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Phylogenetic Framework and Molecular Signatures for the Main Clades of the Phylum *Actinobacteria*

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INTRODUCTION

The phylum *Actinobacteria*, which is comprised mainly of Gram-positive organisms with a high G+C content (>55 mol% in genomic DNA), constitutes one of the largest phyla within the *Bacteria* (76, 103, 192, 193, 283, 284). The different genera that are part of this phylum exhibit enormous diversity in terms of their morphology, physiology, and metabolic capabilities (76, 277, 313). The morphologies of actinobacterial species vary from coccoid (e.g., *Micrococcus*) or rod-coccoid (e.g., *Arthrobacter*) to fragmenting hyphal forms (e.g., *Nocardia*) or highly differentiated branched mycelia (e.g., *Streptomyces*) (8). Spore formation, although common, is not ubiquitous among actinobacteria, and they could range from motile zoospores to specialized propagules (182). The species of this group also exhibit enormous physiological diversity, as evidenced by their production of numerous extracellular enzymes and thousands of metabolic products that they synthesize and excrete (42, 256), many of which are antibiotics (65, 146, 182). The phylum *Actinobacteria* also constitutes one of the earliest lineages within the prokaryotes (119, 122, 168, 179), and the production of antibiotics by them has been indicated to be an important determining factor in the evolution of both the *Archaea* and Gram-negative (diderm) bacteria from Gram-positive (monoderm) bacteria (119, 120, 124, 129, 311).

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The most extensively studied representatives of this group include soil-dwelling *Streptomyces* spp., which are the major producers of antibiotics (18, 41, 145, 146, 219, 314), and important human pathogens of the genus *Mycobacterium* (*M. tuberculosis* and *M. leprae*), which are responsible for the largest number of human deaths from bacterial infections (17, 53, 56, 252, 305). However, the genera *Streptomyces* and *Mycobacterium* constitute only 2 of the genera within this large phylum that contains >300 genera (77, 343). In addition, there are huge populations of poorly studied actinobacteria that are prevalent in soil, water, deep-sea, or extreme environments, such as arctic ice, chemically contaminated sites, and radioactive environments, or that reside with humans, animals, and plants in a friendly or hostile way (14, 35, 85, 202, 205, 270, 307, 314). In recent years, due to rapid advances in genome-sequencing technologies, increasing progress is being made in studying the diversity and biology of *Actinobacteria*. The main focuses of these studies have been on bacteria that either produce or have the potential for the discovery of novel useful natural products (e.g., *Streptomyces*, *Salinispora*, *Saccharopolyspora*, *Cellulomonas*, *Verrucosispora*, *Pseudonocardia*, and *Micromonospora*) (12, 16, 21, 36, 86, 220, 249) or on pathogenic *Actinobacteria* that cause severe human and animal diseases or agricultural losses (e.g., *Mycobacterium*, *Actinomyces*, *Renibacterium*, *Atopobium*, *Gordonia*, *Gardnerella*, *Leifsonia*, and *Clavibacter*) (36, 69, 105, 219, 287). Extensive work has also been carried out on the *Bifidobacteriales*, which form a major component of the microbial flora in the gastrointestinal tracts of humans and other mammals and are believed to exhibit useful probiotic activities (183, 307, 313, 314, 317). In addition, the exploration of other industrially important species (e.g., *Corynebacterium*, *Rhodococcus*, *Micrococcus*, *Cellulomonas*, *Acidothermus*, *Thermobifida*, and *Nocardioides*) and environmentally beneficial species (e.g., *Arthrobacter*, *Kocuria*, *Frankia*, *Kineococcus*, *Pseudonocardia*, and *Rubrobacter*) has been greatly facilitated by the development of technology and the urgency for new biosources (9, 14, 85, 150, 159, 189, 194, 196, 202, 216, 296).

In view of the medical, biotechnological, and ecological importance of the *Actinobacteria*, an understanding of the evolutionary relationships among members of this large phylum and what unique biochemical or physiological characteristics distinguish species of different clades of *Actinobacteria* is of great importance and significance (97, 110, 130, 132, 283, 323, 324). Currently, the phylum *Actinobacteria* is delineated from other bacteria solely on the basis of its branching position in 16S rRNA gene trees. The most recently published taxonomy of *Actinobacteria*, by Zhi et al. (343), divided this phylum at the highest level into four subclasses, namely, *Actinobacteridae*, *Acidimicrobidae*, *Coriobacteridae*, and *Rubrobacteridae*, which together encompassed 219 genera in 50 families (104, 280). In an updated version of this taxonomy in the List of Prokaryotic Names with Standing in Nomenclature, maintained by J. P. Euzeby (http://www.bacterio.cict.fr), the phylum *Actinobacteria* at the highest level is now divided into five subclasses, namely, *Actinobacteridae*, *Acidimicrobidae*, *Coriobacteridae*, *Nitriliruptoridae*, and *Rubrobacteridae*. These subclasses are further subdivided into a number of different orders and suborders (Fig. 1A) (343). It is noteworthy that in this taxonomy, 47 of the 57 families within the phylum *Actinobacteria* are part of a single subclass, *Actinobacteridae*, whereas the other four subclasses together contained only 10 families.

Recently, another update of the taxonomy of the phylum *Actinobacteria* based upon 16S rRNA trees was reported (191), which will form the basis of the section on *Actinobacteria* in the forthcoming *Bergey's Manual of Systematic Bacteriology* (191). Although the phylogenetic information on which this update is based is not posted on the Bergey's Manual Trust website, in the revised taxonomy, the taxonomic ranks of subclasses and suborders are eliminated, and they are now elevated to the ranks of classes and orders, respectively (Fig. 1B). At the highest level, the phylum *Actinobacteria* is now divided into six classes, namely, *Actinobacteria*, *Acidimicrobiia*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria*, and *Thermoleophilia*. The class *Actinobacteria* now contains a total of 15 orders, including both previously proposed orders *Actinomycetales* and *Bifidobacteriales* (343). However, the order *Actinomycetales* is now restricted to the members of the family *Actinomycetaceae*, and the other suborders that were previously part of this order are now designated as distinct orders.

Although the taxonomic classification of the phylum *Actinobacteria* deduced on the basis of 16S rRNA trees represents an important advancement (103, 191, 283, 343), the compact clustering of different actinobacterial orders in the rRNA trees makes it difficult to determine reliably the interrelationships or branching order of the higher taxonomic clades within this phylum. This is especially true for its largest class, *Actinobacteria*, which accounts for >80% of all known actinobacterial families/genera (97, 103). Additionally, in the current classification scheme, all taxa higher than the rank of genus are distinguished primarily on the basis of taxon-specific 16S rRNA signature nucleotides (343). However, these signature nucleotides are based on published 16S rRNA sequences of type strains, and they change when new sequences are added to the databases (283, 343). There is also not much information available regarding the specificity of these signatures or their predictive ability to identify species belonging to these taxa. Although other phenotypic characteristics, such as morphological, physiological, and chemotaxonomic features, are useful for preliminary classifications and identifications of many spore-forming *Actinobacteria*, their levels of congruence are low (76, 103). Thus, in order to develop a reliable and stable understanding of this phylum, novel and more definitive characteristics need to be identified to define and distinguish the phylum *Actinobacteria* and its different lineages in clearer terms.

The rapidly increasing numbers of genome sequences provide an important resource to study *Actinobacteria* from different perspectives (211). This review focuses on the use of available genome sequences to discover novel molecular characteristics that are specific for the phylum *Actinobacteria* and its various lineages and their applications to develop a reliable evolutionary framework for the members of this phylum. However, before focusing on these aspects, a brief overview of some general features of the sequenced actinobacterial genomes is provided.

