Rough Morphological Variants of Mycobacterium avium

CHARACTERIZATION OF GENOMIC DELETIONS RESULTING IN THE LOSS OF GLYCOPEPTIDOLIPID EXPRESSION*

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Previously, a gene cluster, termed ser2, which encodes for the synthesis of the specific oligosaccharide of the glycopeptidolipid antigen of Mycobacterium avium serovar 2 strain TMC 724, was defined. DNA probes from this cloned ser2 gene cluster have now been used to clone and characterize the ser2 region from a strain of M. avium which produces rough and smooth colony forms and to identify the genetic differences between these morphotypes. Interstrain differences were seen to exist between the ser2 gene cluster of M. avium strains TMC 724 and 2151. In addition, two distinct rough (Rg) genotypes of strain 2151 were attributed to a deletion of approximately 28 kilobases from smooth variants, including the entire ser2 gene cluster. This particular deletion is thought to be mediated by recombination between repetitive sequences that flank both sides of the 28-kilobase excised region. The second genotype, seen in M. avium Rg-0 and Rg-1 variants, results from the deletion of an undefined amount of DNA from the right of the ser2 gene cluster. Reported separately (Belisle, J. T., McNeil, M. R., Chatterjee, D., Inamine, J. M., and Brennan, P. J. (1993) J. Biol. Chem. 268, 10510–10516) are the results of biochemical analyses of the glycopeptidolipid/lipopeptide population of the Rg variants which revealed that Rg-0 and Rg-1 possess lipopeptides devoid of all of the sugars of the glycopeptidolipids and are obviously biosynthetically precursors of the glycopeptido- lipids. These studies help formulate a definition of the physiological effects of glycolipid expression, the biosynthetic and genetic mechanisms involved in their formation, and toward an understanding of the role of M. avium as a serious opportunistic pathogen.

Members of the Mycobacterium avium complex form differ-

* The abbreviations used are: SmD, smooth domed; SmT, smooth transparent; Rg, rough; ELISA, enzyme-linked immunosorbent assay; GPL, glycopeptidolipid; LP, lipopeptide; ns-, nonspecific; ss-, serovar-specific; kb, kilobase(s).

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Experimental Procedures

Bacterial Strains and Growth—A list of bacterial strains used in this study is provided in Table I. Mycobacterium smegmatis strain

1 The abbreviations used are: SmD, smooth domed; SmT, smooth transparent; Rg, rough; ELISA, enzyme-linked immunosorbent assay; GPL, glycopeptidolipid; LP, lipopeptide; ns-, nonspecific; ss-, serovar-specific; kb, kilobase(s).
The pellet was suspended in 500 μl of breaking buffer and placed on ice for 15-20 min. Proteinase K (Sigma) was added to the beads, vortexed again for 10 s, and transferred to a new 1.5-ml microcentrifuge tube. Sodium dodecyl sulfate was added to each tube of homogenized cells to a final concentration of 1% and alcohol (24:1). The DNA was precipitated with 0.1 volume of 3 M sodium acetate and 1 volume of isopropl alcohol. The isolated DNA was dissolved in TE (10 mM Tris (pH 8), 0.1 mM EDTA) buffer and fixed on 7H10 agar supplemented with kanamycin (10 mg/ml). E. coli strain XL-I Blue (22) was grown on 7H10 agar or in Luria broth (22). M. avium strains were grown on 7H11 agar supplemented with kanamycin (10 mg/ml). Escherichia coli strains χ2764 and XL-I Blue were grown on LB agar or in Luria broth (22). E. coli strains were grown on 7H11 agar (23). The isolation of colony variants from strain 2151 was described elsewhere (20).

