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Molecular Characterization of Microbacterium Species Recovered from Clinical Ocular and Otic Samples

John G. Bartell, Deidra O. Ward, Linda L. Clark, Celeste H. McLean, David W. Stroman

Contact info:
John Bartell
Microbiology
Alcon Research Ltd.
6201 S. Freeway
Ft. Worth, TX 76134
john.bartell@alconlabs.com

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ABSTRACT

Background: *Microbacterium* have increasingly been recognized as clinical pathogens. A recent publication by Gneiding et al demonstrated extensive diversity within 50 clinical *Microbacterium* isolates belonging to 15 recognized species and 3 undescribed species (1). Our study described 132 *Microbacterium* isolates recovered over an 8 year period in clinical studies.

Methods: All isolates were recovered using standard methods and media at 35°C. Phenotypic characterization was performed using Biolog GP2 MicroPlate (Hayward, CA). DNA-based methods used to characterize the isolates included automated ribotyping (DuPont, Wilmington, DE) and 16S rRNA sequence analysis. The susceptibility profiles were determined for 20 antibiotics using broth dilution methods (per CLSI).

Results: A total of 132 isolates were collected from otic and ocular clinical studies. Molecular analysis determined that these isolates belonged to a total of 45 different *Microbacterium* species. These isolates belonged to 9 of the 18 species reported by Gneiding et al, 4 other species not previously reported from clinical samples and 32 additional undescribed species.

Conclusions: This study demonstrates a greater diversity of clinical *Microbacterium* species than has been previously reported. Antibiotic susceptibility profiles differed among the species. This study also underscores the necessity to take into account subtle differences in 16S rRNA gene sequence when comparative sequencing is utilized to identify *Microbacterium* species.

1. Gneiding, K., Frodl, R., and G. Funke. 2008. Identities of *Microbacterium* spp. encountered in human clinical specimens. J. Clin. Micro. 46:3646-3652.

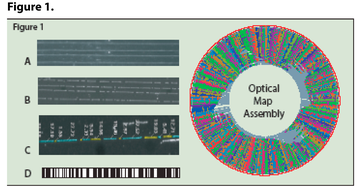
INTRODUCTION

The genus *Microbacterium* is comprised of 61 described species. They are typically considered to be environmental organisms, though recently they have been reported from clinical specimens. In order to better understand the diversity and frequency of this genus in clinical specimens, we have utilized molecular techniques and phenotypic analysis to characterize 137 *Microbacterium* isolates collected in human clinical studies.

MATERIALS AND METHODS

Phenotypic analyses were performed using GP2 MicroPlates (Biolog, Hayward, CA). End-point MICs were determined following CLSI recommended broth dilution methods. Automated ribotyping analysis was performed with EcoR I and Pvu II, using the Riboprinter (DuPont, Wilmington, DE). 16S rRNA gene sequencing was performed using the MicroSeq500 and the MicroSeq Full Gene 16S rDNA Bacterial Sequencing Kits (Applied Biosystems, Foster City, CA). Phylogenetic analysis of sequences was performed using MicroSeq software. Optical Mapping was used for comparative genome analysis. This technique utilizes whole genome restriction mapping and is depicted in Fig. 1.

Figure 1.



Optical Maps were constructed at OpGen (Gaithersburg, MD). (A) High molecular weight DNA from each isolate was immobilized as individual molecules onto an Optical Chip, (B) digested with restriction enzyme Kpn I and fluorescently stained with YOYO-1 (Invitrogen), (C) the stained restriction fragments were analyzed by automated fluorescent microscopy and the size and order of restriction fragments for each molecule were captured with OpGen software. Collections of molecule maps were then assembled according to overlapping fragment patterns to produce whole genome, ordered restriction maps - Optical Maps. (D) Vertical lines in the Optical Maps indicate the locations of cut sites and the space between lines represents the size of the restriction fragments.

RESULTS

137 *Microbacterium* isolates collected in clinical studies over eight years. The organisms were comprised of 18 skin isolates (cheek), 30 nasal isolates, 9 throat isolates, 22 otic isolates and 58 ocular isolates. They were identified to the species level by ribotyping using Pvu II and comparative sequencing of a 500 bp section of the 16S rRNA gene. It was determined that the organisms included 12 validly named species and 53 undescribed species.