OVERVIEW OF GENOMIC CHARACTERISTICS OF *ACTINOBACTERIA*

Genomic characteristics of limited numbers of *Actinobacteria* have been described by various authors (17, 167, 314, 339) (see Table 1 for other references). In the latest comprehensive review on this subject by Ventura et al. (314), the features of 20 actinobacterial genomes that were available in 2007 were summarized. However, since the publication of that review, the number of sequenced actinobacterial genomes has increased more than 8 times (157 complete and 474 in progress), providing an abundant re-

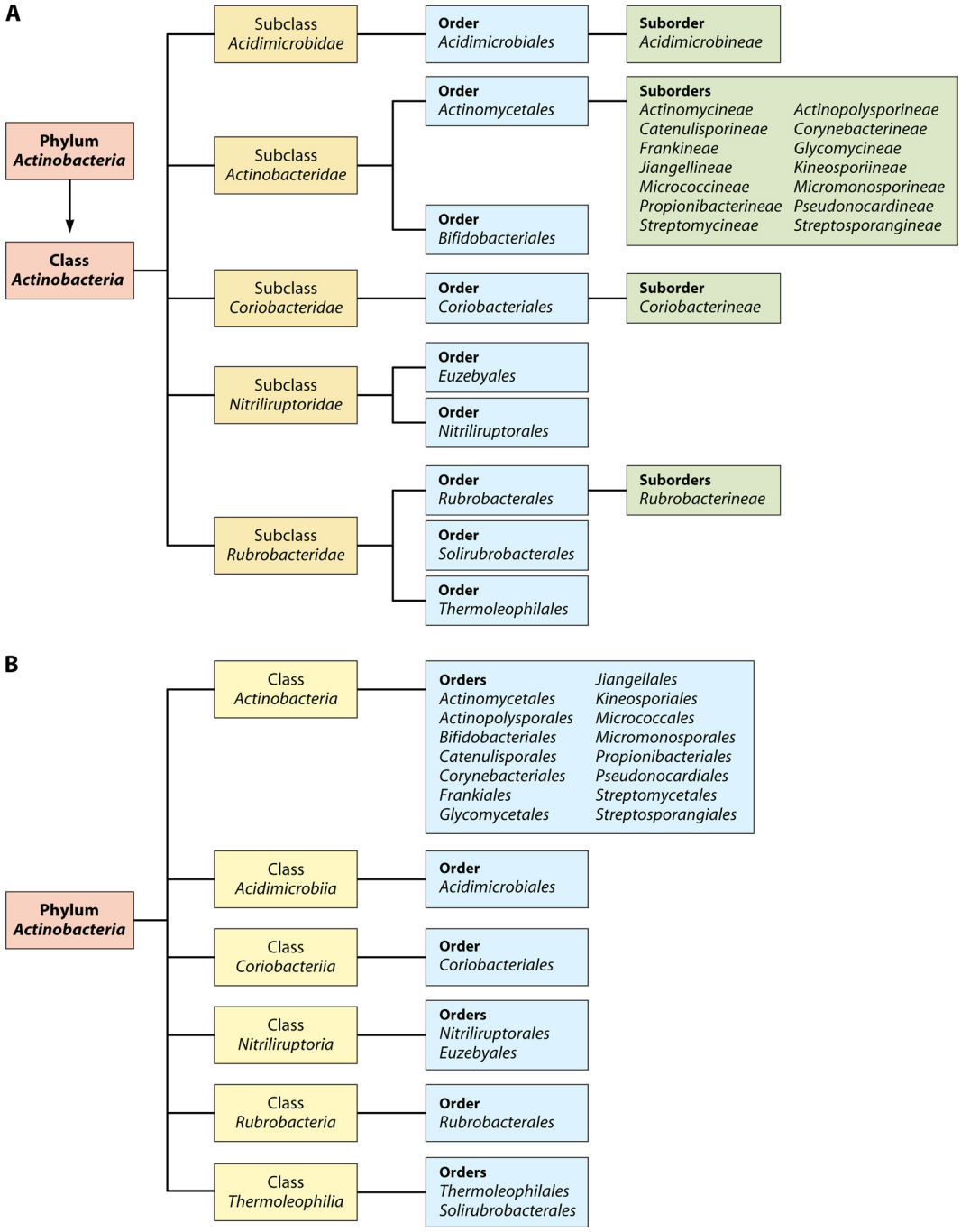

FIG 1 Current taxonomic outline for the phylum *Actinobacteria* based upon the List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.cict.fr/classifphyla.html#Actinobacteria) (A) and proposed taxonomy for *Actinobacteria* in the forthcoming *Bergey's Manual of Systematic Bacteriology* (191) (B).

source for such studies. The enormous phenotypic diversity of the *Actinobacteria* is well reflected in their genotypes. Some features of the completed actinobacterial genomes are summarized in Table 1. The sequenced genomes varied in size from 0.93 Mb (*Tropheryma whipplei*) to 12 Mb (*Streptomyces bingchenggensis*), and their GC contents varied from 41.5% (*Gardnerella vaginalis* ATCC 14019) to 74.2% (*Kineococcus radiotolerans* SRS30216) (9, 20, 30, 196). Interestingly, of these genomes, species of at least 4 genera have linear chromosomes, including *Streptomyces*, *Rhodococcus*, *Gordonibacter*, and *Kineococcus* (9, 44, 165, 196, 257, 261), These linear chromosomes are characterized by a central replication origin (*oriC*) and terminal inverted repeats (9, 47, 196, 257, 314). The mechanism for chromosome linearization was proposed previously to arise from recombination with linear plasmids that have evolved by the integration of bacteriophages (44, 321). Based upon the current taxonomy of the *Actinobacteria* (Fig. 1) and a phylogenetic tree for the sequenced species of this phylum (Fig. 2), the 4 genera containing the linear chro-

TABLE 1 Characteristics of sequenced actinobacterial genomes[c]

Actinobacterial genome	Size (Mb)[e]	% GC content	No. of proteins	GOT (°C)[a]	Habitat[b]	Source or reference[d]
Acidimicrobium ferrooxidans DSM 10331	2.16	68.3	1,964	Ther	S	DOEJGI
Acidothermus cellulolyticus 11B	2.4	66.9	2,157	58	A	14
Actinosynnema mirum DSM 43827	8.25	73.7	6,916	Meso	T	DOEJGI
Amycolatopsis mediterranei U32	10*	—	9,228	Meso	—	341
Amycolicicoccus subflavus DQS3-9A1	4.83*	—	4,557	—	—	COE, Beijing University
Arcanobacterium haemolyticum DSM 20595	2	—	1,731	Meso	H	336
Arthrobacter arilaitensis Re117	3.96*	—	3,376	—	—	203
Arthrobacter aurescens TC1	5.23	62.4	4,041	30	T	202
Arthrobacter chlorophenolicus A6	4.99	66	3,885	Meso	T	DOEJGI
Arthrobacter phenanthrenivorans Sphe3	4.58*	65.7	3,843	30	T	DOEJGI
Arthrobacter sp. strain FB24	5.08	65.4	4,146	Meso	T	DOEJGI
Atopobium parvulum DSM 20469	1.54	45.7	1,353	Meso	H	60
Beutenbergia cavernae DSM 12333	4.7	73.1	4,197	Meso	T	DOEJGI
Bifidobacterium adolescentis ATCC 15703	2.1	59.2	1,631	37	H	Gifu University, Japan
Bifidobacterium animalis subsp. lactis AD011	1.9	60.5	1,528	39	M	164
Bifidobacterium animalis subsp. lactis BB-12	1.9*	60.5	—	Meso	M	102
Bifidobacterium animalis subsp. lactis Bl04	1.9	60.5	1,567	39	M	15
Bifidobacterium animalis subsp. lactis DSM 10140	1.9	60.5	1,566	39	M	15
Bifidobacterium animalis subsp. lactis V9	1.9*	60.5	—	Meso	M	292
Bifidobacterium bifidum PRL2010	2.2*	—	1,706	Meso	—	306
Bifidobacterium bifidum S17	2.2	—	1,783	—	—	344
Bifidobacterium breve ACS-071-V-Sch8b	2.3*	—	—	—	—	JCVI
Bifidobacterium dentium Bd1	2.6*	58.5	2,129	Meso	—	318
Bifidobacterium longum DJO10A	2.41	60.2	1,990	37-41	H	183
Bifidobacterium longum NCC2705	2.26	60.1	1,727	37-41	H	254
Bifidobacterium longum subsp. infantis 157F	2.41	59.9	1,991	Meso	H	93
Bifidobacterium longum subsp. infantis ATCC 15697	2.8	59.9	2,416	37-41	H	262
Bifidobacterium longum subsp. longum BBMN68	2.3*	—	1,806	—	—	141
Bifidobacterium longum subsp. longum F8	2.4*	—	—	Meso	H	MetaHIT
Bifidobacterium longum subsp. longum JCM 1217	2.4*	—	1,924	Meso	—	93
Bifidobacterium longum subsp. longum JDM301	2.5*	—	1,958	37-41	H	326
Brachybacterium faecium DSM 4810	3.6	72.0	3,068	—	T	180
Catenulispora acidiphila DSM 44928	10.47	69.8	8,913	Meso	T	59
Cellulomonas fimi ATCC 484	4.3*	—	3,761	Meso	T	DOEJGI
Cellulomonas flavigena DSM 20109	4.1*	74.1	3,678	Meso	T	2
Clavibacter michiganensis NCPPB 382	3.4	72.5	2,984	25-28	M	105
Clavibacter michiganensis subsp. sepedonicus	3.44	72.4	2,941	25-28	M	19
Conexibacter woesei DSM 14684	6.4*	72.7	5,914	Meso	T	234
Coriobacterium glomerans PW2	2.1*	60	1,768	Meso	H	DOEJGI
Corynebacterium aurimucosum ATCC 700975	2.83	60.6	2,531	Meso	H	304
Corynebacterium diphtheriae NCTC 13129	2.49	53.5	2,272	37	M	39
Corynebacterium efficiens YS-314	3.1*	63.1	2,938	30-45	M	213
Corynebacterium glutamicum ATCC 13032	3.3	53.8	2,993	30-40	M	159
Corynebacterium glutamicum R	3.35	54.1	3,052	30-40	M	339
Corynebacterium jeikeium K411	2.51*	61.4	2,104	Meso	M	297
Corynebacterium kroppenstedtii DSM 44385	2.4	57.5	2,018	Meso	H	298
Corynebacterium pseudotuberculosis 1002	2.3*	—	—	Meso	—	267
Corynebacterium pseudotuberculosis C231	2.3*	—	—	Meso	—	267
Corynebacterium pseudotuberculosis FRC41	2.3*	—	2,110	—	—	267
Corynebacterium pseudotuberculosis I19	2.3*	—	—	—	—	267
Corynebacterium ulcerans 809	2.5*	—	—	—	—	Bielefeld University
Corynebacterium ulcerans BR-AD22	2.6*	—	—	—	—	Bielefeld University
Corynebacterium urealyticum DSM 7109	2.4	64.2	2,024	Meso	H	299
Cryptobacterium curtum DSM 15641	1.6	50.9	1,357	—	—	195
Eggerthella lenta DSM 2243	3.63	64.2	3,070	Meso	H	251
Frankia alni ACN14a	7.5	72.8	6,711	Meso	H	216
Frankia sp. strain CcI3	5.4	70.1	4,499	Meso	M	216
Frankia sp. strain EAN1pec	9	71.2	7,191	Meso	M	DOEJGI
Frankia symbiont of *Datisca glomerata*	5.32*	70.1	—	Meso	—	DOEJGI
Gardnerella vaginalis 409-05	1.6	42.0	1,261	—	—	JCVI