DNA Isolation—DNA was isolated from M. avium and M. smegmatis using a modification of the “bead beater” method (24). Mycobacterial cell pellets that had been stored at −70 °C were thawed and resuspended in breaking buffer (50 mM Tris (pH 8), 10 mM EDTA (pH 8), and 100 mM NaCl). The cells were transferred to graduated 2-ml screw cap conical tubes and pelleted; the target cell pellet volume was 200 μl. The pellet was suspended in 500 μl of breaking buffer and added to a screw cap tube with 1.0 ml of wet 0.5-mm zirconium beads (Bio-spec Products, Bartlesville, OK). The cells and beads were vortexed for 20–40 s at high speed and the supernatant subsequently transferred to a 1.5-ml microcentrifuge tube. Breaking buffer (500 μl) was added to the beads, vortexed again for 10 s, and transferred to a new 1.5-ml microcentrifuge tube. Sodium dodecyl sulfate was added to each tube of homogenized cells to a final concentration of 1% and placed on ice for 15–20 min. Proteinase K (Sigma) was added to a final concentration of 100 μg/ml and incubated at 55 °C for 30–45 min. The suspension was cooled to room temperature and extracted three times with an equal volume of phenol-chloroform-isomyl alcohol (25:24:1) followed by one extraction with chloroform-isomyl alcohol (24:1). The DNA was precipitated with 0.1 volume of 3 M sodium acetate and 1 volume of isopropl alcohol. The isolated DNA was dissolved in TE (10 mM Tris (pH 8), 0.1 mM EDTA) buffer and treated with RNAse (22) and, if necessary, hecadecyltrimethylammonium bromide was used to remove contaminating polysaccharides (23). Cosmid and plasmid DNA were isolated from E. coli by the alkaline lysis method (22). Construction of DNA Probes—The probes were derived from those cosmids used previously to define the M. avium strain TMC 724 ser2 gene cluster (19), with the exception of the 2.8-kb ClaI fragment that was isolated from pJTB232 during this study. The 3.5-, 7.4-, and 7.7-kb HindIII fragments were isolated from pJTBE21 (19), and the 12.5- and 14.8-kb HindIII fragments were isolated from pJTB11 (19). In some cases, the fragments were subcloned into pBluescript II SK− (Stratagene, La Jolla, CA) using E. coli strain XL-I Blue (26) as a host. DNA fragments were purified from preparative agarose gels by electroelution in an Elutrap chamber (Schleicher & Schuell) (27) and then labeled with digoxigenin using the Genius kit (Boehringer Mannheim).

Southern Blot Analysis of Genomic DNA—Genomic DNA isolated from smegmatis strain mc155, M. avium strain TMC 724, and the pure SmD, SmT, Rg-0, Rg-1, Rg-3, and Rg-4 variants of M. avium strain 2151 (20) was digested with HindIII. Approximately 500 ng of each digest was separated by electrophoresis through 0.7% agarose gels using 1 × TBE buffer (22) and transferred to Hybond N+ (International Scientific Products, Natick, MA) by electroblotting at 15 V for 16 h (24). The blots were UV cross-linked and hybridized with the desired probe using directions provided by Boehringer Mannheim for the Genius kit. The hybridization conditions allowed for 20% mismatch, and the washes were high stringency. The positive control on the blots was pJTB21 digested with HindIII.

Cloning and Mapping of the M. avium ser2 Gene Cluster—A cosmid library of M. avium strain TMC 724 SmD DNA was constructed in pYUB18 (24) as described previously (19). The library resulted in 10,000–11,000 E. coli χ2764 clones containing pYUB18.M. avium recombinant cosmids. These E. coli clones were lifted into nitrocellulose filters, lysed, and hybridized with a mixture of the 7.7-, 7.4-, and 3.5-kb probes (22). Colonies that produced positive hybridization signals were picked and grown in superbroth containing kanamycin (25 mg/ml). Recombinant cosmids were isolated (22) and mapped by restriction enzyme analysis using ClaI, DraI, HindIII, and SspI (22). These recombinant cosmids were also electroporated into M. smegmatis strain mc155 (21) and examined by colony dot-blot-ELISA and gas chromatography for expression of the serovar 2 oligosaccharide (19).

