacked the 5.7 and 4.9 kb fragments (see Fig 4A, lanes 4-9).

The correlation between specific RFPs and susceptibility to P1 virus was observed regardless of the order in which the phenotypes were identified. That is, strain KD735-16 and derivatives were initially identified as containing RFPs, and they were subsequently found to have altered virus susceptibilities. In contrast, strain 6a14 was initially identified as a virus-resistant mutant of strain KD735-15 that also had an altered form of V-1 antigen, and it was subsequently determined that this strain contained an RFP. Similarly, strain 6a14/4 was initially identified as a virus-sensitive revertant of strain 6a14, and it was subsequently found to contain an RFP. No correlation was observed between

virus susceptibility and RFPs other than the one summarized in Table 1. For example, the locations of the repetitive element identified by probing chromosomal DNA with pBK85 did not correlate with virus susceptibility. There was also no apparent correlation between RFPs and the previously described phenomenon of colony size switching.

Discussion

In this study, RFPs were frequently detected in subclones of a single strain of M. pulmonis. Southern hybridization data using variant DNA fragments associated with RFPs as probes, indicate that RFPs arose via insertions, deletions, or other rearrangements in the chromosome. The data do not support a model in which RFPs arose as a result of point mutations or changes in DNA methylation affecting restriction enzyme recognition sites.

About 40% of the subclones from strains that had been passaged twice were identified as containing RFPs. Because of the large number of fragments that are obtained by digestion of M. pulmonis DNA with HindIII, many RFPs would not be recognized due to comigration of variable DNA fragments with other DNA fragments of similar size. Variation within HindIII fragments smaller than 5 kb would almost certainly go unobserved using our methods, and we assume that the actual percentage of subclones from the passaged cultures that had RFPs is much greater than 40%.

The rate at which RFPs were generated in the passaged cultures of M. pulmonis can be estimated. As described in

Experimental Procedures, subclones were obtained by picking entire colonies (in the form of agar plugs) and growing them in 1 ml cultures. These cultures reached a titer of about 10^9 CFU, suggesting that about 30 generations of growth occurred ($2^{30}=10^9$). Serial passage of the cells should have resulted in about 16 additional generations of growth; each passage of cells diluted 1:250 required about 8 generations ($2^8=256$) to regain titer. Therefore, cells were grown for about 46 generations prior to subcloning. Because 40%, and possibly many more, of the subclones contained RFPs, we estimate that RFPs were generated at a rate of about 10^{-2} to 10^{-3} variants per CFU per generation. This high rate of DNA recombination would make the mycoplasma chromosome one of the most variable genomes known, rivaling the extreme examples of genetic instability described for halobacteria and streptomyces (Pfeifer and Blaseio, 1989; Sapienza et al., 1982; Leblond et al., 1989, 1990).

The data described here indicate that in one case, strain KD735-26, a RFP contained a copy of a repetitive element. Southern hybridization analysis (data not shown) has indicated that this repetitive sequence is present in most if not all strains of M. pulmonis, including strains unrelated to KD735. The nucleotide sequence of this element has recently been determined (Bhugra and Dybvig, unpublished data), revealing homology with the E. coli insertion sequence IS3. Transposition of IS elements is thought to be one of the major mechanisms by which some genomes (eg. halobacteria)

undergo rearrangements (Sapienza et al., 1982; DasSarma et al, 1988).

Some of the DNA rearrangements that occurred in M. pulmonis correlated with changes in the susceptibility of the cells to mycoplasma virus P1 and, by implication, changes in V-1 antigen. The strongest correlation was with DNA fragments of 6.3 and 5.9 kb. Strains containing these fragments were P1 virus resistant, and strains lacking them were P1 virus sensitive. However, this correlation was complicated by the fact that DNA fragments of 4.9 and 5.7 kb were invariably missing and replaced by a single fragment of higher molecular weight (e.g., 9.5 kb) in those strains that had the 6.3 kb fragment. The connection between the 4.9 and 5.7 kb fragments to the 6.3 and 5.9 kb fragments is unclear. Although all of these fragments have not yet been cloned and used as hybridization probes, those which have been cloned show no crossreaction between these sets of fragments.

DNA rearrangements have been shown to be associated with phenotypic switching in many bacterial systems. DNA inversions, gene conversions, duplications or deletions of tandem homologous blocks of DNA, and movement of transposable elements are frequently employed mechanisms of phenotypic switching in other bacterial systems (Hollingshead et al, 1987; Borst and Greaves, 1987; Hoiseth et al., 1986; Seifert and So, 1988). One or more of these mechanisms are likely to be involved in the phenomenon described here. Phenotypic switching similar to that of M. pulmonis has also been

described in other mycoplasmal species (Olson et al, 1991; Rosengarten and Wise, 1990; 1991; Watson et al, 1990b), and elucidation of the genetic basis of this phenomenon in M. pulmonis may provide insight into how mycoplasmas in general vary their phenotypic traits.

The high frequency of genetic variation in mycoplasmas would allow for the rapid generation of diverse subpopulations which could enable these organisms to survive in a variety of niches or evade immune responses, contributing to the pathogenesis of mycoplasmal infections. In hindsight, a high rate of genetic recombination in these cells is predictable given the small size of the chromosome. The chromosomes of some mycoplasmas are as small as 600 kb (Colman et al, 1990), and creating new coding regions by rearranging the chromosome would increase the coding capacity of the cells without increasing genome size. Ribosomal RNA analysis has revealed that these organisms are evolving much faster than most eubacteria (Woese, 1987), and it will be interesting to learn how DNA recombination contributes to this evolutionary process.

Experimental Procedures

Mycoplasmas

Mycoplasma pulmonis was propagated in mycoplasma medium, and CFUs were assayed on agar as previously described (Dybvig and Cassell, 1987). All M. pulmonis strains used in this study were subclones of strain KD735 (Dybvig et al., 1989), a derivative of strain UAB 6510 that is susceptible to

mycoplasma virus P1 (Dybvig et al., 1988). Subclones were derived by filter cloning methodology, which involves removal of cell aggregates by gently passing cultures through a 0.2 um filter immediately prior to assaying for CFU (Tully, 1983; Dybvig et al., 1989). The filter cloning technique is designed to generate colonies derived from single cells. Well-separated colonies were randomly picked in agar plugs, propagated at 37°C in 1 ml cultures, and assigned a new strain designation. These cultures were stored at -70°C and used as stocks for subsequent experiments.

Electrophoresis of mycoplasma DNA

DNA was isolated from 25 ml cultures of M. pulmonis as described (Dybvig and Alderete, 1988). For agarose gel electrophoresis, about 5 ug of mycoplasma DNA was digested with a restriction enzyme, usually HindIII, and analyzed on a 0.8% gel. Electrophoresis for 16 hours at 2.5 volts/cm was generally sufficient to resolve individual DNA fragments of about 5.5 kb and greater, such that they could be directly observed by staining with ethidium bromide.

DNA manipulations

Restriction enzymes and T4 DNA ligase were used according to the specifications of the supplier (GIBCO/BRL Life Technologies Inc., Gaithersburg, MD). Conditions for agarose gel electrophoresis, transfer of DNA onto nitrocellulose membranes, and labeling of DNA probes with ^{32}P by nick translation were as described (Maniatis et al., 1982). Stringent conditions were used for Southern hybridizations:

the hybridization solution contained 50% formamide and the hybridization temperature was 42°C as described (Maniatis et al., 1982). Plasmid DNA was isolated from E. coli using the alkaline lysis method and further purified by CsCl-ethidium bromide density gradient centrifugation (Maniatis et al., 1982).

For cloning, DNA fragments were recovered from agarose gels by electrophoresis onto DEAE cellulose paper. The eluted fragments were then ligated into the HindIII site of plasmid pUC18, and used to transform E. coli strain JM103 (Messing et al., 1981). The inserts within plasmids pIR95, pIR49, and pBK85 were the 9.5 kb fragment from strain KD735-16, the 4.9 kb fragment from strain KD735-15, and the 8.5 kb fragment from strain KD735-26, respectively.

Susceptibility of host cells to P1 virus

Mycoplasma virus P1 was propagated on M. pulmonis strain KD735. The susceptibility of the subclones of KD735 to P1 virus infection was determined by spotting virus-containing filtrates onto lawns of cells and examining the plates for zones of clearing after incubation at 37°C for 1 or 2 days, as described previously (Dybvig et al., 1987).

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Table 1. Correlation between variable DNA fragments and susceptibility (susc.) of Mycoplasma pulmonis to infection with P1 virus

Strain	Parent	4.9	5.7	5.9	6.3	8.2	9.5	P1 susc.
KD735	UAB 6510	+	+	+	-	-	-	S
KD735-15	KD735	+	+	+	-	-	-	S
KD735-15-53	KD735-15	+	+	-	-	-	-	S
KD735-16	KD735	-	-	+	+	-	+	R
KD735-16-52	KD735-16	-	-	-	-	-	+	S
KD735-16-12	KD735-16	-	-	+	+	+	-	R
KD735-16-12-1	KD735-16-12	-	-	-	-	+	-	S
6a14	KD735-15	-	-	+	+	-	+	R
6a14/4	6a14	-	-	-	-	-	+	S

Numbers in top row refer to the sizes of variable DNA fragments in kb. + and - refer to presence and absence of DNA fragments, respectively. S and R refer to P1 virus sensitive and resistant phenotypes, respectively.

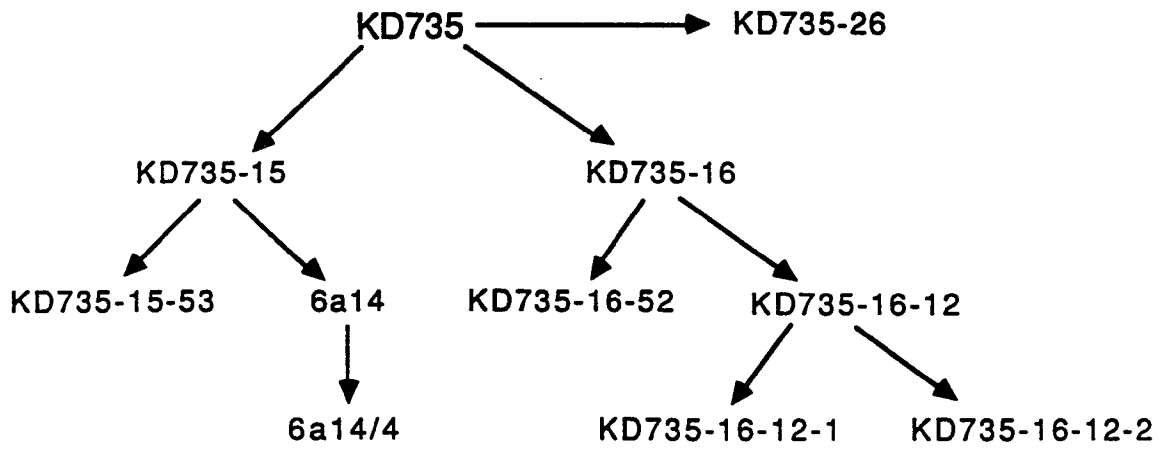


Fig. 1. Schematic diagram showing the relationship of RFP-containing strains to the parental strain KD735.

Fig. 2. RFPs in derivatives of strain KD735. HindIII-digested chromosomal DNA's were analyzed on an agarose gel stained with ethidium bromide. Lane 1, strain KD735-15 (derived from KD735); lane 2, strain KD735-15-53 (derived from KD735-15); lane 3, strain KD735-16 (derived from KD735); lane 4, strain KD735-16-52 (derived from KD735-16); lane 5, strain KD735-16-12-1 (derived from KD735-16-12); lane 6, strain KD735-16-12 (derived from KD735-16-12); lane 7, strain KD735-26 (derived from KD735). Arrows refer to the variant 9.5, 6.3 and 5.9 kb fragments from strain KD735-16 and the variant 8.2, 6.3 and 5.9 kb fragments from strain KD735-16-12.