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TABLE 1 (Continued)

Actinobacterial genome	Size (Mb)[e]	% GC content	No. of proteins	GOT (°C)[a]	Habitat[b]	Source or reference[d]
Gardnerella vaginalis ATCC 14019	1.7*	41.5	1,365	—	—	337
Gardnerella vaginalis HMP9231	1.7*	—	—	—	—	JCVI
Geodermatophilus obscurus DSM 43160	5.3*	74.0	4,810	Meso	T	154
Gordonia bronchialis DSM 43247	5.28	67.1	4,616	Meso	H	153
Gordonibacter pamelaeae 7-10-1-b	3.6*	—	—	—	—	Sanger Institute
Intrasporangium calvum DSM 43043	4*	—	3,563	Meso	—	66
Isoptericola variabilis 225	3.3	—	2,881	—	—	DOEJGI
Jonesia denitrificans DSM 20603	2.75	58.4	2,511	—	—	233
Kineococcus radiotolerans SRS30216	4.99	74.2	4,480	32	M	DOEJGI
Kocuria rhizophila DC2201	2.7	71.2	2,357	Meso	M	296
Kribbella flavida DSM 17836	7.6*	70.6	6,943	Meso	T	235
Kytococcus sedentarius DSM 20547	2.8	71.6	2,554	Meso	—	268
Leifsonia xyli subsp. xyli strain CTCB07	2.58	67.7	2,030	20-25	H	205
Microbacterium testaceum StLB037	4	—	3,676	—	—	207
Micrococcus luteus NCTC 2665	2.5	72.9	2,236	Meso	M	DOEJGI
Micromonospora aurantiaca ATCC 27029	7*	72.9	6,222	Meso	M	DOEJGI
Micromonospora sp. L5	7*	72.9	6,150	Meso	—	DOEJGI
Mobiluncus curtisii ATCC 43063	2.1*	55.6	1,909	—	—	Baylor College
Mycobacterium abscessus ATCC 19977	5.09	64.1	4,920	37	M	242
Mycobacterium avium 104	5.5	69	5,120	37	H	TIGR
Mycobacterium avium subsp. paratuberculosis K-10	4.8	69.3	4,350	37	M	186
Mycobacterium bovis AF2122/97	4.35	65.6	3,920	37	H	101
Mycobacterium bovis BCG strain Pasteur 1173P2	4.4	65.6	3,052	Meso	H	32
Mycobacterium bovis BCG strain Tokyo 172	4.4	65.6	3,947	Meso	H	260
Mycobacterium gilvum PYR-GCK	5.96	67.7	5,241	Meso		DOEJGI
Mycobacterium leprae Br4923	3.3	57.8	1,604	37	H	204
Mycobacterium leprae TN	3.27	57.8	1,605	37	H	56
Mycobacterium marinum M	6.62	65.7	5,423	32	M	287
Mycobacterium smegmatis strain MC2 155	7	67.4	6,716	37	H	TIGR
Mycobacterium sp. JDM601	4.6*	—	4,346	—	—	Shanghai JT University
Mycobacterium sp. strain JLS	6	68.4	5,739	Meso	M	DOEJGI
Mycobacterium sp. strain KMS	6.22	68.2	5,460	Meso	M	DOEJGI
Mycobacterium sp. strain MCS	5.92	68.4	5,391	Meso	—	DOEJGI
Mycobacterium sp. strain Spyr1	5.73*	—	5,130	Meso	T	DOEJGI
Mycobacterium tuberculosis CDC1551	4.4	65.6	4,189	37	H	88
Mycobacterium tuberculosis F11	4.4	65.6	3,941	37	H	The Broad Institute
Mycobacterium tuberculosis H37Ra	4.4	65.6	4,034	37	H	342
Mycobacterium tuberculosis H37Rv	4.4	65.6	3,989	37	H	55
Mycobacterium tuberculosis KZN 1435	4.4	65.6	4,059	37	H	The Broad Institute
Mycobacterium tuberculosis KZN 4207	4.4*	65.4	—	37	H	The Broad Institute
Mycobacterium ulcerans Agy99	5.77	65.4	4,160	32	H	288
Mycobacterium vanbaalenii PYR-1	6.5	67.8	5,979	24-37	—	DOEJGI
Nakamurella multipartita DSM 44233	6.06	70.9	5,240	Meso	T	302
Nocardia farcinica IFM 10152	6.29	70.7	5,683	37	M	151
Nocardioides sp. JS614	5.31	71.4	4,645	30	T	DOEJGI
Nocardiopsis dassonvillei subsp. dassonvillei DSM 43111	6.58*	72.7	4,798	—	M	291
Olsenella uli DSM 7084	2.1*	—	1,739	37	—	108
Propionibacterium acnes 266	2.5*	60	—	—	—	G.-A. University
Propionibacterium acnes KPA171202	2.56	60	2,297	37	H	34
Propionibacterium acnes SK137	2.5	60.1	2,352	Meso	—	JCVI
Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1	2.6	—	2,375	Meso	M	79
Pseudonocardia dioxanivorans CB1190	7.3	—	6,495	30	—	DOEJGI
Renibacterium salmoninarum ATCC 33209	3.2	56.3	3,507	15	H	328
Rhodococcus equi 103S	5*	—	4,512	Meso	M	184
Rhodococcus erythropolis PR4	6.88	62.3	6,030	20	—	261
Rhodococcus jostii RHA1	9.67	67	7,211	30	T	196

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TABLE 1 (Continued)

Actinobacterial genome	Size (Mb)[e]	% GC content	No. of proteins	GOT (°C)[a]	Habitat[b]	Source or reference[d]
Rhodococcus opacus B4	7.9	67.9	7,246	—	T	209
Rothia dentocariosa ATCC 17931	2.5	—	2,217	Meso	H	Baylor College
Rothia mucilaginosa	2.5*	59.6	1,904	Meso	H	Osaka Dental University
Rubrobacter xylanophilus DSM 9941	3.23	70.5	3,140	60	S	DOEJGI
Saccharomonospora viridis DSM 43017	4.3	67.3	3,828	37	H	228
Saccharopolyspora erythraea NRRL 2338	8.2	71.1	7,197	28	T	222
Salinispora arenicola CNS-205	5.8	69.5	4,917	Meso	A	229
Salinispora tropica CNB-440	5.2	69.5	4,536	28	A	309
Sanguibacter keddieii DSM 10542	4.3*	71.9	3,710	Meso	H	155
Segniliparus rotundus DSM 44985	3.2*	68	3,006	Meso	—	266
Slackia heliotrinireducens DSM 20476	3.17	60.2	2,765	Meso	M	DOEJGI
Stackebrandtia nassauensis DSM 44728	6.8*	68.1	6,379	—	—	DOEJGI
Streptomyces avermitilis MA-4680	9.09	70.7	7,580	26	M	148
Streptomyces bingchenggensis BCW-1	12	—	—	—	—	322
Streptomyces coelicolor A3(2)	9.09	72	7,769	25-35	M	18
Streptomyces flavogriseus ATCC 33331	7.62*	71.0	—	Meso	T	DOEJGI
Streptomyces griseus subsp. griseus NBRC 13350	8.5	72.2	7,136	25-35	M	144
Streptomyces scabiei 87.22	10*	—	8,746	Meso	T	23
Streptosporangium roseum DSM 43021	10.03*	70.9	8,945	Meso	T	214
Thermobifida fusca YX	3.6	67.5	3,110	50-55	M	194
Thermobispora bispora DSM 43833	4.2	70	3,546	Thermo	T	188
Thermomonospora curvata DSM 43183	5.6*	71.6	4,890	45-55	S	45
Tropheryma whipplei strain Twist	0.93	46.3	808	37	H	239
Tropheryma whipplei TW08/27	0.93	46.3	783	37	H	20
Tsukamurella paurometabola DSM 20162	4.5*	68.4	4,157	Meso	T	DOEJGI
Verrucosispora maris AB-18-032	6.75*	—	5,956	—	—	CSBL, Korea University
Xylanimonas cellulosilytica DSM 15894	3.79*	72.5	3,337	—	S	89