Detection of Genetic Differences between the Morphological Variants—Separately, we have reported the isolation of two distinct Rg phenotypes of M. avium serovar 2 strain 2151 (20). Both of these phenotypes lacked the nsGPLs and ssGPL-2; however, they differed in that Rg-0 and Rg-1 isolates produced a lipopeptide, an obvious GPL precursor, which was not expressed by the Rg-3 and Rg-4 isolates. Thus it was necessary to determine if the loss of GPL production by all Rg variants was caused by a deletion or inversion of the genomic DNA involved in GPL biosynthesis and to examine the genetic basis for the difference between the two rough phenotypes. To investigate these questions initially, pJTB21 (19) was utilized as the probe in a Southern blot analysis of genomic DNA (Fig. 1); the pJTB21 cosmid contains the entire ser2 gene cluster from M. avium serovar 2 strain 2151 (20). As expected, TMC 724 chromosomal DNA (lane 2) showed hybridization to fragments of 3.5, 7.4, and 14.8 kb. This pattern differed from that of the SmD and SmT morphotypes of strain 2151 (lanes 3 and 4), suggesting that there were restriction fragment length polymorphisms in the ser2 regions of these two serovar 2 strains. In addition, a striking difference was observed with the hybridization patterns of the 2151 Rg-0 (LP+) variant (lane 5) and the 2151 Rg-4 (LP−) variant (lane 6); both Rg variants lacked bands found in 2151 SmD and SmT, but different genomic fragments were missing. Although not shown in Fig. 1, the DNA from the Rg-1 (LP+) isolate produced a hybridization pattern identical to that of Rg-0 DNA, and the hybridization pattern of Rg-3 (LP−) was identical to that of Rg-4.

These initial hybridization data provided three pieces of information. First, minor interstrain differences were ob-

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<tr>
<th>TABLE I</th>
<th>Genomic Deletions and Rough Morphology in M. avium</th>
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<tr>
<td>Bacterial species and strain</td>
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<td>E. coli χ2764</td>
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Genomic Deletions and Rough Morphology in M. avium

The ser2 region of strain 2151 served as a genetic marker for the identification of genomic deletions and the construction of a rough phenotype. Genetic characterization of the ser2 region indicated that a repetitive element was present in this area (see following section), and so the probes were derived from the TMC 724 ser2 region. Fig. 4 shows the results of Southern blot analyses of genomic DNAs digested with HindIII and hybridized to the HindIII probes depicted in the bottom panel of Fig. 3; beginning with the probe from the left of the ser2 region of TMC 724, the panels of Fig. 4 “walk” across the ser2 region of the 2151 variants from left to right. The 12.5-kb probe (Fig. 4A), which represents the left flanking region of the TMC 724 gene cluster, hybridized to an HindIII fragment in the 18-kb region of TMC 724 (lane 1) and to an approximately 20-kb fragment from 2151 SmD DNA (lane 2). The other hybridizing fragments of TMC 724 (lane 2 of panels B–E) and 2151 SmD (lane 3 of panels B–E) were those expected from the comparative maps of the TMC 724 and 2151 SmD ser2 regions (top panel of Fig. 3).

The use of these same probes demonstrated that the Rg-0 or Rg-1 (LP+) and Rg-3 or Rg-4 (LP-) phenotypes arose from separate genomic deletions. Specifically, the Rg-0 and Rg-1 (LP+) isolates have lost the internal 18- and 3.5-kb HindIII fragments (lane 5 of Fig. 4, D and E, respectively; only Rg-0 is shown). At the same time, the two flanking probes of 12.5 and 7.4 kb both hybridized to a fragment of approximately 20 kb (lane 3 in Fig. 4A and lane 4 in Fig. 4D, respectively), whereas the 7.7-kb HindIII fragment was present in the Rg-0 variant just as it was in the SmD variant (lanes 3 and 4 in Fig. 4E). In all it appeared that an intervening sequence of greater than 21 kb, including the ser2 gene cluster, was deleted from the smooth morphotype to form M. avium 2151 Rg-0 (LP-). Analysis of DNA from Rg-3 or Rg-4 (LP+) variants indicated that these isolates have lost the 7.8- and 7.7-kb HindIII fragments (lane 5 of Fig. 4, D and E, respectively; only Rg-3 is shown) but retained the 20-, 18-, and 3.5-kb HindIII fragments present in the SmD variant (Fig. 4, A, B, and C, respectively). Thus the deletion attributed to the formation of the 2151 Rg-3 (LP+) isolate lies to the right of the deletion that gives rise to the Rg-0 (LP+) isolate.

