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Origins of the Mycoplasmas: Sterol-Nonrequiring Mycoplasmas Evolved from Streptococci

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We report the establishment of a phylogenetic relationship between the sterol-nonrequiring mycoplasmas (*Acholeplasma* species) and streptococci. Three specific antisera prepared against purified *Streptococcus faecalis* fructose diphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase and *Pediococcus cerevisiae* glyceraldehyde-3-phosphate dehydrogenase were used for comparative enzyme immunological studies; the Ouchterlony double-diffusion technique and the quantitative microcomplement fixation procedure were employed. The reactions obtained provide evidence showing that all seven *Acholeplasma* species studied (*A. laidlawii*, *A. granularum*, *A. modicum*, *A. oculi*, *A. axanthum*, *A. hippikon*, and *A. equifetale*) are phylogenetically related to streptococci and that they evolved from streptococci. The data strongly suggest that the acholeplasmas comprise a distinct evolutionary group that has diverged from streptococci belonging to Lancefield group D or N. No reactions were observed between these enzyme antisera and cell extracts from six fermentative *Mycoplasma* species. These results support the view that mycoplasmas are derived from various bacteria.

Mycoplasmas are procaryotes that differ from other bacteria by their lack of a cell wall, by the requirement of most species for cholesterol for growth, and by their small size—they are the smallest organisms capable of independent replication. Mycoplasmas are also distinguished from the majority of procaryotes by their small genome sizes, which cluster to form two separate sets of values around 5×10^8 and 1×10^9 daltons (3). Over 60 species have been isolated from humans, animals, plants, and insects, and these have been set apart in the class *Mollicutes*, order *Mycoplasmatales*, and classified into the genera *Mycoplasma*, *Acholeplasma*, *Ureaplasma*, and *Spiroplasma*. We have emphasized the heterogeneity of the microorganisms making up the mycoplasmas, and this heterogeneity extends also to DNA G+C contents which range from 23 to 40% (20). Some of these values are among the lowest known for any procaryotes. In addition, other wall-less procaryotes resembling mycoplasmas have been observed in amphibians, invertebrates, and plants, but not cultivated (28). Consequently, it appears that a large class of wall-less procaryotes occurs widely in nature and that many more mycoplasmas remain to be isolated.

The origin of mycoplasmas, their relationship to one another, and their relationship to other procaryotes came into question when it was recognized that virtually all bacteria can produce wall-deficient growth forms (so-called "L forms") that closely resemble the naturally occurring mycoplasmas (9). Two opposing hypotheses have been offered to explain the relationship of mycoplasmas to other procaryotes (8, 10, 15): one hypothesis holds that mycoplasmas are a true biological class whose members are phylogenetically related through common evolution; the other hypothesis holds that mycoplasmas are an assemblage of wall-less forms derived from various bacteria. It is implicit in the former view that the extant mycoplasmas are the surviving descendants of exceedingly primitive bacteria that existed before the development of a peptidoglycan-based cell wall.

Various workers (25, 31, 35, 37) have pointed out that physiological and metabolic similarities exist between fermentative mycoplasmas and lactic acid bacteria. When it was observed that *Acholeplasma* species possess fructose diphosphate-activated lactate dehydrogenases that are strikingly similar to the unique lactate dehydrogenases found in streptococci (21, 24, 26), a study was initiated to determine whether this large segment of mycoplasmas was phylogenetically related to members of the genus *Streptococcus*. In this paper we report results of comparative enzyme protein studies which establish that the acholeplasmas are phylogenetically related to

streptococci and that they evolved from streptococci. This finding suggests that the acholeplasmas are radically altered descendants of streptococci that possessed the capacity to undergo and survive profound cellular alterations. Analysis of these results indicates that mycoplasmas do not comprise a true biological class, but rather are derived from various bacteria. Part of this work has been described previously (23).

MATERIALS AND METHODS

Organisms and culture conditions. The majority of the mycoplasmas used in this study have been described previously (20); *Acholeplasma equifetale* and *A. hippikon* were kindly provided by J. G. Tully (National Institutes of Health). Media and growth conditions were essentially as described previously (20). No inhibitory agents were employed in growing mycoplasmas. Streptococci and other lactic acid bacteria were obtained from the American Type Culture Collection, the collection of the National Institute of Dental Research, or the collection of Ellen Garvie (N.I.R.D., Reading, England); *Streptococcus pneumoniae* (type I) was provided by Gerald Schiffman (Downstate Medical Center). Streptococci and lactic acid bacteria were grown as described previously (18).