[a] GOT, growth-optimal temperature; Meso, mesophilic; Ther, thermophilic.
[b] A, aquatic; T, terrestrial; H, host associated; M, multiple; S, specialized.
[c] The information in the table was collected from the NCBI website (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). —, information not available.
[d] Abbreviations: DOEJGI, U.S. Department of Energy Joint Genome Institute; COE, College of Engineering; JCVI, J. Craig Venter Institute; TIGR, The Institute for Genomic Research; Shanghai JT University, Jiao Tong University School of Medicine, Shanghai, China; G.-A. University, Georg-August University.
[e] An asterisk indicates that the genome size is estimated; otherwise, the genome size was calculated based on existing sequences.

mosomes belong to 4 distinct suborders, and they are distantly related (103, 283, 343). Thus, the chromosome linearization characteristic has evolved more than once during the evolution of the *Actinobacteria* (165).

Remarkably, even within the same genus of *Actinobacteria*, the genome size differences can be significant. For example, of the 23 sequenced *Mycobacterium* species and strains, *M. smegmatis* strain MC2 155 has a genome of 7.0 Mb, while the intracellular pathogen *M. leprae* TN has a massively reduced genome of 3.27 Mb (Table 1) (54, 56, 287). Interestingly, the genome sizes of the 4 sequenced *Frankia* strains also varied from 5.43 Mb to 9.04 Mb, showing the greatest divergence yet reported for such closely related soil bacteria (97.8% to 98.9% identity in 16S rRNA genes) (216). The bacterial genome is believed to be plastic and dynamic, in which gene gains, gene losses, and lateral gene transfers (LGT) happen all the time to shape the gene repertoire (64, 181, 218, 273). The main driving force for genome expansion or reduction is niche adaptation. In the case of the *Actinobacteria*, most isolated species are free living, and they are from complex and densely populated soil environments. Thus, their genomes are generally large (approximately 5 to 9 Mb) in order to combat environmental changes and species competition (Table 1) (18, 170, 196, 222, 226, 314). However, some species that are parasitic or symbionts have undergone extensive genome reduction, reflecting their adaptation to the much more stable conditions within the host (56, 69, 205). Thus, while host associations favor genome contraction, host diversification leads to genome expansion. As a result, *Frankia* strains or *Mycobacterium* species that have a narrow host range or a broad host range exhibit large differences in their genome sizes (69, 216, 287, 338). Although it is debatable whether genome reduction is a strategy to reduce the energy cost of maintaining genome integrity in extreme environments (48, 91, 237), several actinobacterial species isolated under harsh conditions, such as *Acidothermus cellulolyticus*, *Thermobifida fusca*, *Kocuria rhizophila*, and *Rubrobacter radiotolerans*, etc. (14, 194, 296), have relatively small genomes (approximately 2 to 3.5 Mb) (Table 1). A number of comparative analyses suggested that selection does not act on the genome size; rather, it acts on individual genes and determines the gene repertoire, which in turn influences the genome size (92, 170, 172, 175). Thus, in order to better understand bacterial niche adaptation, it is important to study their diversified gene repertoire, especially the unique gene sets, whose products are the functional executives (regulators), workers (enzymes), and buildings (structural proteins) in the cell. A sound phylogenetic framework for the *Actinobacteria* should prove very helpful in these regards (231, 331).

PHYLOGENY OF *ACTINOBACTERIA* BASED ON COMBINED DATA SETS OF PROTEIN SEQUENCES

Detailed phylogenetic investigations of *Actinobacteria* have thus far been carried out mainly by using 16S rRNA sequences (3, 191,

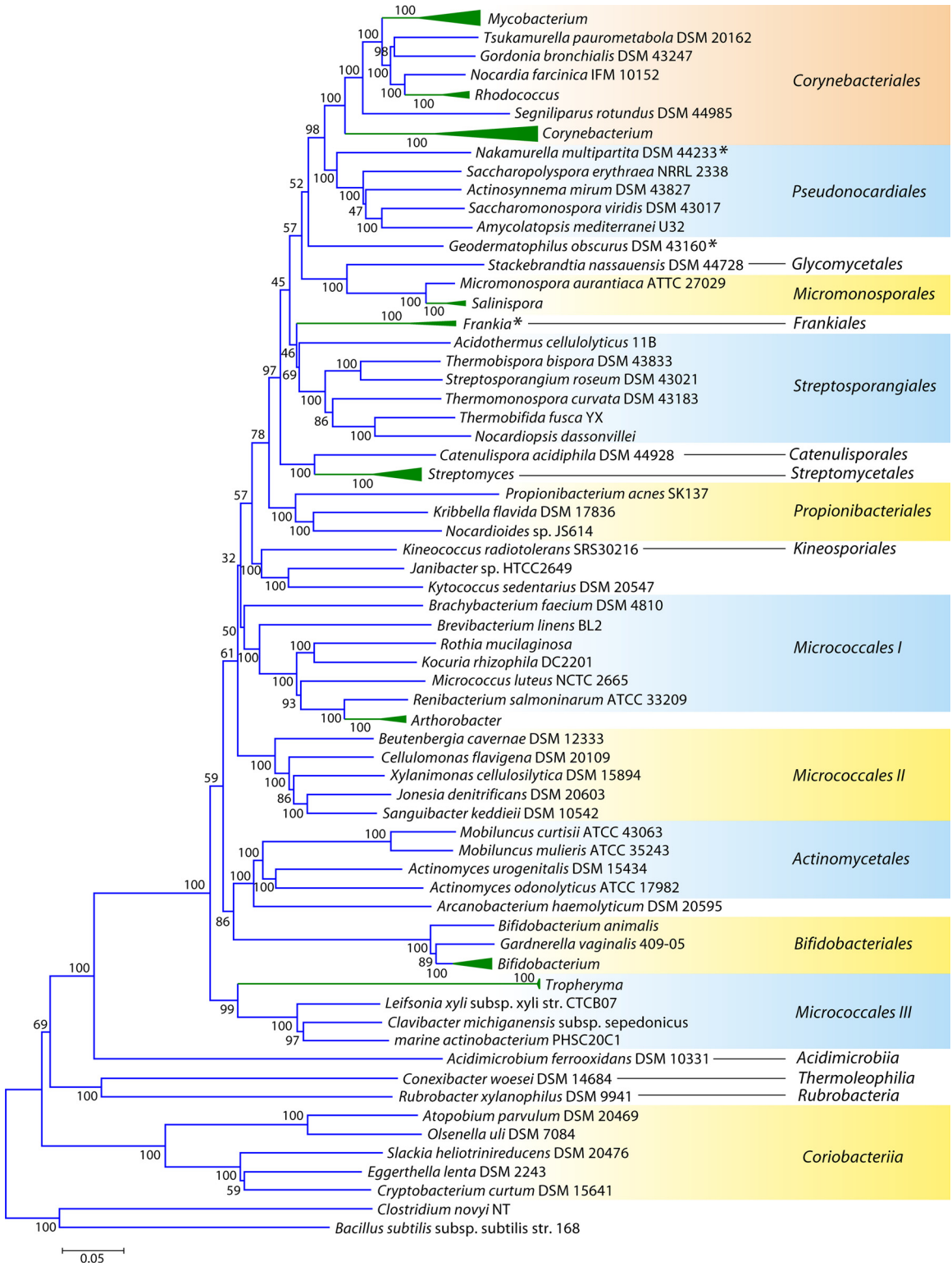

FIG 2 Phylogenetic tree for 98 actinobacterial species whose genomes have been sequenced, based upon concatenated sequences for 35 conserved proteins. Many genera for which sequence information is available from multiple species are represented by triangles in this tree. The sizes of the triangles reflect the number of species that have been sequenced, and more detailed trees for some of these groups are presented in other figures. The tree shown is based on neighbor-joining (NJ) analysis, and the numbers at the nodes represent the bootstrap scores of the nodes. Similar branching patterns for most of these groups can also be observed in a maximum likelihood tree. The asterisks mark the *Frankiales* species that branch in different positions in this tree.