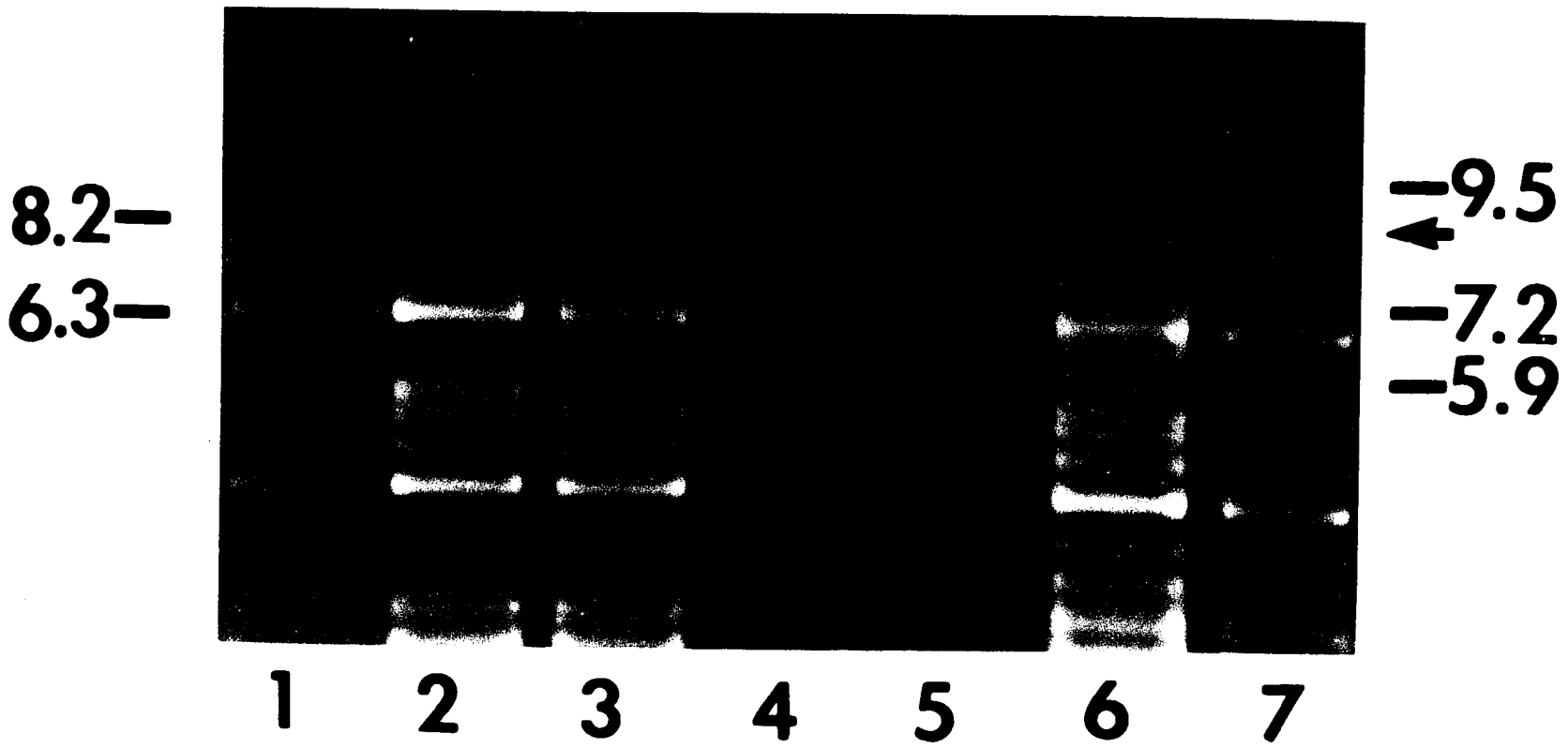

8.2
6.3
9.5
7.2
5.9
1
2
3
4
5
6
7

Fig. 3. Detection of a repetitive DNA element by Southern hybridization. HindIII-digested, chromosomal DNA's were analyzed on an agarose gel, transferred to nitrocellulose, and probed with pBBK85. Analyzed in lanes 1-3 were DNA from KD735-26, KD735-16, and KD735-15, respectively. Numbers in the margins refer to the size (in kb) of selected fragments. The fragment at 3.0 kb is actually a doublet that was poorly resolved in this particular experiment.

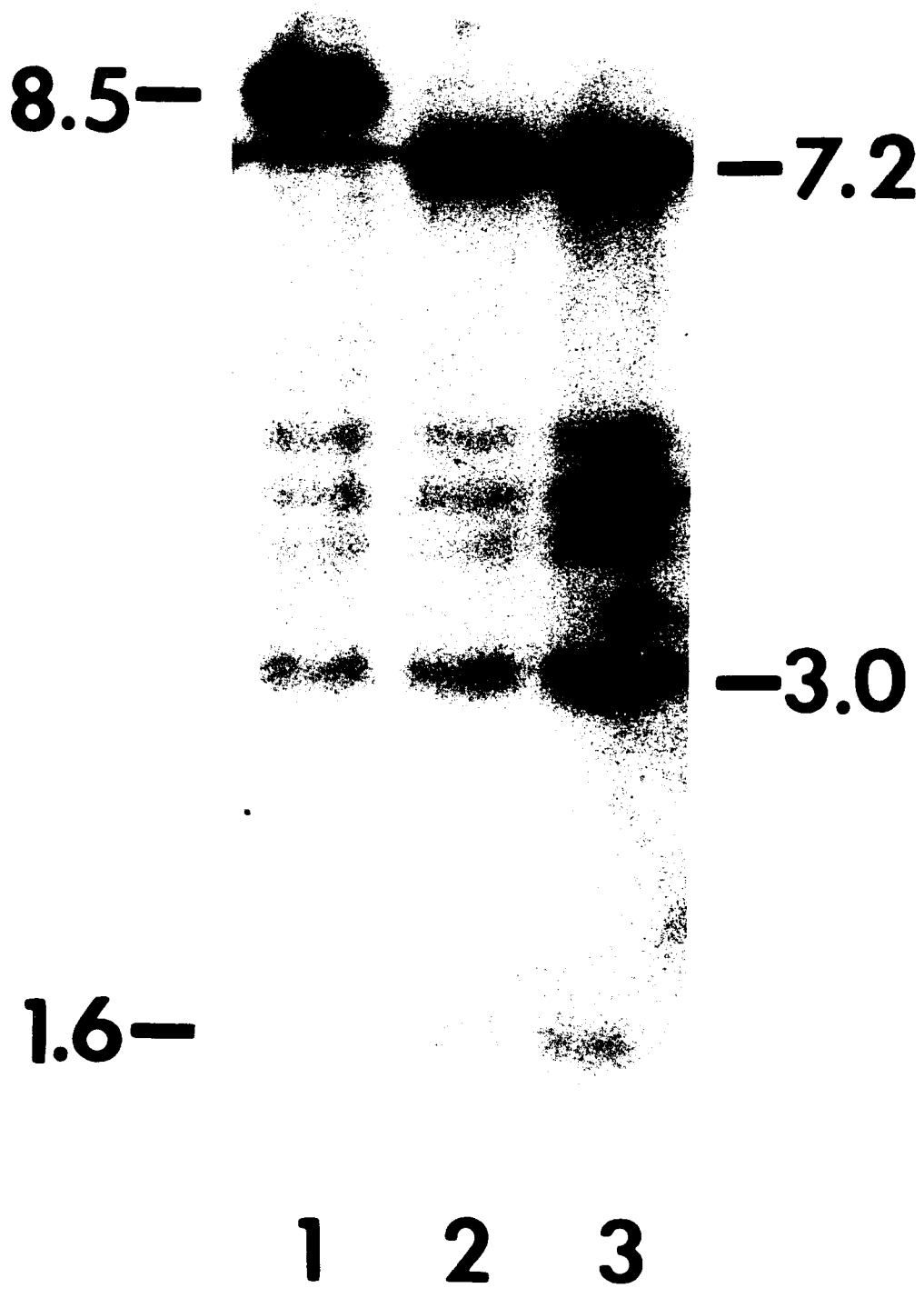
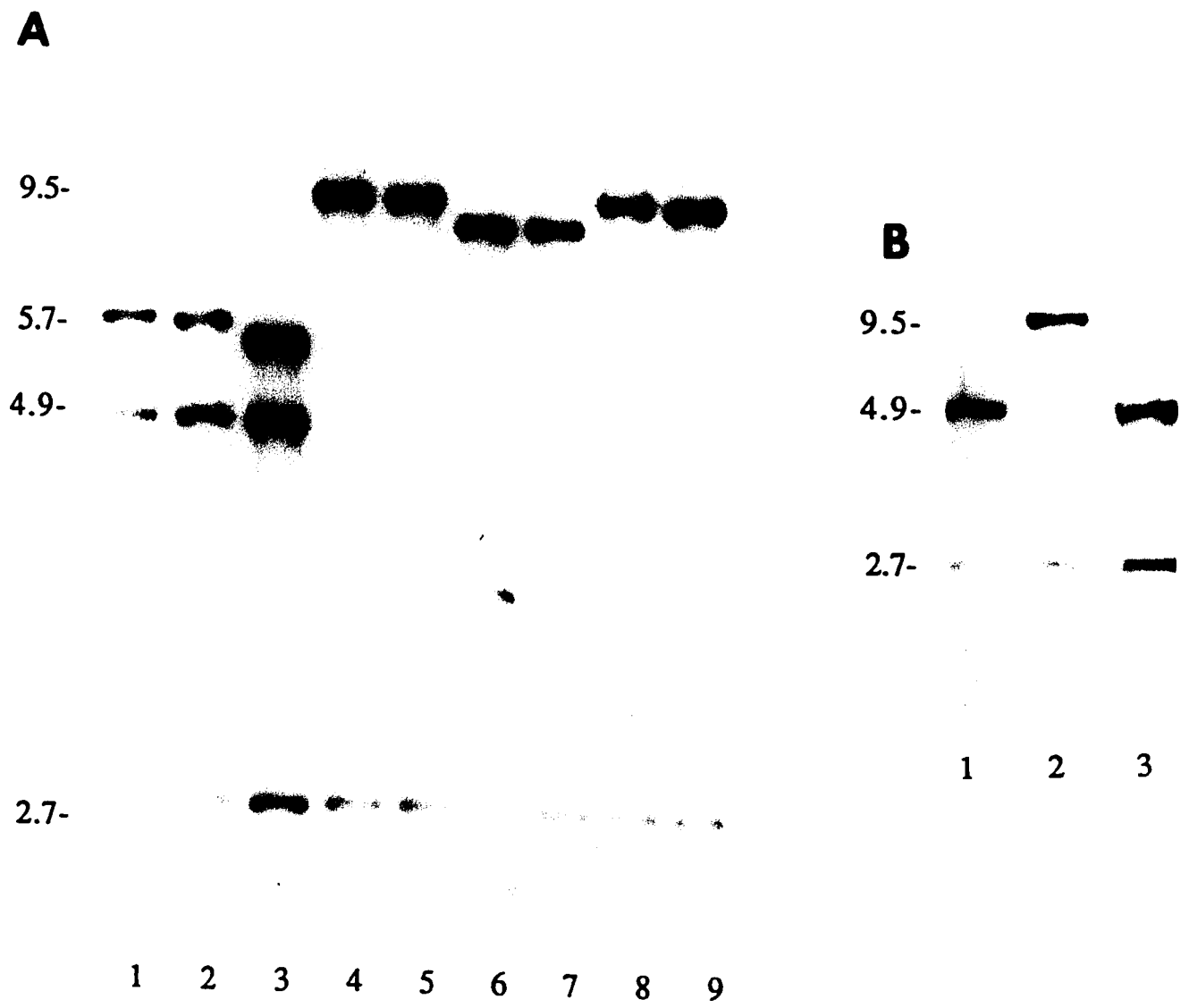

8.5
7.2
3.0
1.6
1
2
3

Fig. 4. Southern hybridization analysis of the variable 9.5, 5.7, and 4.9 kb fragments. Chromosomal DNA was digested with HindIII, analyzed on an agarose gel, and transferred to nitrocellulose. Numbers in the margins refer to the size (in kb) of selected fragments. (A) Southern blot probed with pIR95. Lane 1, KD735; lane 2, KD735-15; lane 3, KD735-15-53; lane 4, KD735-16; lane 5, KD735-16-52; lane 6, KD735-16-12-2; lane 7, KD735-16-12-1; lane 8, 6a14; and lane 9, 6a14/4. (B) Southern blot probed with pIR49. Analyzed in lanes 1-3 was DNA from KD735-15, KD735-16, and KD735-26, respectively.


A
9.5-
5.7-
4.9-
2.7-
1 2 3 4 5 6 7 8 9
B
9.5-
4.9-
2.7-
1 2 3

IDENTIFICATION AND CHARACTERIZATION OF IS*1138*, A TRANSPOSABLE ELEMENT FROM *MYCOPLASMA PULMONIS* THAT BELONGS TO THE IS*3* FAMILY

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Summary

Insertion sequence (IS) elements are mobile genetic elements found in prokaryotes. We have identified a repetitive element from Mycoplasma pulmonis, a murine pathogen, that is similar to eubacterial IS elements. By subcloning a single strain of M. pulmonis, we isolated a variant clone in which the IS element had undergone an apparent transposition event. The nucleotide sequences of the element, designated IS1138, and the target site into which it inserted were determined. IS1138 consists of 1,288 bp with 18 bp perfect terminal inverted repeats. Sequence analysis of the target site before and after insertion of IS1138 identified a 3 bp duplication of target DNA flanking the element. The predicted amino acids encoded by the major open reading frame of IS1138 share significant similarity with the transposases of the IS3 family. Southern hybridization analysis indicates that repetitive sequences similar to IS1138 are present in most, if not all, strains of M. pulmonis, but IS1138-like sequences were not detected in other mycoplasmal species.

Introduction

We have previously reported the identification of restriction fragment polymorphisms (RFPs) in subclones of Mycoplasma pulmonis strain KD735 (Bhugra and Dybvig, 1992). RFPs resulted from DNA rearrangements and occurred at a high rate of about 10^{-2} to 10^{-3} per CFU per generation, suggesting that M. pulmonis has one of the most variable genomes known. Some RFPs correlated with changes in the susceptibility of cells

to infection by mycoplasma virus P1. Described here is another RFP unrelated to the earlier observed changes in phenotypic switching. A rearrangement in the M. pulmonis chromosome due to this RFP resulted from insertion of a copy of a repetitive element. The nucleotide sequence of the element indicates that it is an insertion sequence (IS) element related to the IS3 family.

Bacterial IS elements constitute a large and diverse group of mobile genetic elements (Galas and Chandler, 1989; Iida et al., 1983) that have several characteristic features. They usually are 1 to 2 kb in size and have inverted repeats at their termini. On transposition, most IS elements generate a duplication of target sequences that flank the inserted element. Several families of IS elements have been described, e.g., the IS1, IS2, and IS3 families (Galas and Chandler, 1989; Iida et al., 1983). The IS3 family is particularly widespread, consisting of elements from both Gram-negative and Gram-positive bacteria. Although IS elements belonging to this group share little nucleotide homology, they share extensive amino-acid homology (Schwartz et al., 1988; Prere et al., 1990; Rubens et al., 1989).

This report describes the identification, cloning, and structural characterization of a repetitive element associated with RFPs in M. pulmonis. The element, designated IS1138, contains 1,288 bp and has 18 bp terminal inverted repeats. An apparent transposition event involving IS1138 generated a 3 bp duplication of target sequences that flank

its ends as direct repeats. IS1138 has a long open reading frame (ORF) on the plus strand. The predicted amino acids of this ORF display similarity to those of the IS3 family of elements. Although recent reports have described the isolation of IS-like elements from mycoplasmas (Taylor et al., 1987; Ferrell et al., 1989; Hu et al., 1990), IS1138 is the first element indigenous to mycoplasmas that has been shown to actively transpose within the mycoplasmal chromosome.