Species	N	Source
Microbacterium binotii	1	nose
Microbacterium arborescens	1	ocular
Microbacterium aurum	10	otic (1) ocular (9)
Microbacterium esteraromaticum	3	cheek throat (2)
Microbacterium flavescens	1	nose
Microbacterium foliorum	1	cheek
Microbacterium hydrocarbonoxydans	1	cheek
Microbacterium imperiale	1	ocular
Microbacterium lacticum	1	nose
Microbacterium liquefaciens	1	ATCC strain 43647
Microbacterium maritypicum	1	ATCC strain 19260
Microbacterium oxydans	4	throat (3) DSM Strain 20578
Microbacterium paraoxydans	5	ocular (3) nose otic
Microbacterium testaceum	7	otic ocular nose (3) cheek (2)
Microbacterium sp. nov. "*alconae*"	4	otic (2) ocular (2)
Microbacterium sp. nov. "*harmaniae*"	5	otic (2) ocular (3)
Microbacterium sp. nov. "*otitidis*"	5	otic (2) ocular (3)
Undescribed *Microbacterium* species	90 isolates 50 different undescribed species	otic (12) ocular (36) cheek (13) throat (4) nose (25)

Table 1. Summary of isolates recovered in the studies with the source.

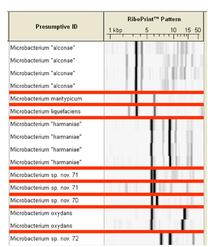


Figure 2. Pvu II ribopatterns of species within the *M. oxydans* cluster (*M. oxydans, M. liquefaciens, M. maritypicum,* and 5 undescribed species).

Figure 3. Neighbor Joining tree of full gene sequences (1,445 bp) of the 16S rRNA gene.

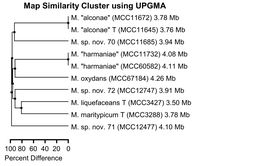


Figure 4. Optical mapping of isolates in the *M. oxydans* cluster.

Species	N	N-acetyl D-glucosamine	L-arabinose	Lactulose	α-methyl D-mannoside	Xylitol	L-lactic acid	mono-methyl succinate	alaninamide	glycyl-L-glutamic acid	L-pyroglutamic acid
M. "alconae"	4	-	+	-	+	-	+	+	+	+	-
M. "harmaniae"	5	-	+	-	+	+	+	-	+	+	-
M. oxydans	4	-	+	-	+	+	+	-	+	-	-
M. liquefaciens	1	-	-	-	-	-	+	-	+	-	-
M. maritypicum	1	+	-	-	-	-	-	-	+	-	-
M. arborescens	2	+	+	v	v	-	+	-	+	-	-
M. aurum	4	+	-	-	-	-	+	-	v	-	-
M. binotii	1	-	+	-	-	-	-	+	v	-	-
M. esteraromaticum	3	+	-	-	-	+	+	-	+	-	-