192, 280, 283, 314, 343). Many studies have utilized alternate gene/protein sequences (e.g., RecA, RpoB, GyrB, DnaK, GrpE, GroEL, and CTP synthase, etc.) to examine actinobacterial phylogeny, but those studies employed only small numbers of *Actinobacteria*, and they were often limited to particular taxa (e.g., mycobacteria or *Bifidobacterium*) (25, 70, 97, 161, 312, 313, 315, 319, 320). The availability of genome sequences has provided new opportunities to examine actinobacterial phylogeny based upon different gene/protein sequences. With genomic sequences, many approaches have been used to infer the evolutionary relationships (10, 26, 247). These approaches include examinations of gene order (5, 173, 174) and shared gene content (29, 63, 98, 99, 118, 134, 274), the construction of supertrees based upon concatenated sequences for large numbers of proteins (49, 247, 333), the use of conserved indels to construct rooted phylogenetic trees (119, 121, 125, 126, 128), the use of character compatibility analysis based upon molecular sequences (134, 135), the construction of trees based on the protein domain content (335), and other methods or a combination of the above-mentioned approaches (5, 140, 334). Of these different approaches, the construction of phylogenetic trees based upon combined sequences of large numbers of protein sequences has proven particularly useful for an understanding of the evolutionary relationships among distantly related taxa (49, 247, 333). Phylogenetic trees based upon large numbers of characters derived from multiple conserved (or slow-evolving) genes/proteins are better able to resolve deeper-branching evolutionary relationships than those based on any single gene or protein (33, 49, 67, 139, 247). Alam et al. (5) recently reported a detailed phylogenetic analysis of 45 *Actinobacteria* using a number of different gene sequences (e.g., 5S rRNA, 16S rRNA, and 23S rRNA) and approaches, including a tree based upon concatenated sequences for 155 proteins. Based upon the results obtained by using different approaches, those authors drew a consensus tree for the *Actinobacteria*. In addition, a phylogenetic tree for the *Actinobacteria* based upon fragments derived from *ychF*, *rpoB*, and *secY* gene sequences was also constructed (3).

Although these trees provide good reference resources, the number of sequenced actinobacterial genomes has now greatly increased. Hence, to acquire a comprehensive view of *Actinobacteria* phylogeny covering different lineages, phylogenetic trees were constructed for 98 actinobacterial species (from 57 genera) whose genomes were either completely sequenced or were at the assembly stages in October 2010, when these analyses were carried out (Table 1). The species in our data set included representatives from 13 of the 15 orders of the class *Actinobacteria* as well as members of four of the other 5 proposed classes of this phylum (*viz.*, *Acidimicrobiia*, *Coriobacteriia*, *Rubrobacteria*, and *Thermoleophilia*). A total of 35 universally distributed proteins, which are involved in a broad range of cellular functions, were extracted from these genomes for phylogenetic analyses (see File S1 in the supplemental material) (49). The sequence alignments for these proteins were concatenated into a single large data set, which, after the removal of all poorly aligned regions, consisted of 9,953 aligned positions. Phylogenetic trees based on this large data set of protein sequences were constructed by using the maximum likelihood (ML) and neighbor-joining (NJ) methods. Both these methods gave similar tree topologies, except for the branching points that were weakly supported in the trees. An NJ distance tree based on this data set is shown in Fig. 2. Compared to the other previously reported phylogenetic trees for *Actinobacteria* (3, 192, 343), where the bootstrap scores were either very low or not indicated, many of the nodes in this tree are supported by high bootstrap scores, indicating that the observed relationship is reliable and that this tree is better able to resolve the interrelationships among actinobacterial species. The important characteristics of this tree are noted below.

In contrast to the 16S rRNA tree, where *Rubrobacter* or a clade consisting of *Rubrobacter* and the *Coriobacteriia* was the earliest-branching lineage within the phylum *Actinobacteria* (343), in the tree based upon concatenated protein sequences, a clade consisting of various *Coriobacteriia* species constituted the deepest branch within this phylum. This clade was separated from all other *Actinobacteria* by a long branch. Following *Coriobacteriia*, a clade consisting of *Rubrobacter xylanophilus* and *Conexibacter woesei* (belonging to the classes *Rubrobacteria* and *Thermoleophilia*, respectively) as well *Acidimicrobium ferrooxidans* (class *Acidimicrobiia*) formed the next two deepest branches in the *Actinobacteria* tree. These species were also separated from all other species belonging to the class *Actinobacteria* by a long branch. Within the class *Actinobacteria*, a number of strongly supported clades that corresponded primarily to a number of known actinobacterial orders were observed. These clades included those corresponding to the orders *Corynebacteriales*, *Pseudonocardiales*, *Micromonosporales*, *Propionibacteriales*, *Streptosporangiales*, *Streptomycetales*, *Actinomycetales*, and *Bifidobacteriales*. In addition, this tree also supported a number of deeper-branching clades consisting of several orders of *Actinobacteria*. One of these clusters consisted of the orders *Corynebacteriales*, *Pseudonocardiales*, *Micromonosporales*, *Streptosporangiales*, *Streptomycetales*, and *Frankiales*. Within this large cluster, a clade consisting of the orders *Corynebacteriales* and *Pseudonocardiales* was also strongly supported by high bootstrap scores. This large clade was also observed in the consensus tree reported by Alam et al. (5). As discussed below, the existence of some of these clusters is also supported by the identification of conserved indels that are specific for them.

In contrast to these orders, the order *Micrococcales*, which is one of the largest orders of the *Actinobacteria*, did not form a phylogenetically coherent cluster, and *Bifidobacteriales* and *Actinomycetales* were interspersed within this order of bacteria (Fig. 2). Based upon the 16S rRNA tree and the consensus tree reported by Alam et al. (5), the order *Bifidobacteriales* formed the deepest-branching lineage within the class *Actinobacteria* (314, 343). However, in recent works by Ludwig et al. (191) and Adekambi et al. (3), it was indicated to branch in a position similar to that seen in the present work. The order *Micrococcales* is the most diverse group within the phylum/class *Actinobacteria*, and the relationships within this order cannot be resolved by the 16S rRNA tree with any degree of confidence (76, 191, 296, 343). In the phylogenetic tree shown in Fig. 2, the species of this order are split into at least three clusters. Of these, cluster I included *Arthrobacter*, *Renibacterium*, *Micrococcus*, *Kocuria*, *Rothia*, *Brachybacterium*, and *Brevibacterium*; cluster II consisted of *Beutenbergia*, *Jonesia*, *Cellulomonas*, *Sanguibacter*, and *Xylanimonas*; and cluster III consisted of *Clavibacter*, the marine actinobacterium PHSC20C1, *Leifsonia*, and the fast-evolving intracellular parasite *Tropheryma*. The relationships of different genera within these clusters is discussed in more detail below in conjunction with signature sequences.

In the phylogenetic tree shown in Fig. 2, species of several genera (e.g., *Geodermatophilus*, *Nakamurella*, *Stackebrandtia*, and *Janibacter*, etc.) branched within clades that do not correspond to their expected position based on the current taxonomic classifica-

tion (103, 215, 343). The branching of these species in the observed positions is also independently supported by several conserved indels that are discussed in later sections. Overall, the phylogenetic tree based on combined protein sequences (Fig. 2) provides a useful reference point to interpret the species distribution patterns of various conserved indels.

USEFULNESS OF CONSERVED SIGNATURE INDELS AND SIGNATURE PROTEINS AS MOLECULAR MARKERS FOR PHYLOGENETIC/SYSTEMATIC STUDIES

The shared derived characters that are unique to particular groups or clades of organisms (i.e., synapomorphies) provide an important means for identifying various clades and also for an understanding of how these clades are related to each other. In the past, this approach has been employed largely by using morphology and other observable traits (271, 272). However, often, such traits either are plesiomorphic (i.e., a particular character is not limited to a given group) or exhibit homoplasy (the derived character state has evolved independently in the given group of organisms), which limits their utility as phylogenetic or taxonomic markers. In recent years, the availability of genome sequences has led to the discovery of large numbers of molecular characteristics that are uniquely shared by different groups of organisms and provide important means for the identification of different clades and an understanding of their evolutionary relationships (11, 13, 119, 121, 123, 126, 128, 131, 243, 246). The molecular characteristics that are ideally suited for evolutionary and systematic studies are those that are specifically present in all species belonging to certain bacterial taxa but that are not found outside those lineages. Due to their specificity for particular taxa or phylogenetically well-defined clades, the genetic events leading to the origins of these molecular characteristics likely occurred in the common ancestors of these clades, and these characteristics were then passed on to their various descendants vertically. Thus, such molecular synapomorphies act as hallmarks recording the divergence of different lineages, which can be used to delineate different taxa and clades at various phylogenetic depths (123, 124, 126, 128). The markers that are ideally suited for evolutionary and systematic studies are those that are generally not affected by factors such as multiple changes at a given site, long-branch attraction effects, differences in evolutionary rates, and lateral gene transfers, etc., which confound the inferences from phylogenetic trees (10, 81, 82, 119, 126, 130, 206). Two types of molecular markers that generally satisfy these characteristics have been identified recently for a number of bacterial phyla, and they are proving to be of great value in our understanding of bacterial phylogeny and systematics (124, 126, 127, 130, 132, 133, 179).