Results

Restriction map of pBBK85

Previously, we have described the identification of a repetitive element (IS1138) present in eight copies in the chromosome of M. pulmonis strain KD735-26, a derivative of strain KD735 (Bhugra and Dybvig, 1992). Additionally, hybridization analysis using plasmid pBBK85 (Table 1) as a probe had shown that the 8.5 kb variable fragment of strain KD735-26 arose from a 7.2 kb precursor fragment in strain KD735. Restriction maps of pBBK85 and two of its subclones, plasmids pBBK25 and pBBK60, are shown in Fig. 1A. When used to probe Southern blots of M. pulmonis DNA, pBBK60 and pBBK25 hybridized to the same set of multiple DNA fragments as did pBBK85 (Fig. 2A and B), indicating that each of these subclones contain a copy of IS1138. Southern hybridization analysis of pBBK85 probed with pBBK25 revealed that one of the IS1138 copies was located on the 2.5 kb HindIII-SstI fragment (Fig. 3A and B, lanes 2 and 3) and the other copy

was located on the 2 kb HincII-HindIII fragment of pBBK60 (Fig. 3A and B, lanes 3 and 4).

Comparison of the 7.2 kb precursor and the 8.5 kb product fragments

Southern blots probed with pBBK25 were used to compare the 7.2 kb precursor fragment from strain KD735-15 to the variant 8.5 kb fragment from strain KD735-26 (Fig 4). Chromosomal DNA from these strains was digested with restriction enzymes HindIII and SstI. Digestion of KD735-26 DNA with these enzymes resulted in cleavage of the 8.5 kb fragment into two fragments of 6.0 and 2.5 kb corresponding to the inserts in pBBK60 and pBBK25, respectively. Both of these cleavage products, as well as several additional fragments (Fig. 4A, lane 3), hybridized with pBBK25. Similarly, DNA from strains with the parental DNA banding pattern, KD735-15 (or KD735-16), had a 6.0 kb restriction fragment that hybridized with pBBK25 (Fig. 4A, lanes 1 and 2). However, these strains did not have the 2.5 kb HindIII-SstI fragment characteristic of KD735-26. Instead, they had a smaller 1.2 kb restriction fragment, indicating that the 7.2 kb precursor fragment from KD735-15 (or KD735-16) was composed of 6.0 and 1.2 kb HindIII-SstI fragments.

Evidence for transposition of IS1138

The precursor 1.2 kb HindIII-SstI fragment from KD735-15 was isolated by polymerase chain reaction (PCR) amplification using oligonucleotide primers specific for the ends of the sequenced insert in pBBK25 (see Experimental procedures). The

amplified product was cloned, resulting in plasmid pBBK12 (Fig. 1B). When used to probe Southern blots of chromosomal DNA digested with HindIII and SstI, pBBK12 hybridized to the 1.2 kb fragment from KD735-15 and the 2.5 kb fragment from KD735-26 (Fig. 4B). However, pBBK12 did not hybridize to any additional fragments from these strains, indicating that it does not contain a copy of IS1138. From these data, a restriction map of the 7.2 kb precursor fragment from KD735-15 has been deduced (Fig. 1B). This fragment contains a single copy of IS1138 located on the 6.0 kb HindIII-SstI fragment. Insertion of a second copy of IS1138 into the 1.2 kb HindIII-SstI region would have generated the variant 8.5 kb fragment of KD735-26.

Characterization of IS1138

IS1138 was identified and characterized by sequencing the entire inserts in both pBBK25 and pBBK12. These sequences were identical except for a 1,288 bp region (IS1138) in the middle of pBBK25 that was lacking in pBBK12. The complete nucleotide sequence of IS1138 is shown in Fig. 5. It has 18 bp inverted repeats at its ends between nucleotides 1-18 and 1270-1288. IS1138 has one ORF on the plus strand and one ORF on the complementary strand. The plus-strand ORF extends from nucleotides 60 to 1262 and could encode a protein of 400 amino acids. There is a potential ribosome binding site (RBS) preceding the start of this ORF by 5 bp. However, the ORF on the complementary strand, located between nucleotides 1147 to

842, lacks a potential RBS preceding a TTG initiation codon, and no significance has been assigned to this ORF.

The sequence of the inserts in pBBK12 and pBBK25 were compared to examine the target site before and after insertion of IS1138. Transposition of IS1138 apparently generated a 3 bp duplication of target sequences (TTG) that flank the copy of IS1138 in pBBK25 as direct repeats.

IS1138 is a member of the IS3 family of elements

A complete search of Genbank and NBRF databases for sequences related to IS1138 was performed using the TFASTA program of the Genetics Computer Group (University of Wisconsin). All of the sequences scored as having the highest homology were IS elements. Prominent among these were IS3 and IS150 from Escherichia coli (Timmerman and Tu, 1985; Schwartz et al., 1988), IS861 from Streptococcus agalactiae (Rubens et al., 1989), IS600 from Shigella sonnei (Matsutani et al., 1987), IS1076 (Huang et al., 1991), IS904 (Rauch et al., 1990), and IS981 (Polzin and McKay, 1991) from Lactococcus lactis, IS911 from Shigella dysenteriae (Prere et al., 1990), IS476 from Xanthomonas campestris (Kearney and Staskawicz, 1990). All of these elements belong to the IS3 family of insertion sequences and share a similar DNA organization.

The putative transposase of IS1138

The plus-strand ORF of IS1138 likely encodes the functional transposase of IS1138. The deduced amino acid sequence of the plus-strand ORF from IS1138 was compared to the predicted amino acids of the putative transposases from members of the

IS3 family using the BESTFIT sequence alignment program of the GCG package. Alignment of the gene product of the IS1138 ORF displayed extensive similarity to the following transposases: ORF1 of IS3 (45.3% similarity, 20.7% identity), ORFB of IS150 (57.8 % similarity, 31.8 % identity), ORFB of IS911 (48.2% similarity, 23.3% identity), ORFB of IS904 (54.3% similarity, 30.9% identity), ORF2 of IS981 (52.5% similarity, 27.5% identity), ORF2 of IS861 (56.8% similarity, 33.3% identity). The alignment of the putative transposase with those of the predicted transposases of IS3, IS150 and IS911 elements is shown in Fig. 6. Several clusters of well-conserved amino acid residues common to all four putative transposases were identified.

IS1138 from pBBK20

The nucleotide sequence of the other copy of IS1138, located on the 2.0 kb HincII-HindIII fragment in plasmid pBBK20, was also determined. Analysis of this copy in pBBK20 (Table 1) reveals a sequence nearly identical to the copy of IS1138 on pBBK25 (see Fig. 5). The sequence differs only at three bases: it has a T instead of G at nucleotide position 179 (changing glutamic acid to aspartic acid), a T instead of A at nucleotide position 539 (no amino acid change), and a G instead of A at position 1065 (changing asparagine to aspartic acid). The second copy of IS1138 is also flanked by a 3 bp direct repeat (TTC), possibly generated by duplication of target sequences via transposition.

Distribution of IS1138 in M. pulmonis strains and other mycoplasma species

We investigated the distribution of IS1138 in various M. pulmonis strains. Twelve different strains of M. pulmonis, isolated from different tissues and host sources (Davidson et al., 1988), were examined by Southern hybridization analysis using IS1138 (in pBBK25) as the probe (Fig. 7). The probe hybridized to several fragments, varying in size and number, among all M. pulmonis strains examined, except strain 66, where it hybridized to only one fragment. Therefore, repetitive elements similar to IS1138 are present in most strains of M. pulmonis. DNA from KD735-15-52 had a novel fragment at 2.8 kb (see Fig. 7, lane 9) that was not present in its parent KD735-15. This may represent another example of transposition of IS1138.

Similar Southern hybridization analysis was used to examine other species of mycoplasma. No significant hybridization was observed between IS1138 and DNA from Mycoplasma gallisepticum, Mycoplasma fermentans, Mycoplasma hyorhinis, Mycoplasma mycoides subsp. mycoides and Acholeplasma laidlawii.

Discussion

IS1138 is the first example of a mobile mycoplasmal element. The RFP identified in KD735-26 was evidently generated by transposition of IS1138. The variant 2.8 kb fragment found in DNA from KD735-15-52 may also have resulted from a transposition event. It would therefore seem that IS1138

transposes in the M. pulmonis chromosome at a high frequency. IS1138 is ubiquitous among strains of M. pulmonis, but it appears for the most part to be species specific. However, repetitive sequences that have features similar to prokaryotic IS elements have been previously identified in M. fermentans and M. hyorhinis (Ferrell et al., 1989; Hu et al., 1990; Taylor et al., 1987), but transposition events involving these elements have not yet been described. Although these elements, like IS1138, are related to the IS3 family, no cross hybridization between these elements was observed.

A 3 bp direct repeat (TTG) flanks the copy of IS1138 located on pBBK25. The parental 1.2 kb fragment in pBBK12, which lacks the 1,288 bp IS1138 sequence, contains only a single TTG in the same region. Therefore, transposition of IS1138 apparently generates duplication of target sequences, as has been observed among several other members of the IS3 family (Iida et al., 1983; Prere et al., 1990).

The deduced amino acid residues from the major ORF of IS1138 show striking similarities to the predicted amino acid sequence of transposases of other IS3 family elements. In fact, several amino acid residues are highly conserved among the putative transposases of IS1138, IS3, IS150 and IS911. A motif consisting of conserved residues in the C-terminal end of the transposases (amino acids 305 to 348 of IS1138), comprising DG_2Y_5 and 26 amino acids later $N_{32}E_{36}K_{43}$, form a signature sequence for the IS3 family. The degree of

conservation suggests that these residues might be functionally important. The region containing this motif shares striking similarity with a region containing the identical motif in retroviral integrases (Fayet et al., 1990). Recent studies based on mutational analysis imply that the amino acid residues in this motif are essential for the binding of integrase to DNA (Eichinger and Boeke, 1988; Quinn and Grandgenett, 1988). The amino acids of the putative transposases of IS3 family may provide a similar DNA-binding function, necessary for transposition. We were unable to identify alternative DNA binding motifs (e.g., helix-turn-helix or leucine zipper) in the putative IS1138 transposase.

The base compositions of IS1138 and the target DNA in pBBK12 were 77% A+T and 76% A+T, respectively, reflecting the AT-rich genome that is characteristic of mycoplasmas. It is therefore not surprising that although amino acids homology with other members of the IS3 family was striking, nucleotide homology was not so pronounced. The high A+T composition also indicates that the acquisition of this IS element by M. pulmonis was not a recent event. The major ORF of IS1138 contains 11 UGA codons, encoding tryptophan in this organism. Because UGA is a termination codon in most eubacteria, IS1138 would presumably be nonfunctional in organisms other than mycoplasmas.

Experimental Procedures

Bacterial strains and plasmids

DNA was cloned using plasmid pUC18 as the vector and *Escherichia coli* strain DH5α as host cells. The recombinant plasmid pBBK85 contains the variant 8.5 kb fragment from strain KD735-26 (Bhugra and Dybvig, 1992). The schematics of subclones of pBBK85 are shown in Fig. 1 and their derivations in Table 1. The characteristics of strains of *M. pulmonis* used in this study are summarized in Table 1. Other mycoplasma species for which the presence of IS*1138* was examined were *M. fermentans* strains K7 (Murphy *et al.*, 1965), PG18 (Edward and Freundt, 1973) and incognitus (Hu *et al.*, 1990), *M. gallisepticum* strain PG31 (Edward and Freundt, 1973), *M. hyorhinis* strain GDL-I (Taylor *et al.*, 1987), *M. mycoides* subsp. *mycoides* strain GM9 (Dybvig and Khaled, 1990) and *A. laidlawii* strains JA1 and K2 (Dybvig and Woodard, 1992).

DNA techniques

Mycoplasmas were grown overnight in mycoplasma medium, and DNA was isolated from 25 ml cultures as previously described (Dybvig and Alderete, 1988). For cloning of specific fragments, restriction enzyme-digested DNAs were purified from agarose gels and ligated into pUC18 as described (Maniatis *et al.*, 1982). Radiolabelling of DNA with ^{32}P by nick translation and Southern blotting techniques were as described (Maniatis *et al.*, 1982), and normal (high)

stringency conditions were used for all hybridization experiments.

PCR amplification

For enzymatic amplification of the parental 1.2 kb fragment from strain KD735-15, oligonucleotide primers were designed to sequences flanking the copy of IS1138 present in pBBK25. The left primer spanned the pBBK25 Sst1 site, and a HindIII site was introduced into the right primer. The left primer was 5'-CTCTTTGTGTTTTGAGGAGCTCC-3' and the right primer was 5'-GGAAGCTTTTTTAGCAAGACAAGC-3'. Reaction conditions for PCR were as described (Dybvig et al., 1992). For amplification, the reaction mixture was subjected to 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C using the Programmable Thermal Cycler from MJ Research (Watertown, MA).

DNA sequencing and sequence analysis

DNA sequencing was performed on both strands of the plasmid DNA template using the dideoxy sequencing technique and the Sequenase 2.0 Kit from United States Biochemical Corp. (Cleveland, OH). DNA primers were supplied by the Oligonucleotide Synthesis Core Facility at the University of Alabama at Birmingham. Computer analysis was performed by using the Genetics Computer Group (GCG) program (University of Wisconsin, Madison) on a VAX-VMS computer at the University of Alabama at Birmingham Cancer Center.

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Table 1. Derivations of the plasmid constructs and of subclones (strains) of Mycoplasma pulmonis strain KD735 used in this study.

