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Nucleic Acid Hybridization Studies of Mycobactin-Dependent Mycobacteria

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Using molecular techniques, specifically, DNA-DNA hybridization in solution and measurement of the thermal stabilities of hybrids, we examined the genetic relationships among mycobactin-dependent mycobacteria and between such organisms and other (mycobactin-independent) mycobacteria. The mycobactin-dependent mycobacteria examined included five strains of *Mycobacterium paratuberculosis*, two wood pigeon isolates, and one bovine isolate that was biochemically identified as *Mycobacterium avium*. All mycobactin-dependent mycobacteria were found to belong to the same hybridization group and to be closely related to organisms in *M. avium* complex serovars 2, 8, and 9, but more distantly related to organisms in serovars 7 and 19. Relatively low levels of hybridization were observed with other mycobacterial species examined. Thus, these results provide genetic evidence to support previous biochemical and cultural evidence that indicated that mycobactin-dependent mycobacteria constitute a closely related group of organisms within the *M. avium* complex.

About 80 years ago, the organism currently known as *Mycobacterium paratuberculosis* was distinguished from the organisms that caused human, bovine, and avian tuberculosis by its inability to grow on media capable of supporting the growth of the other mycobacteria. The difficulty in cultivating *M. paratuberculosis* was traced to a deficiency of mycobactin, an iron-chelating compound used for transport and storage of iron needed for good growth in culture (15). The organism could be successfully cultivated if the medium was enriched with ferric ammonium citrate (23) or mycobactin (24); previously, mycobactin could be provided by addition of mycobacterial cell walls, but purified mycobactin is now available. Absolute mycobactin dependence is often exhibited only on initial isolation. Upon subculture, the organism often is capable of some growth in unsupplemented medium as long as it is not excessively diluted during subculture (17); however, growth of these organisms is usually enhanced by addition of mycobactin to the medium.

Mycobactin dependence is not solely restricted to *M. paratuberculosis* as it has been noted in mycobacteria isolated from wood pigeons and in some *Mycobacterium avium* isolates (2, 17). In many characteristics, *M. paratuberculosis* resembles *M. avium* (22), and a close genetic relationship between *M. paratuberculosis* and *M. avium* has been suspected (13). However, there has been no direct demonstration of such a relationship.

In this paper, we describe experiments that use DNA-DNA hybridization in solution to examine the genetic relationships of mycobactin-dependent mycobacteria to one another and to other mycobactin-independent mycobacteria. Our goals were to investigate the proper taxonomic niche(s) for these organisms and to evaluate the significance of mycobactin dependence in the classification of mycobacteria.

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MATERIALS AND METHODS

Sources of mycobacteria. Strains of *M. paratuberculosis* and other mycobactin-dependent mycobacteria were obtained from R. S. Merkal and D. L. Whipple, National Animal Disease Center, Ames, Iowa, and from P. J. Brennan, Colorado State University, Ft. Collins (Table 1). Organisms belonging to several serovars of the *M. avium* complex (25) were obtained from P. J. Brennan and from the Trudeau Mycobacterial Collection (which has been transferred to the American Type Culture Collection, Rockville, Md.). A reference strain of *Mycobacterium fortuitum* was obtained from the American Type Culture Collection, and clinical isolates of mycobacteria of several species were obtained from J. E. Clarridge, Clinical Microbiology Laboratory, Veterans Administration Medical Center, Houston, Tex.

Growth of mycobacteria. Mycobacteria were grown at 37°C in Middlebrook 7H9 broth containing 0.05% Tween 80 and 100 ml of Dubos oleic albumin complex per liter (11). Media for growth of mycobactin-dependent organisms also contained 2 mg of mycobactin J (Allied Labs, Ames, Iowa) per liter.

Extraction of DNA. Mycobacterial DNA was extracted and purified as described previously (26). Briefly, mycobacteria were disrupted by passage through a French press, treatment with lysozyme, and digestion by proteinase K and sodium dodecyl sulfate. Mycobacterial DNA was then purified by phenol extraction, ethanol precipitation, and RNase treatment. Usual yields were on the order of 1 mg of DNA per g (wet weight) of bacterial pellet.