The first kind of these molecular markers is comprised of conserved signature indels (CSIs) of defined lengths that are present in gene/protein sequences at specific positions and which are uniquely shared by particular groups of organisms (11, 119, 121, 126, 128, 132, 243, 246). Because of the rare and highly specific nature of the genetic event that gives rise to a given conserved indel, such changes are less likely to arise independently by either convergent or parallel evolution (i.e., homoplasy) (119, 121, 126, 246). Furthermore, the presence or absence of CSIs in protein sequences should not be generally affected by factors, such as differences in evolutionary rates at different sites or among different species, that greatly influence the branching patterns of species in phylogenetic trees (82, 83, 114). Hence, when a CSI of a defined size is uniquely found in a phylogenetically defined group(s) of species, its most parsimonious explanation is that the genetic change responsible for this CSI occurred once in a common ancestor of this group and was then passed on vertically to its various descendants. Since both large as well as small CSIs (including 1-amino-acid [aa] indels) are products of single unique genetic events, they both provide reliable phylogenetic markers of a common evolutionary descent (119, 121, 126, 246, 269). Because genetic changes leading to CSIs could occur at various stages during evolution, it is possible to identify CSIs in gene/protein sequences at different phylogenetic depths corresponding to various taxonomic groupings (e.g., phylum, order, family, genus, and even single-species and subspecies levels). Additionally, based upon the presence or absence of these CSIs in the outgroup species, it is possible to infer whether a given CSI represents an insertion or a deletion in a particular clade and which of the two character states of the protein is ancestral (116, 119, 122, 132). Thus, by making use of CSIs that have been introduced at various stages in evolution, it is possible to derive a rooted evolutionary relationship among various taxa under consideration (119, 122, 129). In some cases where a given CSI is present in unrelated groups of organisms, this can be a consequence of lateral gene transfers (LGTs) or due to the independent occurrence of similar genetic events (117, 117, 119).

The second kind of molecular markers that have proven very useful for systematic and phylogenetic studies is whole proteins or conserved signature proteins (CSPs) that are confined to particular lineages (100, 128, 130). In contrast to the orphan open reading frame (ORFan) proteins that are specific for particular species or strains and are subject to rapid loss (64, 175, 265), many proteins of unknown (or even known) functions are unique and distinctive, characteristic of various species from monophyletic clades of different phylogenetic depths (74, 98, 100, 118, 130, 274). The presence of these proteins in a conserved state in all or most species and strains from these clades, but nowhere else, suggests that the genes for these proteins first evolved in a common ancestor of these clades, followed by their retention by various descendants (74, 80, 98, 100, 210). Thus, these proteins represent CSPs that are distinctive characteristics of particular lineages, and they provide useful molecular markers for defining or distinguishing those groups from other bacteria (118). However, when a CSP (or CSI) is confined to certain species or strains, based upon this information alone, it is difficult to determine whether these species form a clade in the phylogenetic sense or not. Hence, to understand the evolutionary significance of these signatures, such studies are generally performed in conjunction with phylogenetic analyses, which provide a reference point for evaluating the significance of various CSIs and CSPs (99, 118). In the work leading to this review, we carried out extensive work on actinobacterial genomes to identify CSIs and CSPs that are specific for all (or most) sequenced actinobacteria or their different groups or clades at various phylogenetic depths. The identification of these molecular markers was carried out as described in our previous work (97, 100, 130), and additional information in this regard is provided in File S1B in the supplemental material.

MOLECULAR MARKERS OF THE PHYLUM *ACTINOBACTERIA*

The phylum *Actinobacteria* is currently identified solely on the basis of the branching patterns of different species in the 16S rRNA tree (103, 110, 191, 283, 343). However, there is no known

unique feature or characteristic that is commonly shared by all or most constituent taxa of this phylum. Because phylogenetic trees have no distinct boundaries, in the absence of any distinctive property of the group of species, it is difficult to delimit a group based solely on the branching in the phylogenetic trees (192, 193, 223, 234, 329). Hence, it is of central importance to determine what unique properties are shared by different species of this phylum that could be employed to more precisely define and circumscribe member species of this phylum (126, 130, 132).

CSIs That Are Uniquely Present in Most *Actinobacteria*

We have previously described two CSIs, consisting of a 2-aa deletion in cytochrome *c* oxidase subunit 1 (Cox1) and a 4-aa insert in CTP synthetase, that were uniquely present in almost all actinobacteria except for the deepest-branching genus, *Rubrobacter* (97). A 5-aa insert in glutamyl-tRNA synthetase (GluRS) was also identified, but it was lacking in several actinobacterial species (97). Additionally, a large insert in the 23S rRNA is also specific for most actinobacterial species (97, 248). Our recent analyses of protein sequences from actinobacterial genomes identified 6 additional CSIs in various proteins that are uniquely shared by most of the sequenced actinobacterial species. These CSIs include a 4-aa insert in the protein glucosamine-fructose-6-phosphate aminotransferase (Gft) (Fig. 3), which catalyzes the formation of glucosamine 6-phosphate and is the first and rate-limiting enzyme of the hexosamine biosynthetic pathway (290); a 3-aa insert in the enzyme glycyl-tRNA synthetase (GlyRS) that is required for protein synthesis (see File S2 in the supplemental material); a 4- to 6-aa insert in the enzyme tRNA (guanine-1)-methyltransferase (TrmD) that methylates guanosine 37 in various tRNAs (see File S3 in the supplemental material) (230); a 4-aa insert in gyrase A, which plays an essential role in DNA replication and transcription due to its ability to make transient double-strand breaks in DNA to maintain appropriate levels of supercoiling (see File S4 in the supplemental material) (185); a 9-aa insert in the enzyme *S*-adenosyl-L-homocysteine hydrolase (SAHH) that hydrolyzes *S*-adenosylhomocysteine, which is an end product of various methylation reactions (see File S5 in the supplemental material) (143); and, finally, a 5-aa insert in the enzyme serine hydroxymethyltransferase (SHMT), which catalyzes the reversible interconversion of serine and glycine (see File S6 in the supplemental material) (117, 238).

A partial sequence alignment of the protein glucosamine-fructose-6-phosphate aminotransferase showing the *Actinobacteria*-specific insert is presented in Fig. 3. The absence of this indel in the *Archaea* as well as other bacterial phyla provides evidence that this indel constitutes an insert in the *Actinobacteria* rather than a deletion in other groups. The sequence alignments for other newly identified CSIs that are uniquely present in most *Actinobacteria* are provided in Files S2 to S6 in the supplemental material. In all of these proteins, the identified CSIs are present in highly conserved regions. Table 2 presents information regarding the specificity as well as the presence or absence of these CSIs as well as the CSIs in Cox1, CTP synthetase, and 23S rRNA in different genera of *Actinobacteria*. As shown in Table 2, most of these CSIs are highly specific characteristics of the phylum *Actinobacteria*. The CSIs in Cox1, CTP synthase, Gft, TrmD, and 23S rRNA are not found in any other bacteria except *Actinobacteria*, whereas for the other signatures, CSIs of similar lengths were also present in a small number of distantly related organisms, which could be due to either LGT or the independent occurrence of similar genetic changes in these lineages. From the species distribution profiles of these CSIs, it is clear that while most of these CSIs are commonly shared by virtually all sequenced genera belonging to the class *Actinobacteria*, they are generally not found in the deeper-branching lineages of *Actinobacteria*. Of these CSIs, only the Cox1 CSI was present in the genus *Acidimicrobium*, while the genus *Conexibacter* contained CSIs in the SAHH and SHMT proteins. However, none of these CSIs were detected in *Rubrobacter* or the *Coriobacteriia*. For some of these proteins, their homologs were also not detected in most of the *Coriobacteriia* (Table 2).

CSPs That Are Specific for the Phylum *Actinobacteria*

In addition to these CSIs, our Blastp analyses of several actinobacterial genomes (*viz.*, *M. leprae* TN, *Leifsonia xyli* subsp. xyli strain CTCB07, *Bifidobacterium longum* NCC2705, and *T. fusca* YX) previously identified 29 CSPs that were indicated to be specific for either all or most genome-sequenced *Actinobacteria* (100). Since the number of sequenced actinobacterial genomes has increased from 25 at that time to >150 at present, the *Actinobacteria* specificities of these proteins were reexamined. Of the 29 proteins that were reported to be specific for all (or most) of the *Actinobacteria*, 24 are still specifically present in all of the sequenced actinobacterial genera, except for a few of the deepest-branching lineages (see File S7 in the supplemental material). A summary of the properties of these proteins and information regarding their *Actinobacteria* specificities are provided in Table 3. Except for *Actinobacteria*, homologs showing significant similarity to these proteins are not found in any other bacterial phyla. Five proteins that were previously retained despite their presence in some other bacterial groups are now excluded from Table 3. The 24 proteins listed in Table 3 are present in virtually all sequenced genera (total of 57) belonging to the class *Actinobacteria* (see File S7 in the supplemental material). The homologs of two of them, *viz.*, ML1306 (GenBank accession number NP_301939.1) and ML1009 (accession number NP_301746.1), were also found in *Rubrobacter xylanophilus*, *Conexibacter woesei*, and *Acidimicrobium ferrooxidans*, belonging to the classes *Rubrobacteria*, *Thermoleophilia*, and *Acidimicrobiia*, respectively. Homologs of four additional proteins (*viz.*, ML0642, ML1029, ML0760, and ML0804) were also present in one or two of these three classes (see File S7 in the supplemental material). However, significantly, of all the CSPs identified by comparative genomic analyses, the homologs of none of them were detected in any of the members of the class *Coriobacteriia*.