Strain or Plasmid	Characteristics	Reference
Plasmids		
pBBK85	8.5 kb HindIII fragment from strain strain KD735-26 in pUC18, contains 2 copies of IS1138.	(Bhugra and Dybvig,1992)
pBBK60	6.0 kb insert in pUC18, contains 1 copy of IS1138.	This work
pBBK25	2.5 kb insert in pUC18, contains 1 copy of IS1138.	This work
pBBK20	2.0 kb insert in pUC18, contains 1 copy of IS1138.	This work
pBBK12	1.2 kb insert in pUC18, lacks IS1138.	This work
M. pulmonis strains		
KD735	Parent strain derived from UAB 6510	(Dybvig and Alderete, 1988)
KD735-15	Derivative of KD735, DNA banding pattern using IS1138 probes is indistinguishable from that of KD735.	(Bhugra and Dybvig, 1992)
KD735-16	Derivative of KD735, DNA banding pattern using IS1138 probes is indistinguishable from that of KD735.	(Bhugra and Dybvig, 1992)
KD735-26	Derivative of KD735 containing the variant 8.5 kb HindIII fragment.	(Bhugra and Dybvig, 1992)

Table 1 (continued)

KD735-15-52	Derivative of KD735, DNA banding pattern using IS1138 probes has a novel 2.8 kb fragment.	(This study)
Other (see Fig.7)	Diverse isolates from a variety of hosts and tissues.	(Davidson et al., 1988)

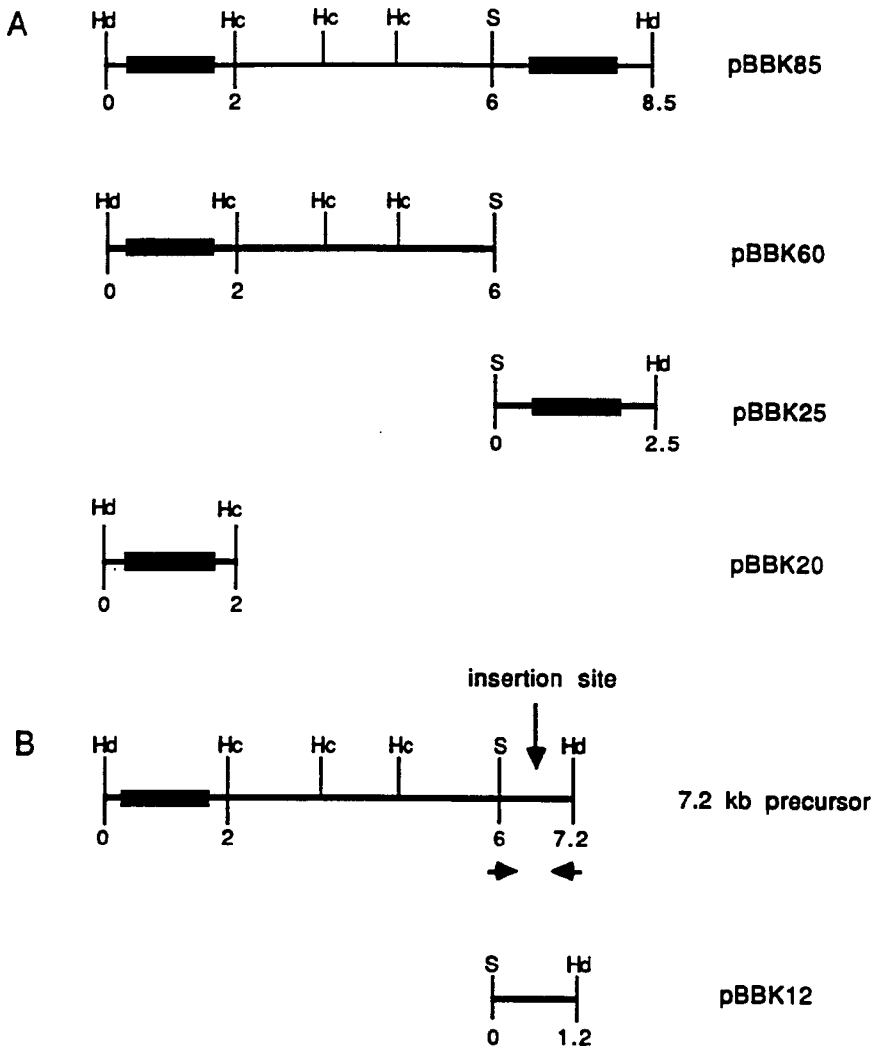


Fig. 1. A. Schematic diagram of recombinant plasmids used in this study. B. Deduced map of the 7.2 kb precursor fragment from strain KD735-15 depicting the insertion site of IS1138 to be within the 1.2 kb HindIII-Sst1 fragment. Arrows show the primer binding sites used for PCR amplification of the 1.2 kb precursor fragment (see Experimental procedures). Black boxes depict IS1138. Abbreviations for restriction enzymes are: Hd for HindIII, Hc for HincII and S for SstI.

Fig. 2. Southern hybridization analysis using (A) pBBK60 and (B) pBBK25 as hybridization probes. Chromosomal DNA was digested with HindIII, analysed on an agarose gel and transferred to nitrocellulose. Lane 1, KD735-15 and lane 2, KD735-26. Numbers in the margins refer to the size (in kb) of selected fragments.

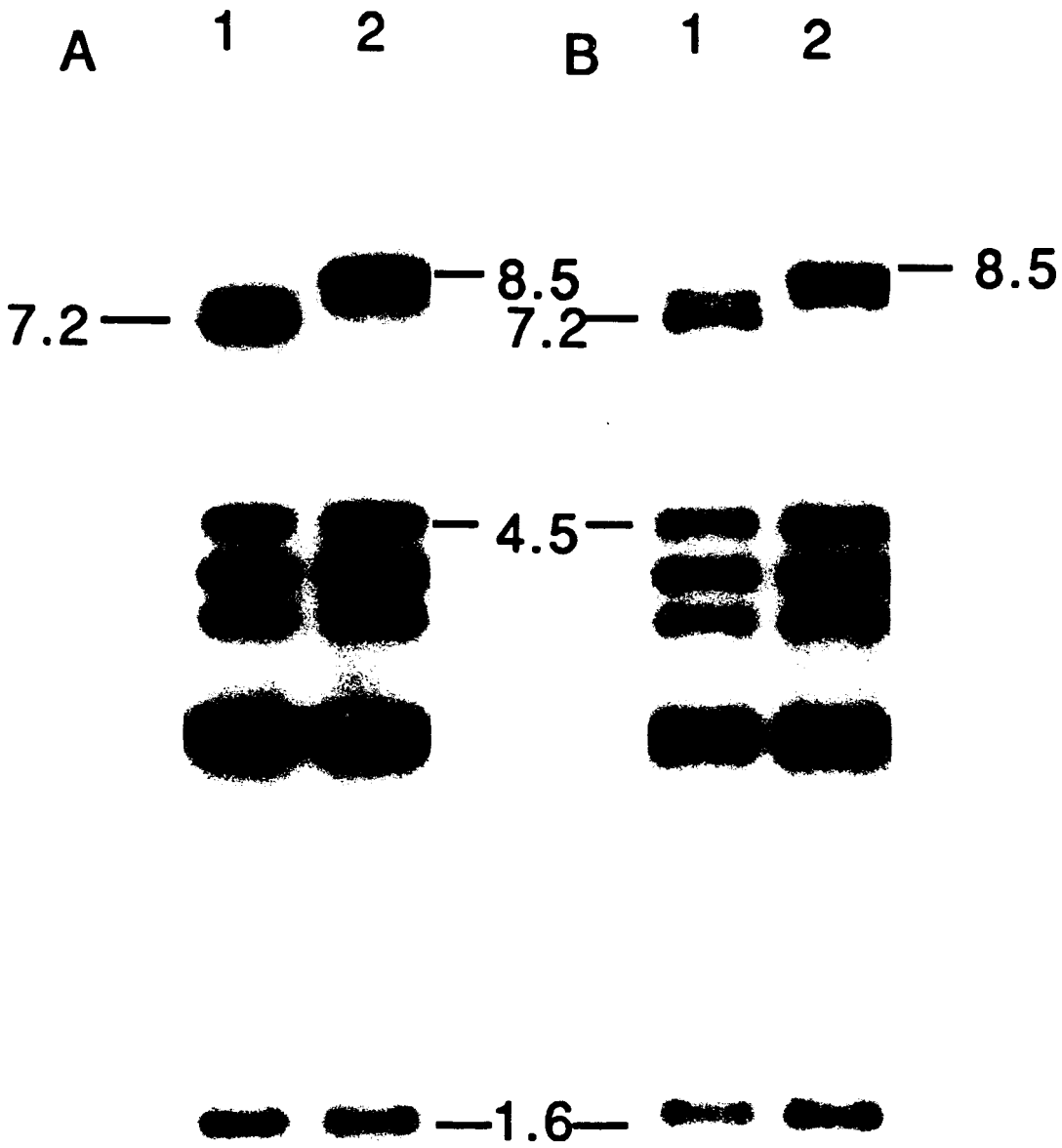

A
1
2
B
1
2
8.5
7.2
7.2
8.5
4.5
1.6

Fig. 3. Mapping of IS*1138* in pBBK85. A. Ethidium bromide stained agarose gel. B. Corresponding Southern blot probed with pBBK25. Lane 1, molecular weight markers consisting of bacteriophage 1 DNA digested with *Hin*dIII; lanes 2 through 4, pBBK85 digested with *Hin*dIII and *Sst*I (lane 2), *Hin*dIII, *Sst*I and *Hin*cII (lane 3), and *Hin*dIII and *Hin*cII (lane 4).

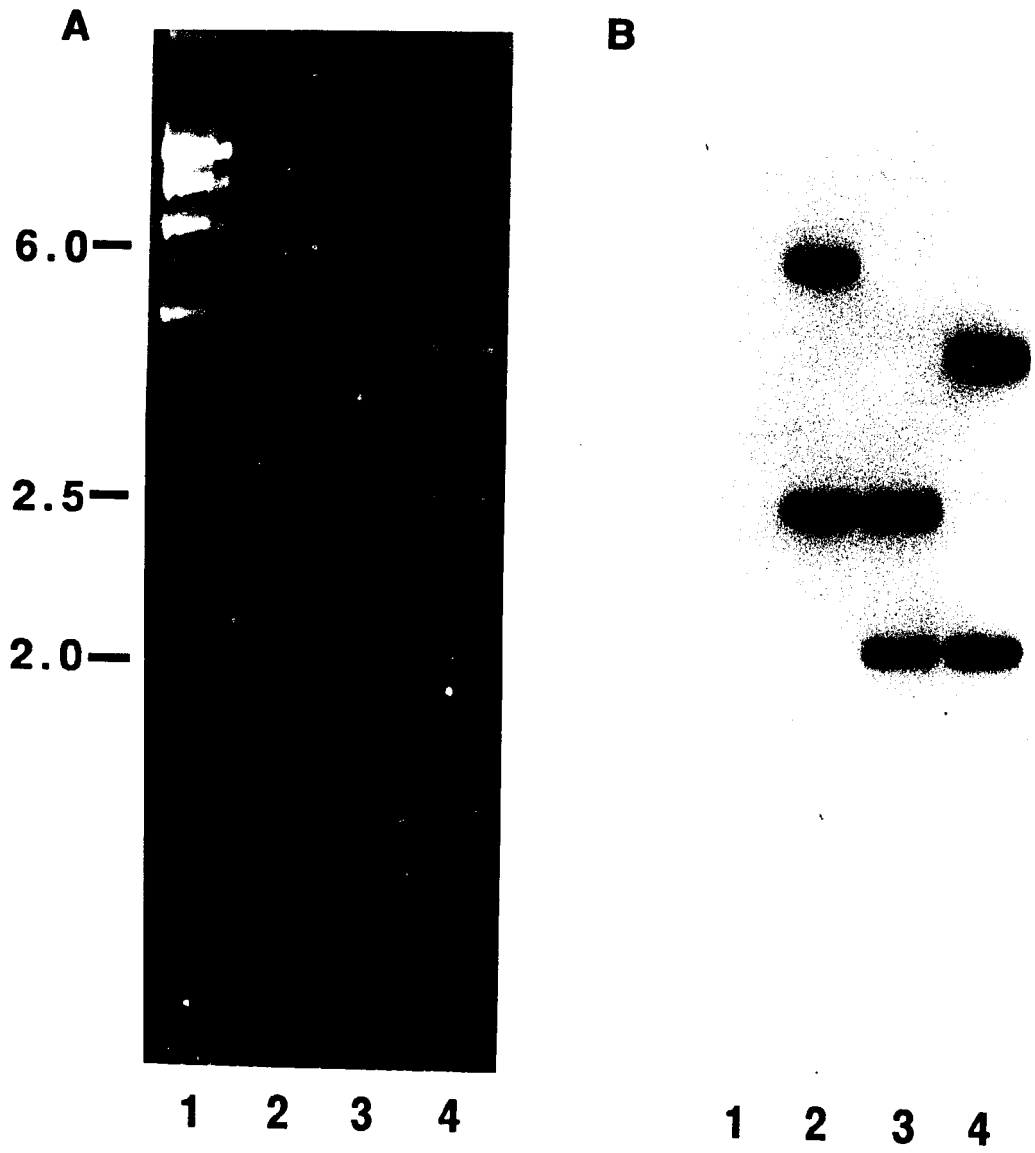

A
B
6.0
2.5
2.0
1 2 3 4
1 2 3 4

Fig. 4. Comparison of DNA from the variant strain KD735-26 (lane 3) to precursor strains KD735-16 (lane 2) and KD735-15 (lane 1) by Southern hybridization analysis. HindIII-SstI digested, chromosomal DNAs were analysed on an agarose gel, transferred to nitrocellulose, and probed with (A) pBBK25 and (B) pBBK12. Numbers in the margins refer to the size of fragments (in kb).