Isolation of a 2.8-Kilobase Repetitive DNA Sequence—As was mentioned above, Southern blot analysis suggested that the ser2 region of strain 2151 contained a repetitive DNA sequence, and, indeed, mapping of overlapping clones revealed that a 2.8-kb Clai fragment appeared to be present in more than one copy (Fig. 2). The Clai digest of pJT226 contained a doublet at 2.8 kb, whereas pJTB231 and pJTB232 possessed only a single fragment of this size (Fig. 5). Analysis of these and other recombinant cosmids placed one of the 2.8-kb Clai fragments in the 7.8-kb HindIII fragment and the other in the 20-kb left flanking region. The distance separating these two Clai fragments was calculated to be 28 kb. Interestingly, these two Clai fragments lie in regions that flank the proposed deletion in the Rg-0 (LP+) variant of M. avium 2151. To confirm that the 2.8-kb Clai fragments were homologous, both

![Fig. 1. Southern blot analysis of HindIII-digested DNAs.](image-url)

Lane 1, pJTB21; lane 2, M. avium TMC 724; lane 3, M. avium 2151 SmD; lane 4, 2151 SmT; lane 5, 2151 Rg-0; lane 6, 2151 Rg-4. The probe used in this Southern blot was derived from total HindIII and EcoRI digests of pJT2B1.
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Fig. 2. Genomic and functional map of the ser2 gene cluster of M. avium 2151 SmD. The M. avium inserts from 10 of the recombinant cosmids are diagrammed along with results from the colony dot-blot-ELISA of M. smegmatis transformants possessing the above cosmids. The numerical designation of the cosmids matches the numerical designation of the recombinant M. smegmatis clones used in the colony dot-blot-ELISA. The expression of the ser2 gene cluster was examined by both colony dot-blot-ELISA and GC of lipid-associated sugars (19). C, ClaI; H, HindIII. +/+, recombinant ssGPL-2 detected by both colony dot-blot-ELISA and GC; -/-, no recombinant ssGPL-2 was detected by either colony dot-blot-ELISA or GC; +/−, recombinant ssGPL-2 expression was detected by GC but not by colony dot-blot-ELISA; ND/−, recombinant ssGPL-2 detection was not done by GC but was negative by colony dot-blot-ELISA.

FIG. 4. Southern blot analyses of HindIII-digested genomic DNA from M. avium TMC 724 and the morphological variants of M. avium 2151. The probes used were generated from HindIII fragments of the M. avium TMC 724 ser2 and flanking regions as shown in Fig. 3. Panel A, hybridization to the 12.5-kb probe. Lane 1, TMC 724; lane 2, 2151 SmD; lane 3, 2151 Rg-0; lane 4, 2151 Rg-4. Panel B, hybridization to the 14.8-kb probe. Lane 1, pJTB21; lane 2, TMC 724; lane 3, 2151 SmD; lane 4, 2151 Rg-0; lane 5, 2151 Rg-4. Panel C, hybridization to the 3.5-kb probe. Lane designations are the same as in panel B. Panel D, hybridization to the 7.4-kb probe. Lane designations are the same as in panel B. Panel E, hybridization to the 7.7-kb probe. Lane designations are the same as in panel B.