Labeling of probes. Whole genome tritiated probes were prepared by nick translation, using [*methyl*-1′,2′-^{3}H]thymidine triphosphate (26). Probes had specific activities of 1×10^6 to 4×10^6 cpm/μg of DNA.

DNA-DNA hybridization in solution. DNA-DNA hybridization in solution was carried out at 68°C in 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered solution (pH 7.0) containing 0.6 M NaCl and 0.002 M EDTA (12, 26). Typical reaction mixtures contained 0.02 μg

TABLE 1. Relative hybridization levels with probes prepared from the neotype strain (ATCC 19698) of *M. paratuberculosis*

Test DNA	Source of organism[a]	Hybridization level (%)[b]
M. paratuberculosis		
ATCC 19698	NADC	100 (0.0)
linda	NADC	92 (2.1)
C286	CSU	90
18	CSU	91
21	NADC	90 (1.3)
Wood pigeon bacillus		
WP M21	NADC	99 (0.0)
WP VI/72	NADC	98 (0.7)
M. avium #7	NADC	97 (0.6)
M. avium complex		
Cardiff (serotype 2)	CSU/TMC	91 (1.5)
TMC 701 (serotype 2)	TMC	96
TMC 1476 (serotype 7)	TMC	49
TMC 1468 (serotype 8)	TMC	92
Watson (serotype 9)	CSU/TMC	92
Darden (serotype 19)	CSU/TMC	35 (9.3)
M. fortuitum ATCC 6841	ATCC	17
Clinical isolates		
M. tuberculosis	VAMC	52
M. phlei	VAMC	19
M. kansasii	VAMC	23
M. gordonae	VAMC	27
M. fortuitum	VAMC	15

[a] NADC, National Animal Disease Center; CSU, Colorado State University; CSU/TMC, strains obtained from P. J. Brennan (Colorado State University), who originally obtained them from the Trudeau Mycobacterial Collection; TMC, Trudeau Mycobacterial Collection (now available from the American Type Culture Collection); ATCC, American Type Culture Collection; VAMC, Veterans Administration Medical Center (Houston, Tex.).

[b] Background was subtracted and results were normalized so that hybridization with homologous test DNA is set at 100%. Values in parentheses are ΔT_ms for hybrids formed in the presence of each test DNA (see Materials and Methods for details).

of probe DNA and 50 μg of test DNA in a volume of 0.8 ml. Under these conditions, hybridizations were allowed to proceed for 90 to 100 h, yielding C_0t values that were 20 times the $C_0t_{1/2}$ value for each probe (approximately 3.5 mol · s/liter). Following hybridization, single-stranded probe was degraded by S1 nuclease, and double-stranded probe was measured by determination of radioactivity in trichloroacetic acid precipitates.

Thermal denaturation of DNA-DNA hybrids. In most hybridization reactions, some hybrids form between sequences that are not exactly complementary to each other. One way to measure the amount of unpaired bases in the hybrids is to measure the thermal stabilities of the hybrids. Thermal stabilities of hybrids were measured as described previously (26). The amount of probe remaining double stranded (resistant to S1 nuclease) after incubation at stepwise increasing temperatures was measured. The melting temperature (T_m) of each hybrid was defined as the temperature at which 50% of the hybridized probe became sensitive to S1 nuclease. Results are presented as ΔT_m, which is the difference between the T_m for hybrids formed with various test DNAs and that for hybrids formed with DNA homologous to the probe (14). Each 1°C in ΔT_m corresponds to unpaired bases in approximately 1% of the hybridized DNA (3, 5).

TABLE 2. Relative hybridization levels with probes prepared from various strains of mycobactin-dependent mycobacteria

Test DNA	Hybridization level (%)[a]							
	M. paratuberculosis					Wood pigeon		*M. avium* #7
	ATCC 19698	linda	C286	18	21	WP M21	WP VI/72	
M. paratuberculosis								
ATCC 19698	100	89	76	84	90	98	95	100
linda	92	100	80	—[b]	101	88	89	92
C286	90	88	100	—	93	85	89	89
18	91	—	76	100	88	—	—	—
21	90	97	76	—	100	—	89	—
Wood pigeon bacillus								
WP M21	99	91	71	84	86	100	98	94
WP VI/72	98	91	80	—	87	—	100	104
M. avium #7	97	85	75	83	—	92	96	100
M. avium complex								
TMC 701	96	88	77	—	90	105	96	101
TMC 1468	92	93	90	—	90	82	87	90
TMC 1476	49	53	40	—	41	51	46	46

[a] Background was subtracted and results were normalized so that hybridization levels with homologous test DNAs are 100%.