Preparation of cell extracts. Cells were harvested and washed once with 0.145 M NaCl–0.05 M potassium phosphate (pH 7.5) and suspended in the same buffer containing 10 mM 2-mercaptoethanol (0.3 to 0.5 g [wet weight] per ml). Cell suspensions in an ice bath were sonicated with a microprobe (Biosonic II sonic oscillator) for several 10-s periods (200 to 400 s total for mycoplasmas) with cooling intervals between sonication periods and centrifuged at an average of 80,800 × *g* for 40 min at 4°C; streptococci required longer sonication with a standard probe. Samples were stored at −20°C until used. This sonication procedure has proved satisfactory for preparing active enzyme extracts from the mycoplasmas and lactic acid bacteria used in this study (17, 18, 24).

Immunological procedures. The preparation of rabbit antisera against electrophoretically homogeneous *Streptococcus faecalis* fructose diphosphate (FDP) aldolase and glyceraldehyde-3-phosphate (GA3P) dehydrogenase and the *Pediococcus cerevisiae* GA3P dehydrogenase has been described previously (17, 18). The specificity of each antiserum was established by the fact that it reacted only with aldolases or GA3P dehydrogenases from streptococci other lactic acid bacteria and closely related allies. Each antiserum upon immunoelectrophoresis produced a single precipitin line against either the purified enzyme or whole cell extracts.

Immunodiffusion experiments were carried out in 0.8% agarose–0.05 M potassium phosphate-buffered saline (pH 7.0) at 4°C by the Ouchterlony technique. The three patterns of immunoprecipitates observed in these studies, (i) confluent lines of precipitation (reactions of identical specificity), (ii) single-spurred lines of precipitation (reactions of partial identity), and (iii) precipitates with crossed spurs (reactions of nonidentity), were used to determine the nature of the antigenic relationships that exist among the various FDP aldolases and GA3P dehydrogenases examined here. Results from the pairwise cross-matches of the two glycolytic enzymes were interpreted according to the conventions of Gasser and Gasser (12).

Microcomplement fixation experiments were carried out by the method of Wasserman and Levine (40) as modified by Champion et al. (6). Microcomplement fixation results are reported as indices of dissimilarity according to the following equation (5): log of the index of dissimilarity = $(Y'_H - Y_h/m) + (\log X_h/X_H)$ where Y'_H and Y_h are the percentages of complement fixed by the homologous and heterologous antigens, respectively, X_H and X_h are the antiserum concentrations used with homologous and heterologous antigens, respectively, and m is the slope of the line resulting from a plot of the log of the antiserum dilution used in the homologous system against the percentage of complement fixed. The immunological distance is the log of the index of dissimilarity × 100 (6, 27). For the anti-FDP aldolase and anti-GA3P dehydrogenase, the values for m were 178 and 143, respectively.

RESULTS

Cell extracts from all of the *Acholeplasma* species (except *A. equifetale*, see below) produced single, strong precipitin bands with the *S. faecalis* FDP aldolase antiserum in immunodiffusion experiments. Typical results are shown in Fig. 1. A fused precipitate was obtained between extracts from *A. laidlawii* and *A. granularum*, and these in turn produced single-spur reactions over extracts of the other acholeplasma species, thus indicating that the aldolases of the former two species form a group of apparent identical specificity and share a greater number of antigenic determinants with the *S. faecalis* aldolase than do the other acholeplasmas. Parenthetically, the immunological relatedness between the *A. laidlawii* and *A. granularum* aldolases con-

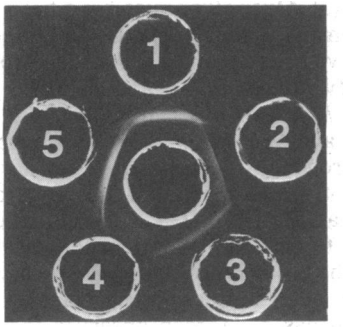

FIG. 1. Ouchterlony gel immunodiffusion reactions of five acholeplasma cell extracts. The center well contained rabbit antiserum to electrophoretically homogeneous *S. faecalis* FDP aldolase. Wells: 1, *A. laidlawii*; 2, *A. modicum*; 3, *A. axanthum*; 4, *A. oculi*; 5, *A. granularum*. Similar comparative experiments were used to determine the antigenic hierarchy of the acholeplasma species relative to the *S. faecalis* aldolase reference.

firms the relatively close relationship between these two mycoplasmas demonstrated previously by nucleic acid hybridization (2, 20).