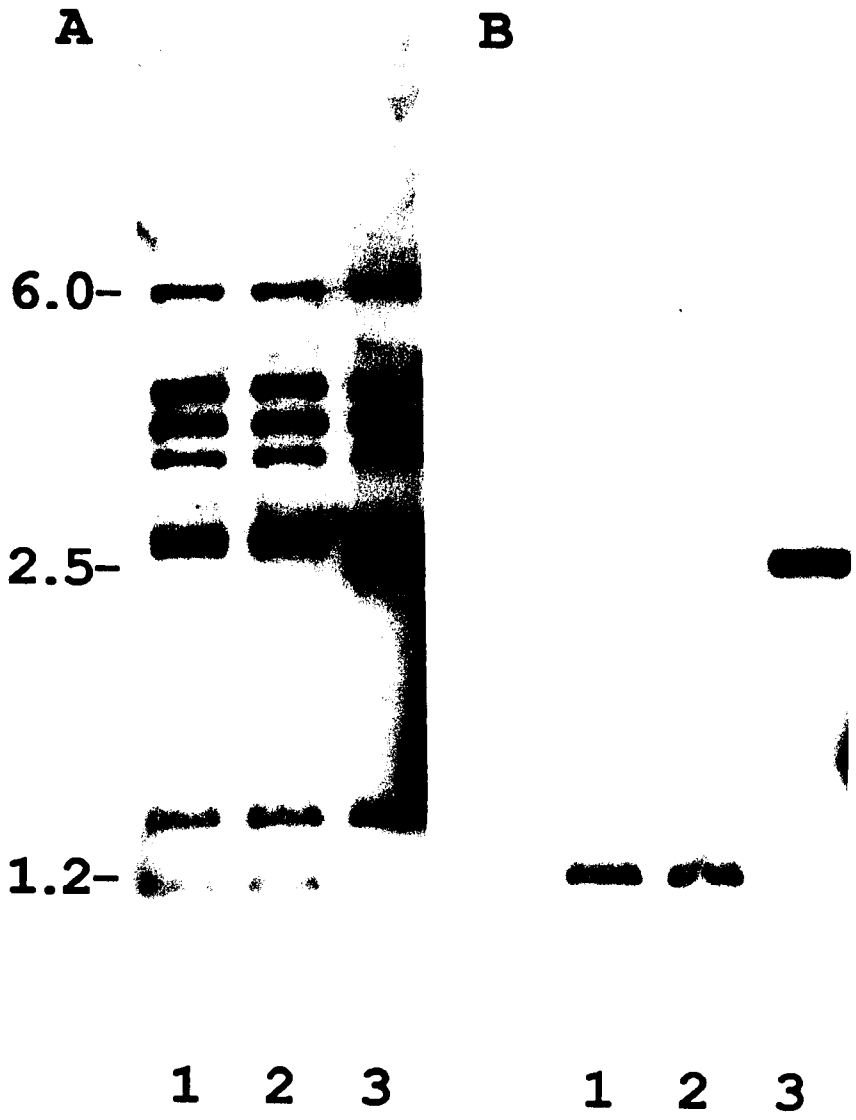

A
B
6.0-
2.5-
1.2-
1 2 3
1 2 3

Fig. 5. Complete nucleotide sequence of IS1138. The inverted repeats at the end of the element are emphasized with arrows on top of the sequence. The deduced amino acids of the putative transposase are indicated under the sequence by single letter codes and a potential RBS, AGGAG, is overlined on the sequence. Underlined positions represent sites where the sequence of IS1138 in pBBK25 is different from the other copy of IS1138 in pBBK20. These differenences are: nucleotide position 179 (G to T), 539 (A to T), and 1065 (A to G). Numbers to the right indicate IS1138 coordinates.

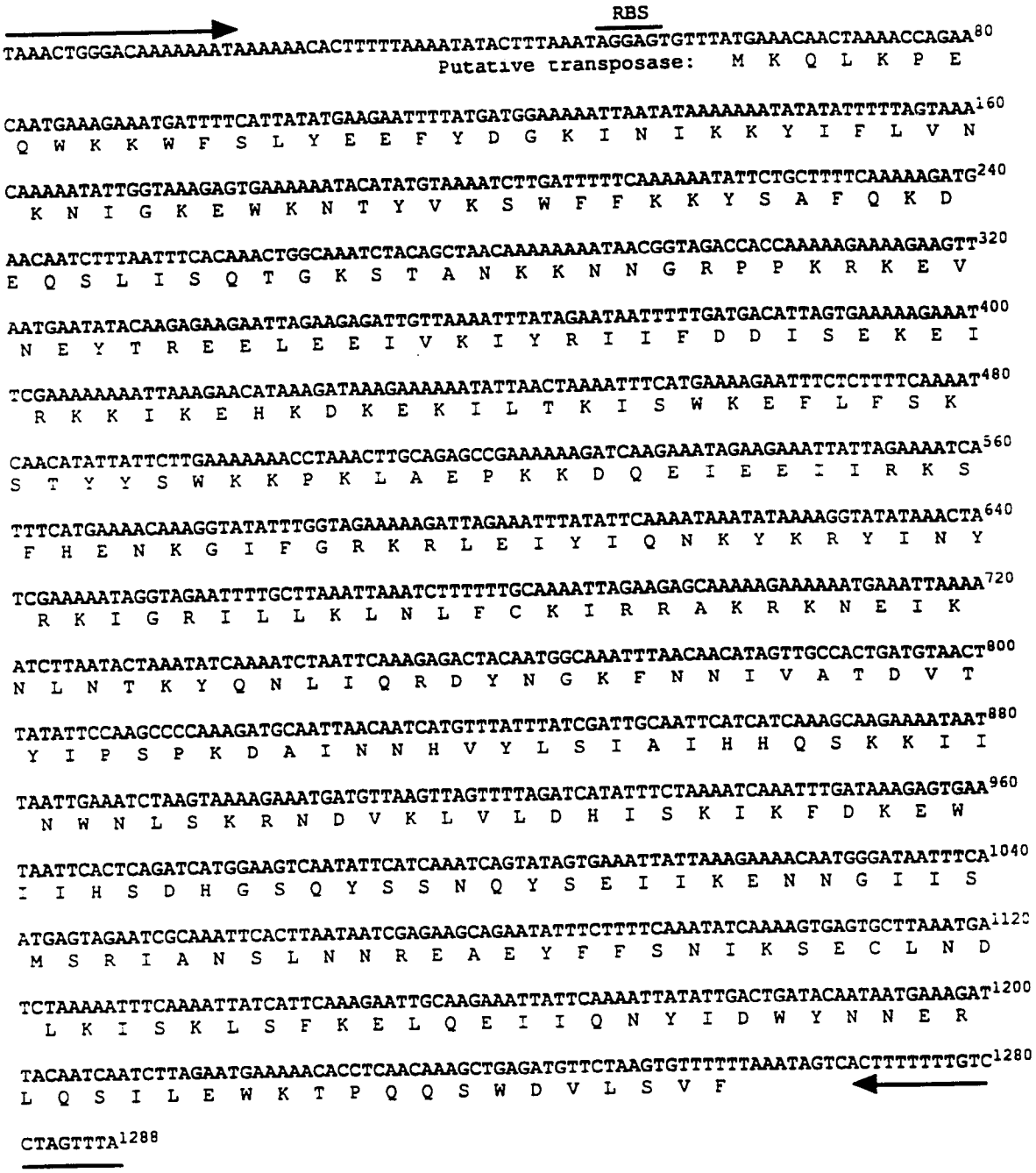

RBS
TAAACTGGGACAAAAAAATAAAAAACACTTTTTAAAATATACTTTAAATAGGAGTGTTTATGAAACAACTAAAACCAGAA 80
Putative transposase: M K Q L K P E
CAATGAAAGAAATGATTTTCATTATATGAAGAATTTTATGATGGAAAAATTAATATAAAAAAATATATATTTTTAGTAAA 160
Q W K K W F S L Y E E F Y D G K I N I K K Y I F L V N
CAAAAATATTGGTAAAGAGTGAAAAAATACATATGTAAAATCTTGATTTTTCAAAAAATATTCTGCTTTTCAAAAAGATG 240
K N I G K E W K N T Y V K S W F F K K Y S A F Q K D
AACAATCTTTAATTTCACAAACTGGCAAATCTACAGCTAACAAAAAAAATAACGGTAGACCACCAAAAAGAAAAGAAGTT 320
E Q S L I S Q T G K S T A N K K N N G R P P K R K E V
AATGAATATACAAGAGAAGAATTAGAAGAGATTGTTAAAATTTATAGAATAATTTTTGATGACATTAGTGAAAAAGAAAT 400
N E Y T R E E L E E I V K I Y R I I F D D I S E K E I
TCGAAAAAAAATTAAAGAACATAAAGATAAAGAAAAAATATTAACTAAAATTTCATGAAAAGAATTTCTCTTTTCAAAAT 480
R K K I K E H K D K E K I L T K I S W K E F L F S K
CAACATATTATTCTTGAAAAAAACCTAAACTTGCAGAGCCGAAAAAAGATCAAGAAATAGAAGAAATTATTAGAAAATCA 560
S T Y Y S W K K P K L A E P K K D Q E I E E I I R K S
TTTCATGAAAACAAAGGTATATTTGGTAGAAAAAGATTAGAAATTTATATTCAAAATAAATATAAAAGGTATATAAACTA 640
F H E N K G I F G R K R L E I Y I Q N K Y K R Y I N Y
TCGAAAAATAGGTAGAATTTTGCTTAAATTAAATCTTTTTTGCAAAATTAGAAGAGCAAAAAGAAAAAATGAAATTAAAA 720
R K I G R I L L K L N L F C K I R R A K R K N E I K
ATCTTAATACTAAATATCAAAATCTAATTCAAAGAGACTACAATGGCAAATTTAACAACATAGTTGCCACTGATGTAACT 800
N L N T K Y Q N L I Q R D Y N G K F N N I V A T D V T
TATATTCCAAGCCCCAAAGATGCAATTAACAATCATGTTTATTTATCGATTGCAATTCATCATCAAAGCAAGAAAATAAT 880
Y I P S P K D A I N N H V Y L S I A I H H Q S K K I I
TAATTGAAATCTAAGTAAAAGAAATGATGTTAAGTTAGTTTTAGATCATATTTCTAAAATCAAATTTGATAAAGAGTGAA 960
N W N L S K R N D V K L V L D H I S K I K F D K E W
TAATTCACTCAGATCATGGAAGTCAATATTCATCAAATCAGTATAGTGAAATTATTAAAGAAAACAATGGGATAATTTCA 1040
I I H S D H G S Q Y S S N Q Y S E I I K E N N G I I S
ATGAGTAGAATCGCAAATTCACTTAATAATCGAGAAGCAGAATATTTCTTTTCAAATATCAAAAGTGAGTGCTTAAATGA 1120
M S R I A N S L N N R E A E Y F F S N I K S E C L N D
TCTAAAAATTTCAAAATTATCATTCAAAGAATTGCAAGAAATTATTCAAAATTATATTGACTGATACAATAATGAAAGAT 1200
L K I S K L S F K E L Q E I I Q N Y I D W Y N N E R
TACAATCAATCTTAGAATGAAAAACACCTCAACAAAGCTGAGATGTTCTAAGTGTTTTTTAAATAGTCACTTTTTTTGTC 1280
L Q S I L E W K T P Q Q S W D V L S V F
CTAGTTTA 1288

Fig. 6. Alignment of the putative transposase of IS1138 with those of IS150, IS3 and IS911. Alignments were generated and displayed using the GCG programs PILEUP and PRETTYBOX. The boxed regions display amino acid residues conserved among the various transposases. The consensus sequence is written in bold letters below the alignment. Gaps introduced in the sequence to display alignment are indicated by dots. The positions of the amino acids residues of each transposase are shown by the numbers on the right.