[b] —, Not done.

RESULTS

In experiments with probes prepared from whole genome DNA from the neotype strain of *M. paratuberculosis* (ATCC 19698), all strains that were initially isolated as mycobactin-dependent organisms exhibited ≥90% hybridization with the probe (Table 1). These strains included five strains of *M. paratuberculosis*, one of which (strain linda) had been isolated from a human patient with Crohn's disease (8) and subsequently characterized biochemically (7) and genetically (18, 26) as a strain of *M. paratuberculosis*. Other mycobactin-dependent strains were the wood pigeon bacilli and *M. avium* #7, a bovine isolate identified biochemically as *M. avium*.

Mycobactin-independent members of the *M. avium* complex fell into at least two hybridization groups when the *M. paratuberculosis* ATCC 19698 probe was used. One group, containing members of serovars 2, 8, and 9, exhibited high levels of hybridization, whereas the organisms from serovars 7 and 19 yielded much lower hybridization levels.

When thermal stabilities of the hybrids were examined, a similar division of tested strains was observed. The organisms yielding high levels of hybridization yielded low (≤2.1°C) values for ΔT_m, while strain Darden, which yielded only 35% hybridization with the probe, had a much higher ΔT_m value, 9.3°C.

Of the other mycobacterial species examined, only one, a clinical *M. tuberculosis* isolate, exhibited appreciable (52%) hybridization with *M. paratuberculosis* ATCC 19698 probe (Table 1). However, this value only approximated that obtained with the more distantly related members of the *M. avium* complex and is much lower than that obtained with mycobactin-dependent isolates.

Results of DNA-DNA hybridization, using probes prepared from whole genome DNA of other mycobactin-dependent organisms, are presented in Table 2. Reproducibility of

results was good, with most repeat determinations differing by 5% or less; the maximum difference seen was 8%. With the exceptions of those hybridizations with probes prepared from DNA from *M. paratuberculosis* C286, relative hybridization levels were always >80%, and reciprocal hybridization results (such as probe ATCC 19698 versus test linda and probe linda versus test ATCC 19698) were in good agreement, ranging from no difference in reciprocal hybridization involving *M. paratuberculosis* ATCC 19698 and *M. paratuberculosis* 21 to 8% difference for reciprocal hybridizations involving *M. avium* #7 and wood pigeon bacillus WP VI/72.

DISCUSSION

These results show that all of the strains of mycobactin-dependent mycobacteria used in these experiments belong to the same hybridization group. The relative hybridization levels and ΔT_m values observed meet the criteria of a genetically defined species as promulgated by Brenner and his associates (4); all relative hybridization levels are >70% and ΔT_m values are <6°C.

In experiments utilizing probes prepared from *M. paratuberculosis* C286, relatively low levels of hybridization with test DNAs from other mycobactin-dependent mycobacteria were always observed. These results were reproducibly obtained with different probes prepared from different DNA preparations. Thus, the results appear to be real and not an artifact of a single erroneous experiment involving a possibly contaminated DNA sample. These results are most consistent with *M. paratuberculosis* C286 having a larger genome than the other mycobactin-dependent mycobacteria.