Because the antialdolase serum was prepared against the *S. faecalis* aldolase, this enzyme is the reference point for all comparisons. The degree of immunological homology of the acholeplasma aldolases relative to the *S. faecalis* aldolase reference was determined by multiple cross-match experiments such as those shown in Fig. 1. From these reactions, it was deduced that acholeplasma aldolases are related to the reference *S. faecalis* aldolase in a sequential order of decreasing similarity as follows: *A. laidlawii* = *A. granularum* > *A. modicum* > *A. oculi* > *A. axanthum* = *A. hippikon*.

To determine the location of the acholeplasma aldolases within the evolutionary sequence of the lactic acid bacteria aldolases (17, 18), cell extracts from acholeplasmas and streptococci representing various Lancefield groups and *S. pneumoniae* (type 1) were cross-matched. Typical immunodiffusion reactions between acholeplasmas and streptococci are shown in Fig. 2. Cross-matches between acholeplasma and streptococcal extracts all produced single-spur precipitates, indicating partial identity between their aldolases. Such reactions of partial identity usually occur when proteins have evolved sequentially in a unidirectional fashion. All of the acholeplasma extracts produced spurs over *S. pneumoniae* and *S. pyogenes*, indicating that the acholeplasma aldolases are more closely related to the *S. faecalis* aldolase than are the aldolases of these streptococci. On the other hand, extracts from *S. cremoris* (Lancefield group N) produced single spurs over most of the acholeplasma extracts, but the *S. cremoris* extract was itself spurred over by the aldolase from *A. laidlawii*. No cross-spur reactions were observed between any of the acholeplasma and streptococcal extracts.

As noted, the *S. faecalis* antialdolase did not react with *A. equifetale* extracts. However, a cross-reaction was produced by the *S. faecalis* anti-GA3P dehydrogenase, and this antiserum demonstrated that *A. laidlawii* extracts produce single-spur reactions over *A. equifetale* extracts. Thus, *A. equifetale* also is related to the streptococci.

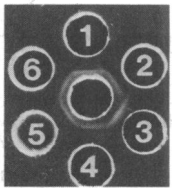

FIG. 2. Example reactions of partial identity between acholeplasmas and streptococci. The center well contained *S. faecalis* anti-aldolase serum. Wells: 1, *A. axanthum*; 2, *A. oculi*; 3, *S. cremoris*; 4, *A. axanthum*; 5, *S. pneumoniae*; 6, *A. modicum*.

All these results clearly demonstrate that the streptococcal and acholeplasma aldolases share a high degree of immunological homology and that the acholeplasmas and streptococci are phylogenetically related. Analysis of the spur reactions indicates that the acholeplasma aldolases are antigenically situated in the region between the *S. faecalis* reference point (Lancefield group D) and the group N streptococci.

The phylogenetic relationship of the acholeplasma cluster within the lactic acid bacteria was estimated more precisely by microcomplement fixation experiments with antisera prepared against the *S. faecalis* FDP aldolase, *S. faecalis* GA3P dehydrogenase, and *P. cerevesiae* GA3P dehydrogenase. The indexes of dissimilarity and the immunological distances for *A. laidlawii*, *A. granularum*, and *A. oculi* are presented in Table 1. There is relatively good agreement between the two immunological distance values obtained with antisera prepared against the *S. faecalis* aldolase and GA3P dehydrogenase. This information permits an initial positioning of the *Acholeplasma* species with regard to the streptococci, and it can be seen that the strains examined in these experiments are closely related to the group D and group N streptococci. Further, the data obtained with the anti-*P. cerevisiae* GA3P dehydrogenase provided a second point of reference that allows the group location to be positioned in a two-dimensional plane, and from this the *Acholeplasma* appear to comprise a distinct evolutionary group that has branched off from the main line of streptococcal evolution. The data available indicate that the acholeplasma group occupies an evolutionary position roughly