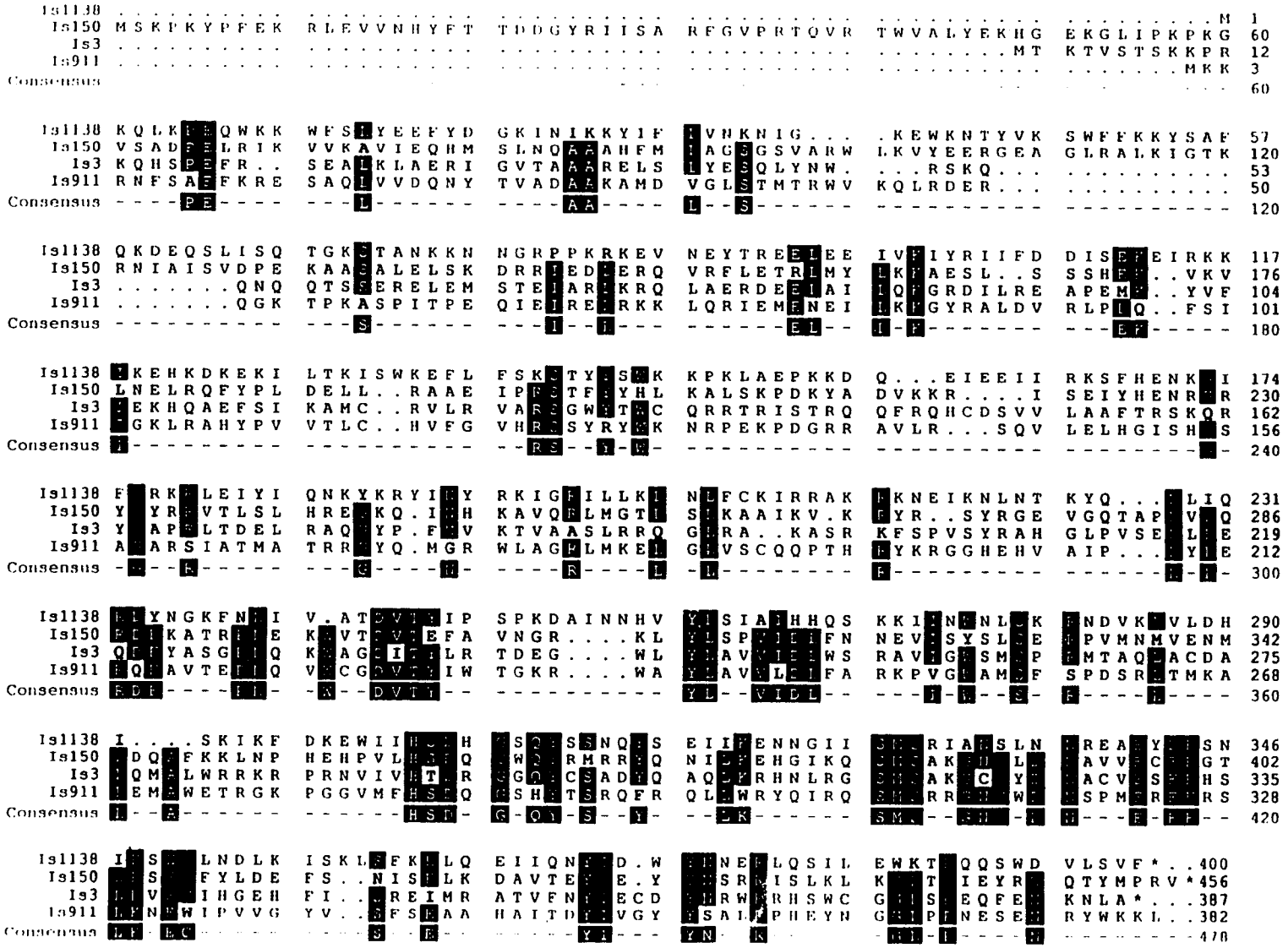
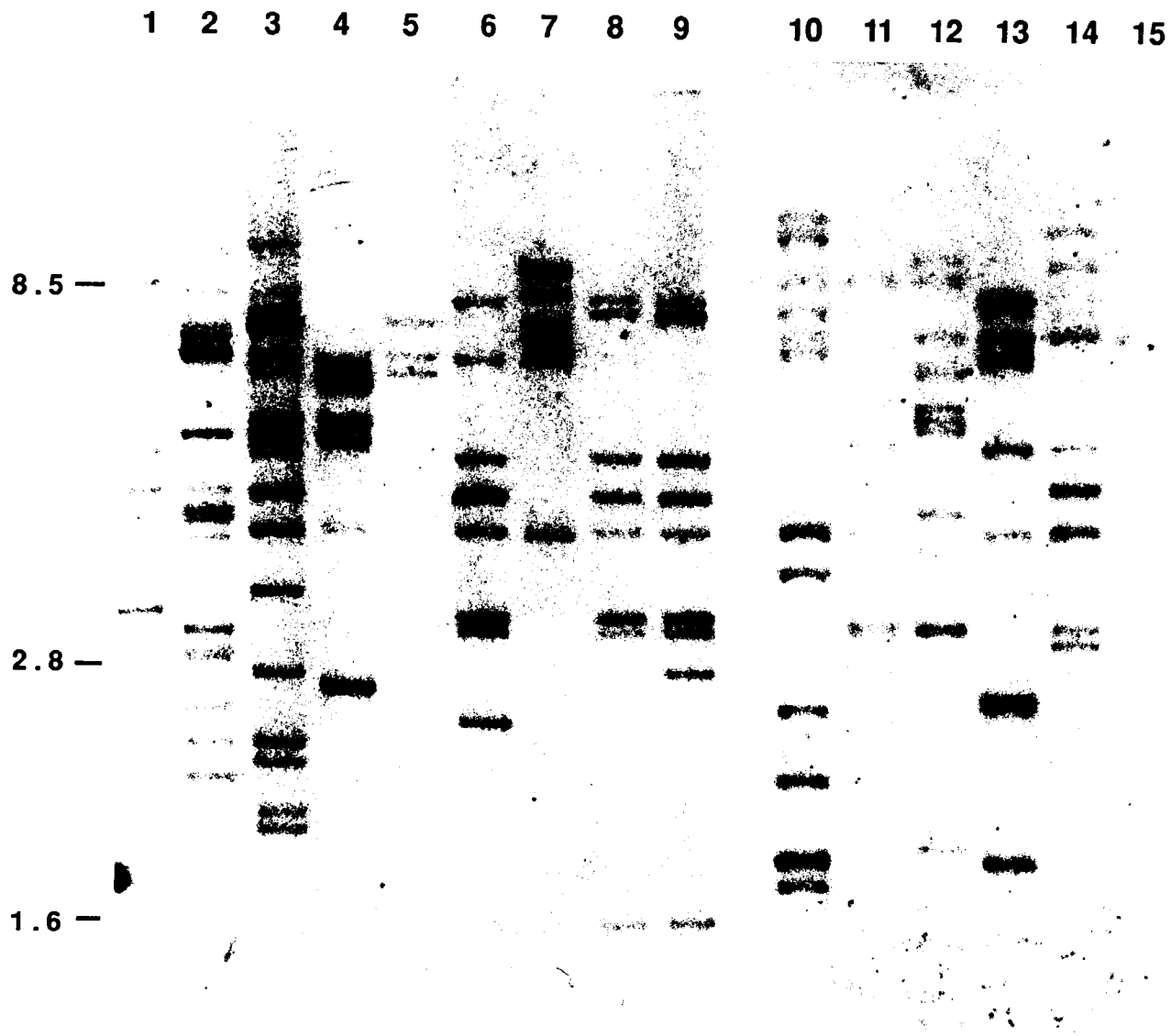

Is1138
Is150
Is3
Is911
Consensus

Fig. 7. Southern hybridization analysis to detect the presence of IS1138-like sequences among various M. pulmonis strains. HindIII-digested chromosomal DNAs were analysed on an agarose gel, transferred to nitrocellulose and probed with pBBK25. Analysed in lanes 1-15 are DNAs from strains KD735-26, Ogata T, 7MCA1, PG34 (ASH), M1, UAB 8145D, Nelson C, KD735-16, KD735-15-52, Ginsburg, Nelson A, UAB 5782C, JB, Negroni and 66. Numbers on the side of the margins indicate the size of the selected fragments in kb.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
8.5
2.8
1.6

HIGH-FREQUENCY GENETIC RECOMBINATION IN *MYCOPLASMA PULMONIS* INVOLVING DNA FRAGMENTS ENCODING EPITOPES OF THE V-1 ANTIGEN

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Introduction

Mycoplasma pulmonis causes chronic respiratory and reproductive tract diseases in laboratory rodents (Cassell et al., 1986). The chronicity of M. pulmonis infections may be related to high-frequency phenotypic and antigenic changes that occur during growth of the organism. Several examples of high-frequency phenotypic switching in M. pulmonis correlate with structural changes in the major surface antigen V-1 (reviewed in Dybvig, 1990). Some of these examples are adherence of the organisms to plastic surfaces and hemadsorption to red blood cells (Watson et al., 1990), growth on agar and in broth (Dybvig et al., 1989), and susceptibility to infection with mycoplasma virus P1 (Dybvig et al., 1988).

Dybvig et al. (1988) have described that changes in the susceptibility of M. pulmonis to P1 virus are affected by changes in the structure of the V-1 surface antigen. On examining whether chromosomal DNA rearrangements correlated with changes in P1 virus susceptibility, we found that a recombinative event generating a variant 9.5 kb fragment from strain KD735-16 correlated to changes in P1 virus susceptibility (Bhugra and Dybvig, 1992). The precursors to the 9.5 kb fragment were two fragments of 5.7 and 4.9 kb. All of the strains examined that contained the parental 5.7 kb and the 4.9 kb fragments, identified by probing with pBBK95, were sensitive to P1 virus infection. P1 virus-resistant strains lacked the 5.7 kb and the 4.9 kb fragments and

instead had a higher molecular weight fragment, similar in size to the 9.5 kb fragment of strain KD735-16.

In an effort to elucidate the mechanism by which the 9.5 kb variant fragment was generated, the 4.9 kb precursor fragment was cloned. However, despite repeated attempts, efforts to clone the 5.7 kb precursor fragment were not successful and a clear understanding of how the 9.5 kb fragment was generated is not yet available.

While several examples of phenotypic switching have been observed that correlate with structural variation in the surface antigen, molecular mechanisms underlying V-1 antigenic or structural changes have not been studied. Because P1 virus resistance has previously been correlated with structural changes in V-1, the cloned 4.9 kb and 9.5 kb fragments were examined for their ability to express V-1 epitopes in Escherichia coli. The 4.9 kb fragment but not the 9.5 kb fragment expressed these epitopes, suggesting that V-1 gene expression may be altered by gene rearrangement.

Results

As described previously in the first chapter of Results section (Bhugra and Dybvig, 1992), strain KD735-16, a subclone of strain KD735, has a variant DNA fragment of about 9.5 kb. Plasmid pBBK95 contains the cloned 9.5 kb variant fragment from this strain. On Southern blots, a pBBK95 probe hybridized to the 9.5 kb fragment from KD735-16 and to two fragments of 5.7 kb and 4.9 kb from the parent strain, KD735. The probe also hybridized to a 2.7 kb fragment common to all

of the strains. Each of the fragments described above was identified as product of restriction endonuclease HindIII digestion. Based on these observations, one hypothesis is that the 9.5 kb variant fragment was generated by the joining of two adjacent parental fragments of 4.9 kb and the 5.7 kb (Fig. 1). This would result in the deletion of about 1.1 kb of parental sequences with the loss of an intervening HindIII site.

Cloning of the parental fragments

To elucidate the mechanism by which the 9.5 kb fragment was generated, cloning of the 4.9 kb and the 5.7 kb precursor fragments was pursued. Despite repeated attempts, the 5.7 kb fragment was not cloned using the approach described in the Experimental Procedures. Maybe, a gene product encoded by the 5.7 kb DNA fragment was deleterious to Escherichia coli. The 4.9 kb fragment was successfully cloned and is contained in the recombinant plasmid pBBK49.

Restriction map and nucleotide sequence analysis of pBBK49 and pBKK95

A restriction map of the cloned 9.5 kb fragment is provided in Fig. 2. Surprisingly, the ends of the fragment had nearly identical enzyme recognition sites for about 1.2 kb, in an inverted orientation. The presence of terminal inverted repeats was confirmed by determining the nucleotide sequence at each end using pUC/M13 universal primers. The terminal sequences were identical for over 150 bp.

The 4.9 and the 9.5 kb fragments were compared at the nucleotide level. First, the nucleotide sequences of the ends of the cloned 4.9 kb fragment were also determined. The sequence at one end was identical to the terminal sequences of the 9.5 kb fragment. However, the sequence at the other end of the 4.9 kb fragment was different from those of pBBK95.

Sequencing on this end of the insert in pBBK49 was continued. In addition to examining pBBK49, each sequential primer derived from sequences of the 4.9 kb insert was also employed with pBBK95, as the template, to determine if the 9.5 kb insert contained a primer binding domain. The initial primers (Fig. 3, primers 1 through 3) applied on pBBK49 as the template did not bind to the 9.5 kb insert fragment in pBBK95, indicating that sequences for about 1.0 kb at this end on the 4.9 kb fragment were not present in the variant 9.5 kb fragment. However, the next primer (primer 4, after 1030 bases from the end of 4.9 kb sequence) derived from sequences on the 4.9 kb fragment bound to the pBBK95 template and yielded identical sequence data as a pBBK49 template (Fig. 3, Primer 4). Next, a primer complementary to both the 4.9 and 9.5 kb fragments was designed (primer 5) to analyze the complementary strand of both the templates and precisely define the recombination junction on the parental and the progeny fragments.

pBBK49 and pBBK95 contain a periodic sequence

Further sequencing of the 4.9 kb insert in pBBK49 revealed a

highly repetitive sequence consisting of a 39 base pair sequence repeated about 40 times (see Fig. 3). This periodic sequence was also found in pBBK95. The consensus for this 39 base pair sequence is shown in Fig. 4. Single nucleotide changes in the sequence of this repetitive 39-mer have been observed among its various copies. If this repetitive element is within a coding region, the resulting protein would have a periodic 13 amino acid structure.

Watson et al. (1988) have shown that V-1 protein, the major surface antigen of M. pulmonis, has an unusual ladder like pattern on immunoblots of one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a strikingly symmetrical distribution of molecular weight and charge. It has been suggested that the multiple banding pattern of the V-1 molecule may be due to the presence of increasing quantities of a repeating unit of small molecular weight, with the ladder pattern representing different synthetic stages of the whole molecule (Watson et al., 1988; 1989). The spacing of the V-1 ladder is consistent with differences of about 13 amino acids (Dybvig, unpublished data). We, therefore examined pBBK49 and pBBK95 to determine whether they expressed V-1 epitopes in E. coli.

pBBK49 expresses V-1 epitopes in E. coli

Total protein was isolated from E. coli cells containing pBBK49 and pBBK95 and analyzed on immunoblots as described in Experimental Procedures. A low level of expression of recombinant protein could be detected in pBBK49-containing

cells using polyclonal antisera to M. pulmonis membranes. Low expression of protein could also be detected using three monoclonal antibodies to V-1 antigen, 7.1-2, 67.2-1, and 85.8-1 (Fig. 5). However, protein extracts from cells containing pBBK95 did not show any reaction to the monoclonal antibodies or the polyclonal antisera. The possibility of enhancement of expression of V-1 epitopes from pBBK49-containing cells by induction with IPTG was examined. There was no noticeable difference (increase) in the V-1 expression levels among cells grown with and without IPTG.

Open reading frames (ORFs) are present in four out of six frames within the 1.6 kb domain comprised of the repeating 39-mer sequence. However, none of these ORFs have convincing ribosome binding site (RBS) preceding potential initiation codons. All of these ORFs end within 100 bps of the 39-mer element.