Predictive Value and Usefulness of the Identified CSIs and CSPs for Delimiting the Phylum *Actinobacteria*

The results obtained with various CSIs and CSPs are significant in a number of respects. First, they provide important information for validating the specificity and reliability of these signatures. Many of these CSIs and all of these CSPs were identified when the number of sequenced actinobacterial genomes was very limited (97, 100). However, despite a large increase in the number of sequenced genomes (between 6- and 10-fold) for both *Actinobacteria* as well as other bacteria, most of these signatures are still specific for *Actinobacteria*. Additionally, most of these signatures are present in virtually all sequenced genera of *Actinobacteria*, except those from the deepest-branching lineages. Thus, the presence of these signatures can be used to distinguish member species belonging to the class/phylum *Actinobacteria* from all other bac-

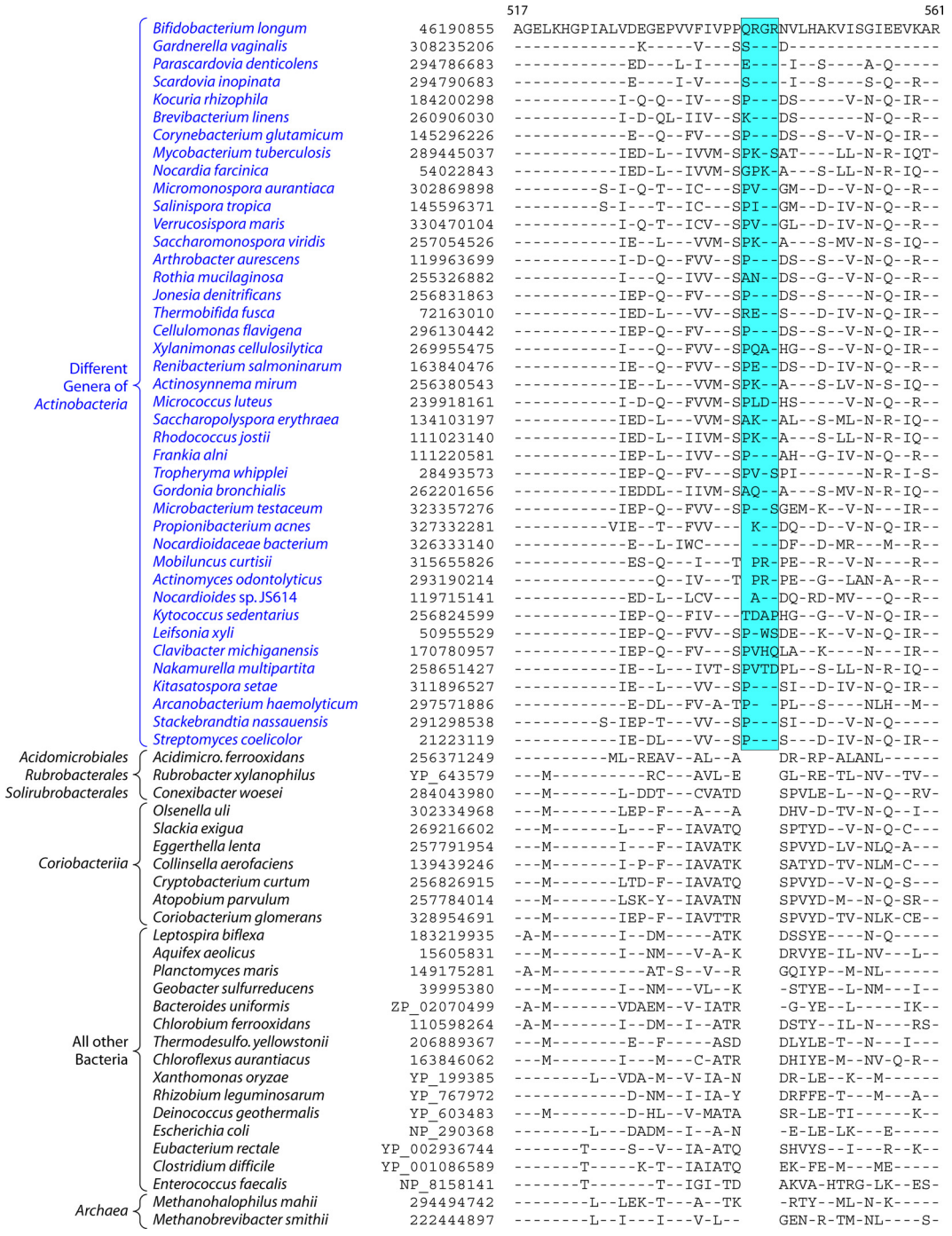

FIG 3 Partial sequence alignment of the protein glucosamine-fructose-6-phosphate aminotransferase (GFT) showing a 4-aa insert that is uniquely present in different genera belonging to the class *Actinobacteria* but is not found in *Coriobacteriia*, *Rubrobacter*, *Acidimicrobiia*, and *Thermoleophilia* or any other prokaryotic organism. Sequence information for several other CSIs that are specifically found in most *Actinobacteria* is presented in Files S2 to S6 in the supplemental material and Table 2. The dashes in this as well as all other sequence alignments indicate identity with the amino acid on the top line. The numbers on the top lines indicate the sequence region where this CSI is found in the species shown at the top. The second column shows the GenBank accession number or GenBank identification (gi) number for the sequences. Sequence information for a limited number of *Actinobacteria* is shown in this alignment. However, detailed information regarding the presence or absence of this CSI in various sequenced genera of *Actinobacteria* is provided in the Table 2.

teria with a high degree of predictive ability. This inference is further strongly supported by our previous work, where the presence of CSIs in Cox1, CTP synthase, and 23S rRNA was examined in a large number of other *Actinobacteria* belonging to different families, whose genomes have not been sequenced, by PCR amplification of the corresponding fragments (97). The results of those studies showed that of the 50 gene fragments for these three genes that were sequenced from diverse members of the *Actino-*

TABLE 2 Presence or absence of various CSIs in different genera of *Actinobacteria*[a]

Genus	Presence of CSI in:								
	CoxI	CTPS	Gft	GlyRS	TrmD	Gyrase A	SAHH	SHMT	23S rRNA
Mycobacterium	+	+	+	+	+	+	+	+	+
Tsukamurella	+	+	−	+	+	+	+	+	+
Gordonia	+	+	+	+	+	+	+	+	+
Nocardia	+	+	+	+	+	+	+	+	+
Rhodococcus	+	+	+	+	+	+	+	+	+
Segniliparus	+	+	+	+	+	+	+	+	+
Amycolicicoccus	+	+	+	+	+	+	+	+	+
Corynebacterium	+	+	+	+	+	+	+	+	+
Nakamurella	+	+	+	+	+	+	+	+	+
Pseudonocardia	+	+	+	+	+	+	+	+	+
Saccharopolyspora	+	+	+	+	+	+	+	+	+
Actinosynnema	+	+	+	+	+	+	+	+	+
Saccharomonospora	+	+	+	+	+	+	+	+	+
Amycolatopsis	+	+	+	+	+	+	+	+	+
Geodermatophilus	+	+	+	+	+	+	+	+	+
Stackebrandtia	+	+	+	+	+	+	+	0	+
Verrucosispora	+	+	+	+	+	+	+	0	+
Micromonospora	+	+	+	+	+	+	+	−	+
Salinispora	+	+	+	+	+	+	+	−	+
Frankia	+	+	+	0	0	+	+	−	+
Acidothermus	+	+	+	+	+	+	+	−	+
Streptosporangium	+	+	+	+	+	+	+	−	+
Thermomonospora	+	+	+	+	+	+	+	+	+
Thermobifida	+	+	+	+	+	+	+	+	+
Nocardiopsis	+	+	+	+	+	+	+	+	+
Catenulispora	+	+	+	+	+	+	+	−	+
Streptomyces	+	+	+	+	+	+	+	+	+
Propionibacterium	+	+	+	+	+	+	0	−	+
Kribbella	+	+	−	+	+	+	+	−	+
Nocardioides	+	+	+	+	+	+	+	−	+
Kineococcus	+	+	+	+	+	+	+	+	+
Janibacter	+	+	+	+	+	+	+	−	+
Kytococcus	+	+	+	+	+	+	0	−	+
Brachybacterium	+	+	+	+	+	+	0	+	+
Brevibacterium	+	+	+	+	+	+	+	+	+
Intrasporangium	+	+	+	+	+	+	+	+	+
Isoptericola	+	+	+	+	+	+	+	+	+
Microbacterium	+	+	+	+	+	+	0	+	+
Rothia	+	+	+	+	+	+	0	+	+
Kocuria	+	+	+	+	+	+	0	+	+
Micrococcus	+	+	+	+	+	+	+	+	+
Renibacterium	+	+	+	+	+	+	+	+	+
Arthrobacter	+	+	+	+	+	+	+	+	+
Beutenbergia	+	+	+	+	+	+	+	+	+
Cellulomonas	+	+	+	+	+	+	0	+	+
Xylanimonas	+	+	+	+	+	+	+	+	+
Jonesia	+	+	+	+	+	+	+	+	+
Sanguibacter	+	+	+	+	+	+	+	+	+
Mobiluncus	0	0	+	+	+	+	0	+	+
Actinomyces	0	+	+	+	+	+	0	+	+
Arcanobacterium	0	0	+	+	+	+	−	+	+
Gardnerella	0	+	+	+	+	+	0	0	+
Bifidobacterium	0	+	+	+	+	+	0	+	+
Tropheryma	+	+	+	0	+	+	0	+	+
Leifsonia	+	+	+	+	+	+	+	+	+
Clavibacter	+	+	+	+	+	+	+	+	+
Marine actinobacterium	+	+	+	+	+	+	0	+	+
Acidimicrobium	+	−	−	−	0	−	−	−	−
Conexibacter	−	−	−	−	−	−	+	+	−
Rubrobacter	0	−	−	−	−	−	−	−	−