TABLE 1. Immunological distances among the FDP aldolases and GA3P dehydrogenases of acholeplasmas, streptococci, and pediococci

Source of enzyme	Immunological distance units and indexes of dissimilarity obtained with antisera prepared against:		
	Anti-*S. faecalis* aldolase	Anti-*S. faecalis* GA3P dehydrogenase	Anti-*P. cerevisiae* GA3P dehydrogenase
A. laidlawii	102 (10.5)[a]	129 (19.6)	112 (13.2)
A. granularum	ND[b]	ND	114 (13.7)
A. oculi	110 (12.8)	ND	ND
S. diacetylactis	113 (13.5)	134 (22)	174 (55)
S. faecalis	0 (1)	0 (1)	103 (10.8)

[a] Numbers within parentheses indicate the index of dissimilarity.

[b] ND, Not determined.

midway between the two genera of spherical lactic acid bacteria, *Streptococcus* and *Pediococcus*, but the acholeplasmas clearly exhibit a greater degree of relatedness to the streptococci. The data at hand strongly suggest that the acholeplasmas recently diverged from members of the genus *Streptococcus* belonging to Lancefield group D or N.

No precipitin reactions were observed against any of the following fermentative *Mycoplasma* species: *M. pneumoniae*, *M. capricolum*, *M. felis*, *M. mycoides* var. *mycoides* (UM30847), *M. species* (California calf), or *M. neurolyticum*.

DISCUSSION

The immunological results presented here demonstrate a significant degree of amino acid sequence homology between enzymes of acholeplasmas and streptococci. Previous studies by Prager and Wilson (27) established that amino acid substitutions amounting to between 25 and 40% of the total sequence resulted in a complete loss of immunological cross-reactivity between related proteins. Therefore, the degree of structural homology among the aldolases and among the GA3P dehydrogenases is probably 60% or greater. If the findings of Ibrahimi et al. (13) can be extended to other proteins, namely, that a change of 1 immunological distance unit is equivalent to an amino acid sequence difference of 0.2%, then the streptococcal and acholeplasma aldolase subunits which are composed of approximately 166 amino acids may differ by as few as 30 amino acids.

A phylogenetic map (Fig. 3) based on studies with FDP aldolases and GA3P dehydrogenases (17, 18) depicts the natural relationships between the acholeplasmas and the three major genera of lactic acid bacteria. These three genera diverge or radiate outward from a single point of origin, where they appear to be related through a common ancestor. The evidence, as summarized by the map, indicates that acholeplasmas as a group diverged from the essentially sequential evolutionary path formed by the streptococci and are most closely related to the group N and D streptococci. It would appear, therefore, that the acholeplasmas cannot be some primitive form of procaryote, but instead descended from streptococci. When the acholeplasmas diverged from streptococci is uncertain, but the close similarity between their shared enzymes suggests that this occurred during the relatively recent evolutionary history of these enzymes, and that divergence probably occurred since streptococci established residence in higher organisms.

Other similarities can be found between the acholeplasmas and the streptococci. In addition to the structural homology shared by their FDP aldolases and GA3P dehydrogenases, these two groups possess L-(+)-lactate dehydrogenases that strikingly resemble one another in their requirement for FDP as a positive effector (21). Also, we noted previously (23) that information on mycoplasma lipids (32) is in accord with our results, and that lipids from *A. laidlawii* closely resemble or are identical to those of streptococci (33); in addition at least two acholeplasmas, *A. laidlawii* and *A. granularum*, accumulate free phospholipids with structure similar to that of the lipid anchor for glycerol teichoic acid in streptococci (34).