Discussion

Antigenic variation has been identified in several bacterial systems (DiRita and Mekalanos, 1989; Seifert and So, 1988). In the case of pili from Neisseria gonorrhoea, phase and antigenic variation involves rearrangements within multigene families (Bergstorm et al., 1986; Blake and Gotschlich, 1983). DNA rearrangements can also cause antigenic variations in the surface proteins of Borrelia hermsii, an arthropod-borne pathogen that causes relapsing fever in humans (Barbour et al., 1991). In these organisms, recombination introduces new DNA sequences within the structural gene for the surface

protein present in the expression locus by replacing sequences with partially homologous sequences from other silent copy genes.

It has been suggested that differences exist between regulatory signals of mycoplasmas and eubacteria since different translation initiation sites may be used for expression of gene products in E. coli compared to in mycoplasmas (Notarnicola et al., 1990). Therefore, the low level of expression of a protein epitope in E. coli may result from promiscuous transcriptional and translational initiation sites, not present in mycoplasmas. It is probable that several copies of the V-1 gene might exist on the M. pulmonis chromosome. pBBK49 and pBBK95 may contain either complete or incomplete V-1 genes that may be in either an expression locus or a silent locus in M. pulmonis.

DNA rearrangements are used by many microorganisms to control gene expression (Borst and Greaves, 1987).In addition to the N. gonorrhoeae and B. hermsii (see above), other examples of phenotypic switching mediated by gene rearrangements are the flagellar phase variation in Salmonella typhimurium, mating type switch of yeast, and multiphasic antigenic variation of surface glycoproteins in African trypanosomes. In all of these organisms, a complete or near-complete gene copy undergoes recombination resulting in altered gene expression. S. typhimurium activates one or another of two flagellin genes through promoter addition, but unlike B. hermsii, S. typhimurium achieves this through

invertible elements (Glasgow et al., 1989). In Saccharomyces cerevisiae, a switch in mating type is the result of a duplicative gene-conversion event, which provides for expression site control (Klar, 1989). A similar recombination event occurs for the VSG gene activation In Trypanosoma brucei (Burman et al., 1990).

In several of these systems, it has been suggested that gene rearrangements are promoted by recombination between homologous sequences. These sequences may involve conserved, semi-variable, or hypervariable coding regions (Borst and Greaves, 1987; Burman et al., 1990; Seifert and So, 1988).

Experimental Procedures

Mycoplasmas

M. pulmonis strains used in this study were subclones of strain KD735 (Dybvig et al., 1989), a derivative of UAB 6510 that is susceptible to mycoplasma virus P1 (Dybvig et al., 1988). M. pulmonis was grown in Hayflick broth at 37°C as previously described (Dybvig et al., 1988).

Cloning strategy

For cloning, DEAE cellulose paper was used to recover the DNA fragments by electrophoresis from agarose gels. The gel eluted fragments were ligated into the HindIII site of plasmid pUC18 and used to transform E. coli strain JM103 (Messing et al., 1981).

DNA manipulations

Plasmid DNA was isolated from E. coli using the alkaline lysis method (Sambrook et al., 1989). The inserts within the

plasmids pBBK95 and pBBK49 were the 9.5 kb fragment from strain KD735-16 and the 4.9 kb fragment from strain KD735-15, respectively, cloned in the HindIII site of plasmid vector pUC18.

DNA sequencing

DNA sequencing was done on both strands of the plasmid DNA template by dideoxy sequencing using Sequenase 2.0 kit from United States Biochemical Corp. (Cleveland, OH). The primers for sequencing were made at the Oligonucleotide Synthesis Core Facility at the University of Alabama at Birmingham. Initial sequencing of the ends of the template was obtained using the universal pUC primers. Additional primers were made about every 200 bases sequentially along the template.

Electrophoretic analysis

Organisms were pelleted for 15 min (15,000 g) at 4°C, washed three times in phosphate buffered saline (PBS), and the final pellet resuspended in SDS-dissociation buffer. The samples were either directly used for electrophoretic analysis or stored at -20° C. Samples used for one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were utilized as basically described previously (Watson et al., 1989). In brief, ten to twenty micrograms of organism protein were loaded per well of a SDS-PAGE gel. Samples were concentrated on 4% stacking gels and separated on 10% resolving gels. Separated proteins were either visualized with Coomasie blue stain (O'Farrell, 1975) or immunoblotted as described below.

Immunoblotting

Separated proteins were transferred to nitrocellulose sheets (Biorad) and the non-specific binding sites were blocked by overnight incubation at room temperature in Blocking buffer which contains PBS with 10% fetal bovine serum and 0.05% polyoxyethylene sorbitan monolaurate (Tween 20, Sigma). All immunological reactions were performed at RT with anti M. pulmonis antisera or monoclonal antibodies specific for V-1 protein. Bound antibody was detected by reaction with 4-chloro-1-naphthol (HRP color development reagent, Biorad).

Acknowledgements

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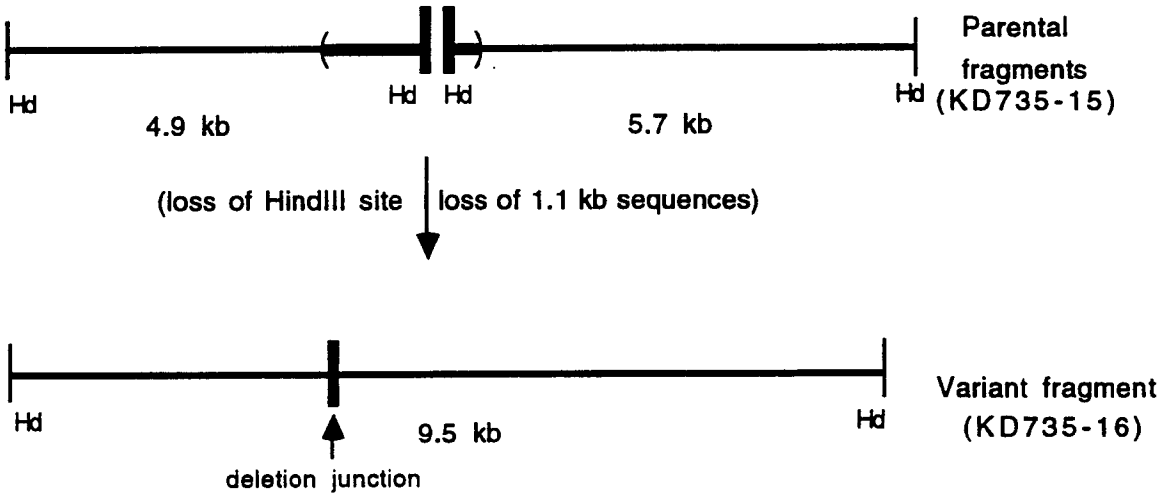


Fig. 1. Deletion model for the formation of the variable 9.5 kb fragment. Hypothesis for the formation of the variable 9.5 kb fragment in strain KD735-16 from the precursor 4.9 kb and 5.7 kb fragments in the parent strain KD735-15. Abbreviations used: Hd for restriction enzyme HindIII.

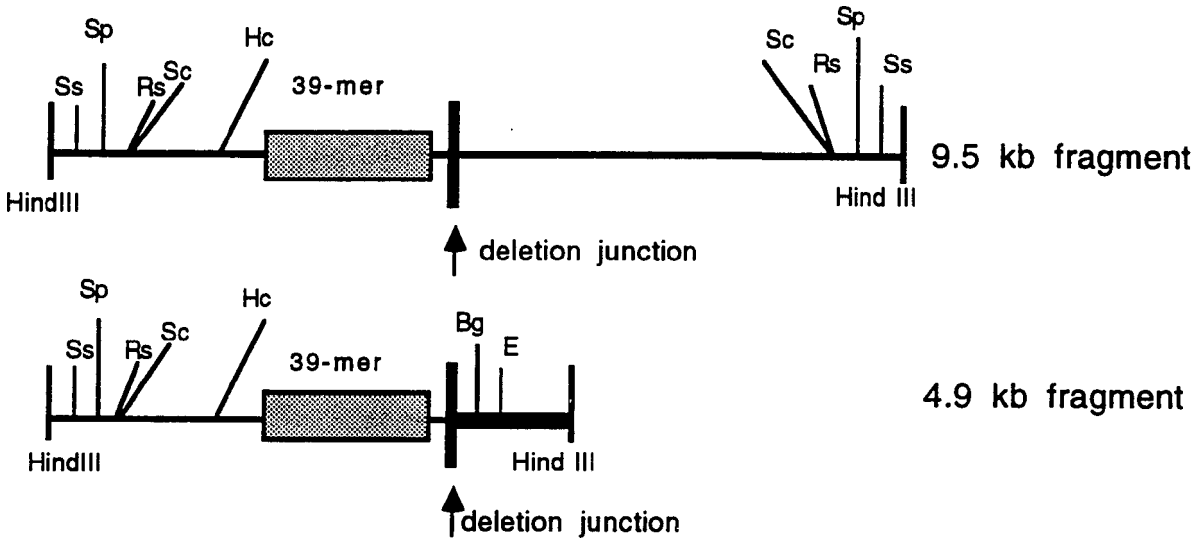


Fig. 2. Comparison of the restriction maps of the variant 9.5 kb fragment with the 4.9 kb precursor fragment. Abbreviations used for restriction enzymes are: Ss for Sst1, Sp for Ssp1, Rs for Rsa1, Sc for Sca1, Hc for HincII, Bg for BglII and E for EcoR1.

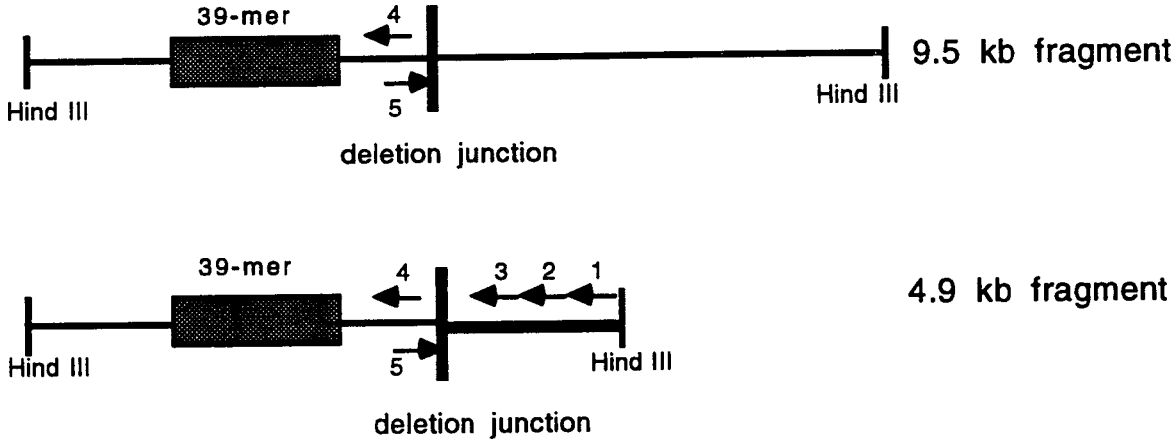


Fig. 3. Schematics of sequencing strategy. Schematic diagram showing the strategy for sequencing the 9.5 kb fragment and the 4.9 kb fragment in plasmid templates pBBK95 and pBBK49, respectively. Arrows on the sequence denote primers used for sequencing, boxed region displays the position of the 39-mer periodic repeat and the vertical line denotes the deletion junction.

39-mer repeat sequence

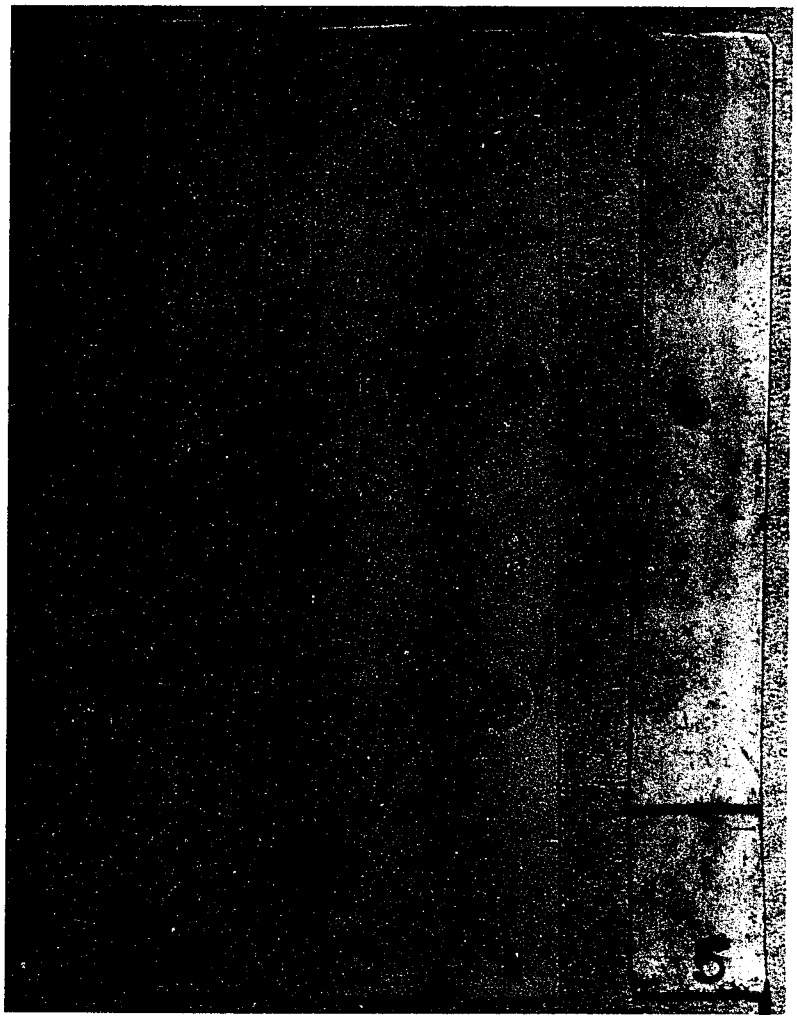
CCGGA GATGC AAATG CAGGA GAGAT GGGCA AAAAT AATG

Alternative 39-mer repeat sequence

CCGGA GATGC AAATG CAGGA GACAT GGGCA AAAAT AATG

Fig. 4. Consensus sequence for the 39-mer repeat. Consensus sequence for the 39-mer repeated sequence present in the 4.9 kb (from strain KD735-15) and the 9.5 kb fragments (from strain KD735-16). Also shown is the alternative sequence of the 39-mer repeat, with one nucleotide change from a G to a C.