homologous (Fig. 6B, lanes 6 and 7). As expected, two HindIII fragments of 7.8 and 7.5 kb from pJTB226 (Fig. 6B, lane 2) hybridized strongly to the 2.8-kb probe. Additionally, the probe hybridized to the 7.8-kb fragment from pJTB231 (Fig. 6B, lane 3) and the 10-kb fragment from pJTB232 (Fig. 6B, lane 4). These observations established that the 2.8-kb ClaI fragment comprised all or part of a repetitive element and that copies were present in the locations indicated by restriction enzyme analysis. In addition, the 2.8-kb ClaI fragment hybridized weakly to the large fragments that contain portions

were isolated and used in hybridization studies. Fig. 6A shows an agarose gel of HindIII-digested cosmids and the 2.8-kb ClaI fragments isolated from pJTB232 and pJTB233; Fig. 6B shows the results obtained when a blot of this gel was hybridized with the labeled 2.8-kb ClaI fragment from pJTB232. This analysis revealed that these two 2.8-kb fragments were
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Fig. 5. An agarose gel of ClaI-digested recombinant cosmids which shows the duplication of a 2.8-kb ClaI fragment. Lane 1, 1-kb ladder (Bethesda Research Laboratories); lane 2, pJTB226; lane 3, pJTB231; lane 4, pJTB232; lane 5, high molecular weight DNA marker (Bethesda Research Laboratories). The white arrow points to the 2.8-kb doublet of pJTB226.

Fig. 6. Southern blot analysis demonstrating homology between the 2.8-kb repeats. Panel A, a 0.7% agarose gel of HindIII-digested recombinant cosmids and the purified 2.8-kb fragments from pJTB232 and pJTB233. Lane 1, 1-kb ladder; lane 2, HindIII-digested pJTB226; lane 3, HindIII-digested pJTB231; lane 4, HindIII-digested pJTB232; lane 5, HindIII-digested pJTB245; lane 6, the left 2.8-kb ClaI fragment isolated from pJTB232; lane 7, the right 2.8-kb ClaI fragment isolated from pJTB233; lane 8, high molecular weight DNA marker. Panel B, a Southern blot produced from the gel in panel A using the 2.8-kb ClaI fragment from pJTB232 as the probe.

of the 18-kb HindIII fragment of the M. avium 2151 ser2 gene cluster (Fig. 6B, lanes 2–4) and also to a calculated 28-kb HindIII fragment of pJTB245 (Fig. 6B, lane 5), which corresponded to the right flanking region of the 60 kb map (Fig. 2). This weak hybridization implied that either a smaller portion of the 2.8-kb ClaI fragment was repeated elsewhere or that these fragments contained sequences with limited homology to the 2.8-kb ClaI fragment.

To determine whether this 2.8-kb ClaI fragment comprised a highly repetitive sequence, Southern blot analysis of EcoRI-digested genomic DNA was performed using the 2.8-kb ClaI fragment as a probe (Fig. 7). There is one EcoRI site in the middle of the 2.8-kb ClaI fragment, and so the probe should hybridize to two EcoRI fragments per copy of the repeat. Hybridization was observed to multiple bands in DNA from both the smooth and rough variants of M. avium strain 2151. The patterns produced by hybridization to Rg-0 and Rg-3 (lanes 4 and 5 in Fig. 7) clearly differed, and these patterns could also be distinguished from those of the smooth variants. Together these observations lend further strength to the theorem that the 2.8-kb ClaI repeats may mediate the deletions that lead to rough isolates. Despite the fact that identical patterns were obtained for 2151 SmD and SmT when ser2-specific sequences were used as the probe (Fig. 1), hybridization with the 2.8-kb ClaI fragment produced different patterns (lanes 2 and 3 in Fig. 7), the significance of which is not known at this time. Of note is the observation that the M. avium serovar 2 laboratory strain TMC 724 possessed a smaller number of EcoRI fragments that hybridized to the 2.8-kb ClaI repeat. The nature of this repetitive sequence has not yet been determined. Restriction site comparisons indicate that the 2.8-kb ClaI fragment does not contain the M. avium insertion sequences IS901 (37) or IS1141; also, the latter is not related to the 2.8-kb ClaI repeat as judged by Southern blot hybridization.2

DISCUSSION

The bacterial world possesses numerous mechanisms by which gene expression is regulated. Many of these mechanisms, which work at the level of transcription, utilize genetic rearrangement (29). The work described herein has revealed that the genetic events responsible for the conversion of smooth morphological variants of M. avium strain 2151 to rough variants involved the deletion of large genomic regions. Extensive genomic mapping and hybridization studies have defined a deletion of approximately 28 kb which gave rise to rough morphological variants (Rg-0 and Rg-1) that were devoid of the ssGPL and the nsGPLs, as shown elsewhere (20), but were still capable of expressing lipopeptide intermediates. Interestingly, this 28-kb deletion encompasses the ser2 gene cluster and appears to be defined by two 2.8-kb repeats that flank the left and right side (Fig. 8). Previous