M. flavescens	1	-	+	-	-	-	-	-	-	-	-
M. folionum	1	+	-	-	-	-	-	-	-	-	-
M. hydrocaboxonoxydans	1	+	-	-	-	-	-	+	+	+	+
M. imperiale	1	+	+	+	+	-	+	-	+	-	-
M. lacticum	1	+	-	-	-	-	-	-	-	-	-
M. paraoxydans	5	-	-	-	+	-	+	+	+	+	+
M. testaceum	7	-	+	-	-	-	+	+	+	-	-
M. "otitidis"	5	+	+	+	-	+	+	-	-	-	-
M. sp. nov. 1	1	+	-	-	+	-	+	-	+	+	-
M. sp. nov. 2	4	-	-	-	+	+	+	-	+	+	-
M. sp. nov. 4	1	-	+	-	-	-	-	+	+	-	-
M. sp. nov. 5	1	-	+	-	-	-	+	-	+	-	-
M. sp. nov. 7	1	-	-	-	-	-	+	-	+	+	-
M. sp. nov. 8	4	-	+	-	-	-	+	-	+	-	-
M. sp. nov. 9	5	+	-	-	-	-	+	v	-	-	-
M. sp. nov. 10	1	+	-	-	-	-	+	-	+	+	-
M. sp. nov. 11	3	-	+	-	-	-	+	-	+	-	-
M. sp. nov. 12	1	+	+	-	-	-	+	-	+	-	-
M. sp. nov. 13	1	-	+	-	-	-	+	-	+	-	-
M. sp. nov. 16	1	+	+	+	+	-	+	+	+	+	-
M. sp. nov. 20	1	+	+	-	-	-	+	+	+	-	-
M. sp. nov. 23	1	-	-	-	-	-	+	-	+	-	-
M. sp. nov. 24	1	+	-	-	-	-	+	-	-	-	-
M. sp. nov. 25	1	-	-	-	-	-	+	-	+	-	-
M. sp. nov. 29	1	+	+	-	+	-	-	-	+	v	-
M. sp. nov. 31	1	-	-	-	-	-	+	-	+	-	-
M. sp. nov. 32	2	-	-	-	+	+	+	+	+	+	+
M. sp. nov. 33	1	+	+	+	-	-	+	-	+	-	-
M. sp. nov. 34	3	-	+	-	-	-	-	-	+	-	-
M. sp. nov. 35	1	+	+	-	-	-	+	-	+	-	-
M. sp. nov. 36	3	+	+	-	-	-	+	-	+	-	-
M. sp. nov. 37	1	-	+	-	-	-	+	-	+	-	-
M. sp. nov. 40	1	-	-	-	-	-	+	+	-	-	-
M. sp. nov. 42	1	-	+	-	-	-	+	-	+	-	-
M. sp. nov. 43	1	-	+	-	-	-	-	-	+	-	-
M. sp. nov. 46	1	-	+	-	-	-	+	-	+	-	-
M. sp. nov. 48	1	-	+	-	+	+	+	-	+	+	-
M. sp. nov. 51	1	-	+	-	-	-	-	+	-	-	-
M. sp. nov. 56	1	-	+	-	-	-	+	-	+	-	-
M. sp. nov. 58	1	-	+	-	-	-	-	-	+	-	-
M. sp. nov. 59	2	-	+	-	-	-	-	+	+	-	-
M. sp. nov. 61	1	+	-	-	-	-	-	+	-	-	-
M. sp. nov. 62	1	-	-	-	-	-	+	-	+	+	-
M. sp. nov. 63	1	-	+	-	-	-	+	-	+	-	-
M. sp. nov. 64	1	+	+	-	-	-	+	+	+	-	-
M. sp. nov. 66	1	-	+	-	-	-	+	+	+	-	-
M. sp. nov. 70	1	+	+	-	+	-	+	+	+	+	+
M. sp. nov. 72	2	-	-	-	+	-	+	+	+	+	v
M. sp. nov. 73	1	-	-	-	+	+	-	-	+	-	-
M. sp. nov. 74	1	-	+	-	-	-	-	+	+	-	-
M. sp. nov. 75	1	-	-	-	-	-	+	-	-	+	-