It is apparent that the DNAs of the mycobactin-dependent mycobacteria exhibit a great deal of sequence homology with the DNAs of certain members of the *M. avium* complex. Specifically, they appear to belong to the same hybridization group as organisms in serovars 2, 8, and 9, but in different hybridization groups from the serovar 7 and serovar 19 organisms. With the possible exception of the classification of serovar 9 organisms, these groupings are in agreement with the findings of Baess (1), whose work has led to the suggestion that all *M. avium* complex organisms in serovars 1 to 6 and 8 to 11 should be assigned to one species and designated *M. avium*. Our results also agree with those of McFadden et al. (19), who also observed very high (102 to 107%) relative hybridization values in DNA-DNA solution hybridization experiments with test DNAs from *M. paratuberculosis* ATCC 19698 and *M. avium* complex serovars 2 and 5 and a probe prepared from a mycobactin-dependent mycobacterium (strain Ben) isolated by Chiodini et al. (7) from a Crohn's disease patient. McFadden et al. (18) also used a different method to examine the genetic relationships among Chiodini's isolates, *M. paratuberculosis*, and *M. avium* complex serovar 2 and 5 organisms. This method utilized statistical analyses of the uniformity of restriction endonuclease cleavage sites in mycobacterial genomes. They found that four Crohn's disease isolates, *M. paratuberculosis* ATCC 19698, and *M. avium* complex serovar 2 and 5 organisms were very closely related.

Other studies have also demonstrated similarities between *M. paratuberculosis* and *M. avium* complex organisms. For example, a recent study by McIntyre and Stanford (20), using immunodiffusion techniques, demonstrated that mycobactin-dependent mycobacteria, including *M. paratuberculosis*, were variants of *M. avium* and that they could be differentiated from *M. intracellulare*. However, the mycobactin-dependent organisms were not identical in these experiments; instead of falling into one single group yielding identical antigen patterns, they were distributed between two of the three defined immunodiffusion types (21). Furthermore, in detailed structural analyses of specific antigenic determinants from numerous strains of mycobacteria, Camphausen et al. (6) found that certain strains of *M. avium* and *M. paratuberculosis* contained identical haptens and suggested that several strains of *M. paratuberculosis* are rough variants of *M. avium*.

The proper classification of the mycobactin-dependent mycobacteria is further complicated by previous classifications of mycobacteria based on the animals from which they were isolated and their virulence for various species, especially chickens and calves. For example, mycobacteria isolated from birds were called *M. avium*; these organisms were not virulent in cattle and could also be classified serologically into a number of clearly defined serovars (25). Organisms isolated from cattle with Johne's disease were called *M. paratuberculosis*; these organisms were typically not pathogenic for chickens and could not be recognized by specific *M. avium* serotyping antisera. However, exceptions have been discovered. For instance, some mycobacteria isolated from wood pigeons demonstrated the mycobactin dependence of *M. paratuberculosis* and could not be classified by specific *M. avium* serotyping schemes (10); some of them were also found to be capable of causing a Johne's-like disease in cattle (16). Some otherwise typical *M. avium* isolates (including *M. avium* #7) were mycobactin dependent upon initial isolation. In addition, some organisms have been designated *M. paratuberculosis* because of their initial isolation from cattle, but have subsequently been found to be virulent for chickens (9).

Thus, the exact taxonomic status of *M. paratuberculosis* and other mycobactin-dependent organisms remains unclear. However, evidence is accumulating to indicate that *M. paratuberculosis* should not be considered a separate species. Indeed, it has already been proposed, based on similarities in cultural, biochemical, and serological characteristics, that *M. paratuberculosis* may represent a subspecies of *M. avium* (13, 15). The exact criteria to use in assigning isolates to such a classification remain to be established; problems are likely to arise in that classification schemes based on different properties (e.g., genetic homology, seroagglutination, host range, and immunodiffusion types) may conflict with one another. Our results suggest that mycobactin dependence may be useful in allowing the assignment of organisms to a genetically defined subspecies of *M. avium*. However, if mycobactin dependence is to be useful in the classification of mycobacteria, uniform methods that take into account the effect of inoculum size (17) and criteria for determination of mycobactin dependence must be developed and accepted.

Even if mycobactin dependence proves to be of limited value in mycobacterial taxonomy, it may be an important property to consider in assessing the involvement of clinical isolates in disease processes. Since mycobactin-dependent organisms would be incapable of existence as free-living environmental organisms, their recovery from clinical specimens would suggest that they were occupying ecological niches in which mycobactin was not required for growth. Such a niche may be as intracellular organisms resident in macrophages, a condition which may be significant in the pathogenesis of disease.

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