2 L. Via and J. Falkinham, personal communication.
between direct repeats of defined insertion (IS) elements (30). This type of mechanism can be readily attested for in the *M. avium* SmD. The proposed mechanism for the deletion of the 28-kb region which gives rise to *M. avium* 2151 Rg-0 is also depicted.

![Fig. 8. Location of the 2.8-kb Clal repeats (lined boxes) in relationship to the ser2 gene cluster (slashed box) of *M. avium* 2151 SmD. The proposed mechanism for the deletion of the 28-kb region which gives rise to *M. avium* 2151 Rg-0 is also depicted.](image)

The hypothesized mechanism by which the 28-kb genomic region of Rg-0 and Rg-1 is deleted is diagrammed in Fig. 8. As depicted in this figure, general recombination between the regions defined by the 2.8-kb Clai fragments would yield a deletion of 28 kb. However, for this to occur the 2.8-kb fragments need to be oriented as direct repeats. The basis for this type of mechanism can be readily attested for in the literature. In a number of large plasmids, such as the fertility (F) and the resistance (R) plasmids of Gram-negative bacteria, similar deletions are mediated through recombination between direct repeats of defined insertion (IS) elements (30). A paradigm of this mechanism is the 89.3-kb R100.1 plasmid of *E. coli* which loses a 23-kb region, termed the resistance determinant (r-det), through general recombination between two IS1 elements that are situated as direct repeats on opposite ends of the r-det (31). Further studies demonstrated that RecA and RecC are essential for the deletion of this 23-kb r-det region (32). Overall, the depicted mechanism for the 28-kb deletion in Rg-0 and Rg-1 is only speculative. Thus it is important not to exclude the possibility of site-specific recombination between small repetitive fragments. Site-specific recombination has been demonstrated as the means by which 11- and 55-kb regions of the genome of *Anabaena* spp. are deleted upon activation of nitrogen fixation and heterocyst formation (33-35).

A second significant finding from the present work is that there are interstrain differences between the ser2 gene clusters of *M. avium* serovar 2 strains TMC 724 and 2151. The ser2 gene cluster of TMC 724 was defined previously as a 22-27-kb region (19), whereas the current study demonstrated that only a 17.8-kb region was required for the 2151 ser2. Additionally, size variations occurred between a number of homologous restriction fragments in *M. avium* TMC 724 and 2151. The 7.4-kb HindIII fragment of TMC 724 is homologous to a 7.8-kb HindIII fragment of 2151. Both of these fragments are located between conserved 3.5- and 7.7-kb HindII fragments. The 14.8-kb HindIII fragment of TMC 724 is homologous to an 18-kb HindII fragment of 2151, and both of these fragments lie to the left of the conserved 3.5-kb fragment. The cause of these polymorphisms cannot be explained from the current data. However, a possible explanation may be that the size differences are a result of the activity of mobile genetic elements. Numerous insertion elements and transposons have been identified and characterized in other mycobacterial species (36), and an atypical insertion element designated IS901 was recently characterized in *M. avium* (37). It is further possible to speculate that these elements have been lost from the laboratory strain of *M. avium* (TMC 724) because of multiple passages. The presence of insertion or other mobile genetic elements may also explain the origin and evolution of the 28 serovars that make up the *M. avium* complex.

It is now clear that continued analysis of the *M. avium* rough and smooth variants is needed to define the precise mechanism of the above deletions and the origins of the observed polymorphisms. Additionally, the isolation and characterization of genes encoding the biosynthesis of the lipopeptide core will shed further light on the biosynthetic pathway of GPLs. The use of the above mutants as well as genetically engineered mutants should provide further valuable tools in deciphering the biological function of the GPLs.

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