Table 2. Phenotypic characteristics of *Microbacterium* species.

Species	N	Ciprofloxacin	Moxifloxacin	Gentamicin	Erythromycin	Clindamycin	Tetracycline	Minocycline	Trimethoprim	Sulphamethoxazole	Vancomycin	Penicillin
M. "alconae"	4	1.0 – 2.0	0.25 – 0.25	0.50 – 0.50	1.0 – 2.0	2.0 – 4.0	1.0 – 2.0	0.06 – 0.06	1.0 – 1.0	2.0 – 4.0	16 – 16	1.0 -1.0
M. "harmaniae"	5	2.0 – 4.0	0.25 – 0.25	0.50 – 4.0	0.06 – 0.25	1.0 – 2.0	1.0 – 2.0	<0.03 – 0.06	0.50 – 1.0	1.0 – 8.0	8.0 – 8.0	2.0 – 2.0
M. liquefaciens	1	1.0	0.25	0.25	<0.03	0.50	0.50	≤0.03	0.50	>512	0.25	0.5
M. maritypicum	1	1.0	0.12	0.12	0.06	0.25	0.50	<0.03	1.0	>512	2.0	1.0
M. oxydans	4	2.0 – 4.0	0.25 – 0.25	0.25 – 1.0	0.50 – 1.0	2.0 – 4.0	64 – 64	0.06 – 0.12	2.0 – 2.0	8.0 – >512	8.0 – 8.0	2.0 – 2.0
M. arborescens	1	1.0	0.12	2.0	1.0	1.0	2.0	0.12	>256	128	2.0	0.50
M. aurum	3	0.25 – 1.0	0.12 – 0.12	0.03 – 2.0	0.06 – 32	0.03 – 1.0	0.12 – 2.0	<0.03 – 0.12	0.25 – 2.0	8.0 – 256	0.12 – 128	0.25 – 1.0
M. binotii	1	4.0	0.50	1.0	0.25	0.50	4.0	0.12	>256	64	0.50	2.0
M. esteraromaticum	3	1.0 – 4.0	0.25 -0.50	0.50 – 1.0	<0.03 – 0.12	0.50 – 4.0	1.0 – 1.0	0.06 – 0.12	1.0 – 4.0	>512 –>512	0.12 – 0.25	0.25 – 8.0
M. folionum	1	2.0	0.12	0.12	0.06	0.25	32	0.50	<0.06	0.25	0.50	0.50
M. hatmannsis	1	1.0	0.25	2.0	0.06	1.0	1.0	0.12	16	64	0.25	0.25
M. hydrocarbonoxydans	1	1.0	0.12	16	<0.03	<0.015	4.0	0.12	0.50	2.0	1.0	0.50
M. imperiale	1	0.5	0.12	2.0	2.0	4.0	1.0	0.06	>256	256	0.50	0.50
M. lacticum	1	2.0	0.12	0.50	<0.03	1.0	0.50	0.12	1.0	1.0	0.25	2.0
M. paraoxydans	5	2.0 – 2.0	0.25 – 2.0	1.0 – 2.0	0.06 – 2.0	2.0 – 4.0	4.0 – 4.0	0.06 – 0.12	1.0 – 2.0	4.0 – >512	4.0 – 8.0	1.0 – 2.0
M. testaceum	7	2.0 – 4.0	0.25 – 0.50	0.50 – 4.0	0.06 – 32	2.0 – 8.0	2.0 – 4.0	0.12 – 0.25	2.0 – 32	>512	2.0 – 8.0	2.0 – 8.0
M. sp. nov. 1	1	1.0	0.50	0.06	<0.03	0.50	2.0	<0.03	1.0	<0.03	0.25	<0.015
M. sp. nov. 2	4	1.0	0.50	0.12	<0.03	1.0	1.0	0.25	2.0	16	0.25	1.0
M. sp. nov. 4	1	0.50	0.12	0.12	<0.03	1.0	1.0	0.12	0.25	0.50	0.25	0.50
M. sp. nov. 5	1	1.0	0.25	0.25	32	2.0	2.0	1.0	0.06	1.0	64	0.25	1.0
M. sp. nov. 7	1	0.50	0.12	2.0	<0.03	4.0	0.50	0.06	0.50	128	0.25	1.0
M. sp. nov. 8	4	1.0 – 2.0	0.12 – 0.12	0.50 – 0.50	0.25 – 0.25	1.0 – 1.0	2.0 – 4.0	0.06 – 0.06	0.50 – 1.0	2.0 – 2.0	8.0 – 16	1.0 – 1.0
M. sp. nov. 9	5	2.0	0.25	1.0	0.25	0.03	2.0	0.06	1.0	32	2.0	1.0
M. sp. nov. 10	1	2.0	0.50	1.0	0.25	1.0	4.0	0.12	>256	32	8.0	2.0
M. sp. nov. 11	3	0.50	0.12	2.0	<0.03	1.0	1.0	0.06	1.0	32	0.50	<0.015
M. sp. nov. 12	1	4.0	0.12	16	0.25	2.0	1.0	0.06	2.0	2.0	0.50	0.50
M. sp. nov. 13	1	1.0	0.12	0.12	<0.03	1.0	1.0	0.06	0.50	32	0.50	<0.015
M. sp. nov. 16	1	4.0	0.12	0.12	<0.03	1.0	2.0	0.12	>256	32	8.0	2.0
M. sp. nov. 20	1	1.0	0.12	0.12	<0.03	1.0	1.0	0.12	0.50	0.50	0.50	0.50
M. sp. nov. 23	1	1.0	0.12	0.12	<0.03	0.50	1.0	0.06	0.50	128	0.25	1.0
M. sp. nov. 24	1	1.0	0.25	2.0	0.50	2.0	2.0	0.06	1.0	64	4.0	2.0
M. sp. nov. 25	1	2.0	0.25	1.0	0.25	0.03	2.0	0.06	1.0	32	2.0	1.0
M. sp. nov. 29	1	2.0	0.50	1.0	0.25	1.0	4.0	0.12	>256	32	8.0	2.0
M. sp. nov. 31	1	0.50	0.12	2.0	<0.03	1.0	1.0	0.06	1.0	32	0.50	<0.015