The demonstration that acholeplasmas de-

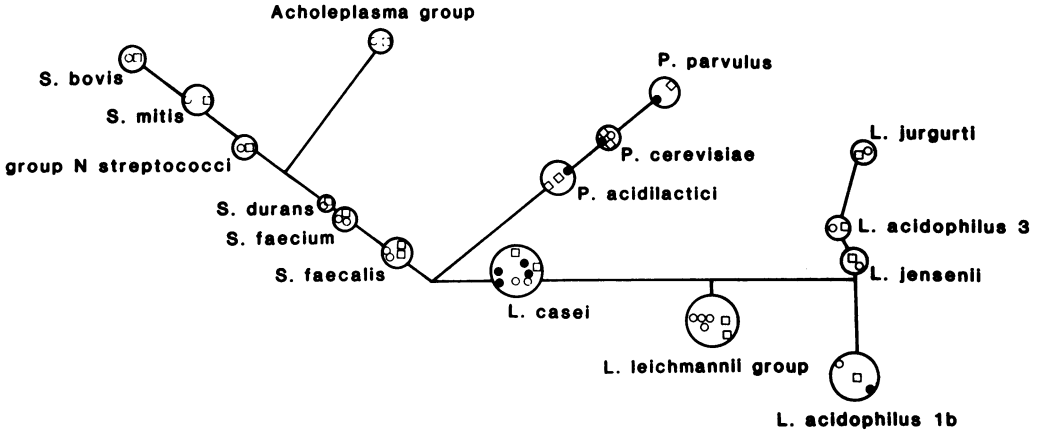

FIG. 3. Phylogenetic map depicting the relationship of acholeplasmas to the lactic acid bacteria. Symbols: ●, positions determined with anti-*P. cerevisiae* aldolase; ○, positions determined with anti-*S. faecalis* aldolase; □, positions determined with anti *P. cerevisiae* GA3P dehydrogenase. The locations of the various species were determined by plotting the respective immunological distance values (0.59 mm equals 1.6 immunological distance units). Branching was established by immunodiffusion (18).

scended from streptococci raises several important questions. First, what is the relationship of other mycoplasmas to bacteria? The fact that acholeplasmas descended from streptococci strongly suggests that the various other mycoplasmas similarly evolved from other bacteria, and one of us proposed this (22). The only alternative would be that mycoplasmas are exceedingly primitive cells—the ancestors or descendants of ancestors of walled bacteria, but this is highly unlikely. For example, if mycoplasmas were ancestral to walled bacteria, it is implicit that cell wall glycopeptide synthesis must have evolved sometime in the course of evolution between mycoplasmas and bacteria. If various mycoplasmas gave rise to various bacteria, the repeated independent evolution of cell wall synthetic machinery would be required, and this is untenable if one accepts the almost certain proposition that wall glycopeptide synthesis evolved only once during the course of bacterial evolution. Similarly, any argument that one mycoplasma could have been the progenitor of all walled bacteria also fails (22). Rather, all of our evidence supports the conclusion that mycoplasmas descended from various bacteria, and consequently mycoplasmas do not comprise a true biological class (22, 23).

Eventually it should be possible to identify the specific bacterial antecedents of each of the broadly different subgroups that make up the mycoplasmas. As already noted, the lipid composition of those mycoplasmas that have been examined is akin to that of gram-positive bacteria (32, 33). Also, it has been pointed out that the $tRNA^{Phe}$ from *M. capricolum* (14) and $tRNA_f^{Met}$ from *M. mycoides* var. *capri* (39) share a higher nucleotide sequence homology with *Bacillus subtilis* than with *Escherichia coli*, and Walker concluded that these mycoplasmas could not be evolutionary primitive bacteria (38). Whether all mycoplasmas will prove to be descendants of gram-positive bacteria remains to be determined because only fermentative mycoplasmas have been examined in detail.

Another important question is by what evolutionary mechanisms did mycoplasmas evolve from their progenitor bacteria. The detection of the specific progenitor-derivative relationship between streptococci and acholeplasmas makes it possible to approach this question by identifying the existing cellular differences between these two groups and comparing them. Although acholeplasmas are closely related to streptococci, it is also clear that they are radically altered from streptococci. One of the most striking and fundamental differences between the two groups is their genome size. Genome size values known for streptococci range from 1.2×10^9 to 1.47×10^9 daltons (4), and acholeplasma genome sizes, which cluster around 1×10^9 daltons, are 20 to 30% smaller than these streptococcal genomes. Evidently, some streptococci must have undergone massive reductions in genome size to produce acholeplasmas.