Fig. 5. Expression of V-1 epitopes from E. coli cells containing pBBK49. Shown in the immunoblot in lanes 1 to 4 are reactions with hyperimmune antisera to M. pulmonis (lane 1) and anti-V-1 monoclonal antibodies 85.8-1, 67.2-1 and 7.1-2, respectively. Lane 5 contains E. coli cells only (control) with no insert reacted to hyperimmune antisera to M. pulmonis.


5

DISCUSSION

A mycoplasmal cell, being the smallest of the self-replicating prokaryotes (Razin, 1985), probably makes the most of its coding capacity by rapidly varying its chromosome. This would provide mycoplasmas the potential to enhance their genetic capacity by creating new coding regions. Because mycoplasmas are apparently undergoing evolution at a rate more rapid than most bacteria (Weisburg et al., 1989), it has been suggested that the small mycoplasma genome may be able to withstand a higher mutation rate (Woese, 1987). It is also apparent from numerous examples (see Introduction) that the surface properties of mycoplasmas are constantly changed as a result of genetic variation. The mechanisms of these high-frequency genetic variations in mycoplasmas are unknown, though their outcome may dictate the interaction of mycoplasmas with their hosts. To gain insight into the unusual evolution of mycoplasmas, we chose the rodent pathogen, Mycoplasma pulmonis, as a model system for studying mechanisms that generate variablity in these organisms.

To examine genetic variation in the M. pulmonis genome, DNA from subclones of one single M. pulmonis strain was examined. Restriction fragment polymorphisms (RFPs) were identified among first generation subclones by comparing the

ethidium bromide-stained DNA banding patterns of the subclones to that of the parent strain, and additional RFPs were similarly identified in second and third generation subclones. It was shown that M. pulmonis has a dynamic chromosome that undergoes rearrangements at a high frequency.

About 40% of the subclones, that had been propagated for about 46 generations, were identified as containing RFPs. Variants arose at a rate of about 10^{-2} to 10^{-3} variants per CFU per generation, making the mycoplasmal chromosome one of the most variable genomes known. Some of the variant fragments associated with the RFPs were cloned and used to probe Southern blots. The results indicated that RFPs arose via insertions, deletions or other rearrangements in the chromosome and not as a result of point mutations.

Analysis of the RFPs indicated that they are generated by multiple, unrelated phenomena. Some of the RFPs had phenotypic differences, which has led to the identification of specific DNA rearrangements that correlate with P1 virus susceptibility and V-1 antigen structure. The mechanisms that generate these RFPs are not understood. One possibility is genetic recombination involving deletion of sequences from parental DNA fragments, as is discussed in the third chapter in the Results section. Not all of the DNA rearrangements that were analyzed are related to phenotypic switching. Another RFP apparently resulted from insertion of a copy of a repetitive element into a target sequence. The nucleotide sequence of two copies of the repetitive element isolated

from different regions of the chromosome has been determined, revealing that this element is an insertion sequence (IS) element related to the IS3 family of Escherichia coli. Other rearrangements in the M. pulmonis chromosome may be due to DNA inversions that generate additional RFPs at a high frequency (Dybvig, unpublished data).

The high rate of variation observed in mycoplasmas rivals the extreme examples of genetic instability that have been described for halobacteria and streptomyces (Pfeifer and Blaseio, 1989; Sapienza et al., 1982; Leblond et al., 1989; 1990). DNA rearrangements that correlate with phenotypic switching, as seen in M. pulmonis, are common in eubacterial systems (Borst and Greaves, 1987; Finlay and Falkow, 1989; Seifert and So, 1988). In fact, the evolutionary success of a number of bacterial systems can be credited to their ability to alter gene expression and vary surface antigens. Mechanisms for these variations are diverse, but several common themes include DNA inversions, gene conversions, duplications or deletions of tandem homologous blocks of DNA, and movement of transposable elements (Barbour et al., 1991; Borst and Greaves, 1987; Hollingshead et al., 1987; Leblond et al., 1990; Seifert and So, 1988). Examples of each of these mechanisms are discussed below.

Frequent DNA rearrrangements catalyzed by transposable elements are thought to result in the unusually high rate of phenotypic variation in Halobacterium halobium (DasSarma et al., 1988). These rearrangements yield readily identifiable

and unstable mutants at a frequency of 10^{-4} to 10^{-2} mutants per CFU (Sapienza et al., 1982). The genome of H. halobium contains many families of repeated sequences, dispersed on both chromosome and plasmids, and most if not all of these are involved in spontaneous rearrangements.

Phenotypic instabilities observed in Streptomyces species are also frequently associated with genomic instabilities (Leblond et al., 1989). The majority of the instabilities are the result of extensive chromosomal deletions which, interestingly, are frequently accompanied by intense DNA amplifications (Birch et al., 1990). A similar phenomenon involving loss of nodulation ability in the soil bacterium, Rhizobium phaseoli, has been described (Soberon-Chavez et al., 1991).

Recombination plays an important role in creating genetic diversity for surface proteins of several bacterial species. Size variation in M protein, the antiphagocytic surface structure of Group A streptococci, results from intragenic recombination between highly homologous, tandem elements within the protein coding sequence (Hollingshead et al., 1987). This also has the potential to generate antigenic diversity in this protein (Jones et al., 1988).

There probably are several pathways of DNA rearrangements in N. gonorrhoeae that are responsible for switches in pilin gene expression. Pilin gene variation in N. gonorrhoeae reflects extensive recombination involving the pilus expression locus (pilE), much of this has been

attributed to the introduction of silent, partial pilin gene (pilS) sequence into pilE (Swanson and Koomey, 1989). Non-reciprocal gene conversion (Segal et al., 1986), transformation-mediated recombination (Gibbs et al., 1989; Seifert et al., 1988; Seifert and So, 1988) and repair-driven recombination (Hill et al., 1990) are some of the mechanisms proposed for gonoccocal pilus variation. The DNA repair-driven model is particularly appealing as a principal mechanism underlying both pilus phase and antigenic variation. However, recent evidence suggests that hypervariant pilin sequences are subject to horizontal exchange and interstrain recombination (Haas et al., 1992).

Multiphasic antigenic variation has been described in eukaryotes as well as prokaryotes. Borrelia hermsii, the cause of relapsing fever, uses a mechanism similar to N. gonorrhoeae to express variable major proteins (Vmps) and thereby undergo antigenic variation. Through recombination between linear plasmids a formerly silent vmp gene replaces another vmp gene at the telomeric expression locus downstream of a promoter causing an antigenic switch (Barbour et al., 1991). The mechanism of gene activation by recombination of silent genes into an expression site is also known in eukaryotes. The switching of the coat composition of the African trypanosome, Trypanosoma brucei, is accompanied by gene rearrangements, occurring at a rate of 10^{-7} to 10^{-6} per trypanosome division, by a mechanism strikingly similar to B. hermsii antigenic variation (Borst and Greaves, 1987; Burman

et al., 1990). A binary switch exists in yeast, where the mating type varies by transposition into the MAT locus, by a process very similar to the one described for T. brucei (Borst and Greaves, 1987)

A number of site-specific recombination systems in prokaryotes have been described in which the inversion of a DNA segment is responsible for alternating expression of one or more genes (Plasterk and van de Putte, 1984). The classical example of the inversion of a DNA segment is provided by the phase variation of Salmonella, which can alternate between two types of flagellar proteins, H1 and H2. Switching requires a specific recombinase called Hin (Johnson and Simon, 1985). Other Hin-related inversion systems have been described, including Gin-mediated G-loop inversion in Mu and Cin-mediated C-loop inversion in bacteriophage P1 (Plasterk and van de Putte, 1984). The DNA inversion in these two systems determines expression of alternate tail fiber antigens and allows the phages to extend their host range. A rearrangement in E. coli involves an invertible 314-base-pair segment of DNA, immediately upstream of the fimbrial structural gene (Abraham et al., 1985). The inversion of this segment apparently results in the on-and-off activation of a promoter that controls the state of fimbrial expression.

The genetic basis for phenotypic switching in M. pulmonis is unknown, but DNA rearrangements are likely to be involved. Some chromosomal rearrangements correlated with changes in the susceptibility of the cells to mycoplasma

virus P1, an example of phenotypic switching involving changes in surface antigen structure (Dybvig et al., 1988). The strongest correlation was observed with DNA fragments of 6.3 and 5.9 kb, although DNA fragments of 5.7 kb and 4.9 kb were also involved. The 6.3, 5.9, 5.7 and 4.9 kb fragments did not cross-hybridize, suggesting that multiple genes may be involved in generating these rearrangements.

As described above, many examples exist where DNA rearrangements have been used successfully by pathogens to vary surface antigens. Chromosomal rearrangements may bring about change in surface properties of M. pulmonis allowing it to survive within multiple niches in the host, thus giving it an advantage in establishing infection. In M. pulmonis, DNA rearrangements that correlated to phenotypic switching involve several different variable DNA fragments, and hence appears to be multifactorial and quite complicated. One or more of the mechanisms used frequently by other bacterial systems is likely to be involved in the phenomenon described here. Phenotypic switching similar to that of M. pulmonis has also been described in other mycoplasma species (Olson et al., 1991; Rosengarten and Wise 1990; 1991; Watson et al., 1990a). Elucidation of the genetic basis of this phenomenon in M. pulmonis may provide insight into how mycoplasmas in general vary their phenotypic traits.

Dybvig et al. (1992) recently reported the isolation and cloning of the recA gene from M. pulmonis. This would enable the construction of mycoplasmal mutants deficient in

homologous recombination, and therefore allow the examination of the role of homologous recombination in high-frequency chromosomal and phenotypic variation in M. pulmonis.

The second paper in the Results section describes the identification and characterization of a repetitive element from M. pulmonis that has structural features of an insertion sequence element (IS element). The nucleotide sequence of the element, designated IS1138, and the target site into which it inserted were determined. This identified a 3 bp duplication of target sequences flanking IS1138 as a direct repeat. The RFP generated in the M. pulmonis chromosome due to insertion of a copy of the IS element are unrelated to the earlier described RFPs that correlate to phenotypic switching.

IS1138 is the first example of a mobile mycoplasmal element. Repetitive sequences that have features similar to prokaryotic IS elements have been described for M. fermentans and M. hyorhinis (Ferrell et al., 1989; Hu et al., 1990), but transposition events involving these elements have not been described. Therefore, IS1138 is the only element intrinsic and native to mycoplasmas described so far.

IS elements are discrete segments of DNA with a dynamic capability to transpose to numerous sites on bacterial plasmids and chromosomes. The inverted repeats at the termini of IS elements are essential for both transposition and cointegration (Galas and Chandler, 1989). In recent years interest in IS elements as agents of chromosomal rearrangements has been growing. It is well known that pairs

of elements can act to mobilize gentic functions lying between them (Iida et al., 1983). As regions of portable homology IS elements can also act as substrates for host recombinative pathways, giving rise to large scale deletions, duplications and inversions. Transpositions of IS elements is thought to be one of the major mechanisms by which some genomes (e.g., halobacteria) undergo rearrrangments (Sapienza et al., 1982; DasSarma, 1988). In addition, another fascinating aspect of bacterial IS elements is their capacity to modulate the expression of genes into which they insert or adjacent genes.

Numerous repetitive elements have been described in mycoplasmas (Ferrell et al., 1989; Mouches et al., 1983; Su et al., 1988; Wenzel et al., 1990). These may play a role in promoting mutational pathways, such as homologous recombination, transposition, and other chromosomal rearrangements. Recombination events catalyzed by these repetitive sequences would have an immense potential for increased mutation and phenotypic changes in these organisms. Some repetitive sequences may directly provide the organism with a selective advantage. Other elements may serve an evolutionary function in providing an increased potential for genetic diversity, thus favoring cell survival. Numerous examples of antigenic diversity have been described in mycoplasmas, some of which may be created by recombinational events mediated by these elements.

IS1138 is ubiqitous in M. pulmonis. It is present among distant and diverse strains of M. pulmonis, that are isolated from varied tissues of different hosts, representing isolates from several different geographical locations. This element, appears to be species specific in that it does not show significant hybridization with DNA from many different mycoplasmal species that have recently been reported to contain prokaryotic IS-like elements. Therefore, IS1138 as a species-specific probe (in PCR amplification) for diagnostics studies involving M. pulmonis infections in laboratory rodents has a tremendous potential. The currently available methods e.g., culturing or immunoblotting, are not highly species-specific or sensitive for detecting low-dose experimental infections of M. pulmonis in rodents (Davidson et al., 1981; Lindsey et al., 1986). Additionally, there is no sensitive method of confirming a mycoplasma-free colony of rodents. IS1138 as a probe, in a PCR amplification reaction, will be an advantageous and useful tool.

Integrative vectors have not been described in M. pulmonis so far, and IS1138 can be used for developing integrative vectors in M. pulmonis. Given its several copies in the M. pulmonis genome, it can insert into the chromosome by transposition or homologous recombination and thereby help in the advance in mycoplasmal genetics in several ways.

Regardless of the mechanism of variation that is being employed, a high rate of genetic recombination may provide the impetus for rapid mycoplasmal evolution. Because of the

high rate of genetic variation, mycoplasmal cultures must be viewed as being dynamic, in that subpopulations must always be present. As much of the documented examples of genetic variation affect mycoplasmal surface properties, these populations will significantly affect the ability of an organism to adapt to changing environments in the host. High-frequency genetic recombination may be one important mechanism for the chronic nature of most mycoplasmal infections.

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