M. sp. nov. 32	2	1.0	0.25	0.25	0.03	2.0	0.06	0.06	2.0	2.0	0.50	0.50
M. sp. nov. 33	1	16	8.0	0.50	<0.03	<0.015	1.0	0.06	>256	32	0.50	2.0
M. sp. nov. 34	3	32 – 64	1.0 – 2.0	1.0 – 2.0	0.03 – 0.06	0.015 – 0.25	1.0 – 1.0	0.06 – 0.12	4.0 – 16	>512	0.50 – 1.0	0.06 – 0.12
M. sp. nov. 35	1	32 – 128	4.0 – 16	1.0 – 2.0	<0.03 – 64	0.06 – 0.25	1.0 – 4.0	0.06 – 0.25	1.0 – 32	4.0 – >512	0.25 – 5.0	0.50 – 1.0
M. sp. nov. 36	3	1.0	0.12	4.0	1.0	2.0	4.0	0.12	>256	64	0.50	1.0
M. sp. nov. 37	1	16	2.0	8.0	0.25	2.0	2.0	0.06	4.0	256	2.0	1.0
M. sp. nov. 40	1	32 – 128	4.0 – 8.0	1.0 – 4.0	≤0.03 – 1.0	0.12 – 1.0	1.0 – 32	0.06 – 0.50	2.0 – 16	1.0 – > 512	0.25 – 0.50	0.12 – 1.0
M. sp. nov. 42	1	32 – 64	4.0 – 8.0	1.0 – 4.0	<0.03 – 0.06	0.03 – 0.06	1.0 – 4.0	0.12 – 0.25	4.0 – 8.0	16 – 512	0.25 – 0.50	0.50 – 0.50
M. sp. nov. 43	1	64	4.0	2.0	<0.03	0.06	1.0	0.06	0.12	32	0.25	0.12
M. sp. nov. 46	1	64	4.0	2.0	<0.03	0.06	1.0	0.06	4.0	64	0.25	0.25
M. sp. nov. 48	1	64	4.0	2.0	16	0.06	2.0	0.12	1.0	16	0.50	0.50
M. sp. nov. 51	1	16 – 64	4.0 – 8.0	0.25 – 0.50	<0.03 – 4.0	0.06 – 0.25	1.0 – 1.0	0.06 – 0.12	4.0 – 8.0	1.0 – > 512	0.25 – 0.50	0.25 – 0.50
M. sp. nov. 56	1	32	4.0	2.0	≤0.03	1.0	2.0	0.06	>256	32	0.25	0.25
M. sp. nov. 58	1	128	2.0	2.0	≤0.03	0.06	1.0	0.12	2.0	256	0.25	0.06
M. sp. nov. 59	1	32	2.0	4.0	0.12	2.0	2.0	0.12	1.0	512	0.50	1.0
M. sp. nov. 61	1	32	4.0	2.0	0.06	0.25	1.0	0.06	4.0	512	0.50	0.50
M. sp. nov. 62	1	32	4.0	2.0	0.12	0.50	1.0	0.06	>256	512	0.50	0.25
M. sp. nov. 63	1	64	4.0	128	0.25	0.50	1.0	0.06	>256	512	0.50	0.25
M. sp. nov. 64	1	8.0	1.0	0.50	0.12	0.12	0.25	0.25	64	512	0.25	1.0
M. sp. nov. 65	1	128	4.0	32	0.12	4.0	4.0	0.06	8.0	512	0.25	0.50
M. sp. nov. 66	1	32	4.0	2.0	<0.03	0.50	1.0	0.06	4.0	64	0.25	0.50
M. sp. nov. 67	1	8.0	2.0	0.25	<0.03	0.06	0.50	0.06	>256	512	0.12	0.50
	1	32	2.0	4.0	0.12	2.0	4.0	0.12	>256	512	1.0	0.25

Table 3. Susceptibility profiles. Several *Microbacterium* species exhibit intrinsic resistance to fluoroquinolones. Resistance to sulphamethoxazole is frequent in *Microbacterium*. Acquired resistance to erythromycin, trimethoprim, penicillin and vancomycin was observed occasionally. High level vancomycin resistance was observed in one of three *M. aurum* isolates.

CONCLUSIONS

- The *Microbacterium* genus contains many undescribed species recovered from human specimens.
- Pvu II ribotyping provided species level differentiation of all *Microbacterium* species in this study. DNA from most *Microbacterium* is not cut by EcoRI, though ribopatterns were generated using this enzyme with isolates from *M. testaceum* and 7 undescribed species.
- Accurate differentiation and identification of *Microbacterium* species by 16S rRNA gene sequence should take into account that the sequence difference between two species can be as low as 2 nucleotides in this genus.
- Optical mapping has proven to be a powerful tool to demonstrate the genomic differences between isolates that appear to be closely related by conventional analysis.

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