The absence of cell walls is the most conspicuous phenotypic character of mycoplasmas, and probably a significant portion of the lost genome segments was devoted to coding for wall structures or wall-associated processes. Losses of proteins that function in these processes have been recorded. Acholeplasmas (and mycoplasmas), in contrast to streptococci, lack all penicillin-binding proteins (19), and losses of other proteins related to membrane function also appear to have occurred in acholeplasmas since they lack a functional phosphoenolpyruvate-dependent phosphotransferase system (7). The capacity to synthesize all wall-related components may not have been lost, however; the occurrence in certain acholeplasmas of free phospholipids that resemble the streptococcal lipid anchor for glycerol teichoic acid suggests the possible accumulation of a metabolic intermediate resulting from the inability to synthesize wall polymers (34).

In addition to genes coding for wall structure, it appears that rRNA genes were altered in the course of formation of mycoplasmas. Reff et al. (29) reported that the 16S (but not 23S) rRNAs from all the mycoplasmas examined appeared to be smaller than the 16S RNAs from bacteria (including *S. faecalis* and an L form). Also, missing 16S rRNA sequences have been reported by Woese et al. (41) (see below). Any explanation for the genesis of mycoplasmas will have to account for the rRNA sequences that appear to be missing from mycoplasma 16S rRNAs. The apparent universality of missing 16S rRNA sequences in mycoplasmas suggests that alterations in rRNA genes together with envelope losses could have been primary events in the formation of mycoplasmas.

Our conclusion that mycoplasmas are not a true phylogenetic class is supported by others. Woese and co-workers (41) compared 16S rRNA oligonucleotide "catalogs" from four mycoplasmas to those of bacteria; they concluded that mycoplasmas arose by degenerative evolution, and that within the gram-positive spore-forming bacteria, the mycoplasmas form a cluster that is peripherally related to a specific subgroup defined by the genera *Bacillus*, *Lactobacillus*, and *Streptococcus*. The broad conclusion of Woese et al. (41) that mycoplasmas evolved from walled bacteria is in agreement with ours, and they cite our work in support of theirs; however, their analysis did not detect the close phylogenetic relationship between acholeplasmas and streptococci. Further, their conclusion (11, 41)

that *A. laidlawii* is more closely related to *Clostridium ramosum* and *C. innocuum* rather than to streptococci differs markedly from ours. (We were able to demonstrate with the *S. faecalis* anti-GA3P dehydrogenase antiserum a double-spur precipitate or reaction of nonidentity between acholeplasma and *C. innocuum* cell extracts. Studies on the phylogenetic relationship between lactic acid bacteria and these clostridia will be described elsewhere.) Woese and coworkers (41) encountered difficulties in analyzing the mycoplasma 16S rRNA data because a relatively high proportion of the highly conserved oligonucleotide sequences that commonly occur in every eubacterial 16S rRNA were not found in mycoplasma 16A rRNA, and this prevented application of their usual analysis. They attempted to circumvent this difficulty by carrying out a different analysis of the data, and these factors may be responsible for the discrepancy in results.

How large reductions in genome size occurred to produce the acholeplasmas is unknown, and why mycoplasma genome sizes do not occur over a range, but rather all cluster closely around the values 5×10^8 or 1×10^9 daltons, also remains to be explained. Large reductions in genome size could have come about either through a series of individually small deletions or through losses of very large DNA segments. One can imagine the occurrence of irregularities in chromosome replication, in rearrangements of large chromosome segments (30), or in the function of transposable elements (36), and any one of these could have contributed to large losses. As already noted, certain acholeplasmas lack a phosphoenolpyruvate-dependent phosphotransferase system (7), and examination of the closely related group N streptococci which have a propensity for carrying important metabolic genes on plasmids (1) may provide a clue to a possible mechanism for the loss of this group of functions (16).

Because mycoplasmas are poorly equipped to survive free in nature, it seems reasonable to propose that they all arose from progenitors that resided in hosts. Infectious agents are under continual selective pressure to evolve new surface antigens which can circumvent the innate and acquired immunity of their hosts. The loss of the cell wall to produce a mycoplasma appears to be the ultimate morphological change available to bacteria for altering their surface antigens. The ability of mycoplasmas to adhere very closely to host cell membranes appears to be associated with cell wall loss, and mycoplasmas thus may have gained over their parental species an ability to occupy new ecological niches in hosts. The fact that several mycoplasmas are agents of human, animal, and plant diseases for which there are no counterpart bacterial diseases suggests the possibility that certain bacteria in their transition into mycoplasmas may have gained new capacities for pathogenecity.

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