(Continued on following page)

TABLE 2 (Continued)

Genus	Presence of CSI in:								
	CoxI	CTPS	Gft	GlyRS	TrmD	Gyrase A	SAHH	SHMT	23S rRNA
Olsenella	0	−	−	0	0	−	0	0	−
Slackia	0	−	−	0	0	−	−	−	−
Eggerthella	0	−	−	0	0	−	0	−	−
Cryptobacterium	0	0	−	0	0	−	0	0	−
Coriobacterium	0	−	−	0	0	−	0	0	−
Non-*Actinobacteria*	None	None	None	*Magnetospirillum* + few planctomycetes	None	Some *Firmicutes*, 1 *Bacteroides* sp., 1 *Agrobacterium* sp.	*Anaeromyxobacter*, *Fibrobacter succinogenes*	Some fungi	None

[a] The presence or absence of various CSIs in different genera of genome-sequenced *Actinobacteria* was determined by means of Blastp searches. The symbols + and − indicate whether the indicated CSI is present or absent in the species of various genera. The symbol "0" indicates that no homologs of these proteins were detected in these genera. The abbreviations for the proteins are as follows: Cox1, cytochrome oxidase subunit 1; CTPS, CTP synthetase; GFT, glucose fructose 6-PO_4 aminotransferase; GlyRS, glycyl-tRNA synthetase; TrmD, tRNA (guanine-1)-methyltransferase; SAHH, *S*-adenosyl-L-homocysteine hydrolase; SHMT, serine hydroxymethyltransferase. The sequence alignments for Cox1, CTP synthetase, and 23S rRNA showing the presence of the CSIs in these genes/proteins were described in previous work (97, 100). Information for other CSIs is provided in the Fig. 2 and Files S2 to S6 in the supplemental material. Besides *Actinobacteria*, in some cases, CSIs of similar lengths can also be found in an isolated or limited number of species of other groups of organisms. This could be due to LGT, or it could also result from independent genetic events.

bacteria, all contained the indicated indels, thereby providing strong evidence that these CSIs are distinctive characteristics of various *Actinobacteria*, even those for whom sequence information is not available at present (97).

Based upon the presence of these CSIs and CSPs, the class *Actinobacteria*, which comprises more than 90% of the known actinobacterial genera, can now be delimited and circumscribed in clear molecular terms based upon large numbers of independent molecular markers that are unique characteristics of different members of this class (Fig. 3 and Tables 2 and 3, and see Files S2 to S6 in the supplemental material). Based upon the two CSPs that are uniquely found in the class *Actinobacteria* and members of the classes *Acidimicrobiia*, *Rubrobacteria*, and *Thermoleophilia*, a case can also be made that these bacterial groups are specifically related to the class *Actinobacteria* and that they should thus be part of the phylum *Actinobacteria*. However, detailed analyses of the genomes of *Actinobacteria* have not identified any CSP or CSI that is commonly shared by the above-mentioned classes *Actinobacteria* and *Coriobacteriia*, which is now represented by five sequenced genomes (Table 1) (108, 195, 251). This observation in conjunc-

TABLE 3 Signature proteins that are uniquely found in all (or most) *Actinobacteria*[a]

Gene	GenBank accession no.	Protein function (reference[s])	Length (aa)	Species specificity
ML1306	NP_301939.1	ParJ, chromosome segregation (71)	274	All except *Coriobacteriia*
ML1009	NP_301746.1	Hypothetical	326	All except *Coriobacteriia*
ML0642	NP_301530.1	Hypothetical	479	All except *Acidimicrobiia* and *Coriobacteriia*
ML1029	NP_301762.1	Hypothetical	273	All except *Acidimicrobiia* and *Coriobacteriia*
ML0760	NP_301589.1	*whiB*-like, sporulation (31, 90)	89	All except *Coriobacteriia* and *Rubrobacter*
ML0804	NP_301614.1	*whiB*-like, sporulation(31, 90)	84	All except *Coriobacteriia* and *Rubrobacter*
ML0857	NP_301645.1	Hypothetical	250	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML0869	NP_301656.1	Hypothetical	124	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML1016	NP_301752.1	Hypothetical	107	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML1026	NP_301759.1	Hypothetical	100	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML2137	NP_302410.1	Hypothetical	251	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML2204	NP_302445.1	Hypothetical	62	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML0013	NP_301140.1	Septation inhibitor protein (31)	93	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML0007	NP_301135.1	Hypothetical	303	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML0580	NP_301492.1	Hypothetical	265	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML0921	NP_301704.1	Hypothetical	96	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML1439	NP_302017.1	Hypothetical	111	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML1610	NP_302109.1	Hypothetical	101	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML2207	NP_302448.1	Hypothetical	131	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML0775	NP_301599.1	LpqB, cell wall-related process (212)	589	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML0761	NP_301590.1	Hypothetical	167	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML0814	NP_301620.1	Hypothetical	82	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML1649	NP_302131.1	Hypothetical	140	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*
ML2142	NP_302413.1	Hypothetical	269	All except *Coriobacteriia*, *Rubrobacter*, and *Acidimicrobiia*

[a] All significant Blast hits for these proteins (barring an isolated exception) were observed for *Actinobacteria*. The first and second columns indicate the gene identifications for these proteins from *M. leprae* and their accession numbers. Most proteins are of unknown functions; however, in a few cases where some information is available, it is noted in the third column. The last column indicates the different classes of *Actinobacteria* where these proteins are found. Homologs of most of these proteins are present in virtually all genome-sequenced species of the class *Actinobacteria*. However, their presence or absence in other classes of *Actinobacteria* is noted in the last column. As noted, none of these proteins are found in any of the species of the class *Coriobacteriia*.

tion with the fact that the *Coriobacteriia* are separated from all other members of the *Actinobacteria* by a long branch in the phylogenetic tree (Fig. 2) makes a strong case for the exclusion of the *Coriobacteriia* from the phylum *Actinobacteria*. It should be noted in this context that the absence of various CSIs and CSPs in *Symbiobacterium thermophilum*, which was previously placed into the phylum *Actinobacteria*, argued against its inclusion within this phylum (97, 100). This inference was later strongly supported by its genome sequence and other lines of evidence (174, 310), and this species is now grouped with the *Firmicutes* (77). No sequences are available at present for the two genera (*viz.*, *Euzebya* and *Nitriliruptor*) that make up the class *Nitriliruptoria* (77, 176, 191). Hence, the affiliation of *Nitriliruptoria* with other classes of the phylum *Actinobacteria* (*viz.*, *Actinobacteria*, *Acidimicrobiia*, *Rubrobacteria*, and *Thermoleophilia*) cannot be confirmed at present.

MOLECULAR SIGNATURES OF THE ORDER *CORYNEBACTERIALES* AND SOME OF ITS FAMILIES

The order *Corynebacteriales* represents one of the largest groups within the actinobacteria in terms of the numbers of genomes that have been sequenced (Table 1). Forty-eight of the sequenced genomes, representing about one-third of the total actinobacterial genomes, are from this order. This is also due to the fact that species of many genera within this order (*viz.*, *Mycobacterium*, *Nocardia*, *Corynebacterium*, and *Gordonia*) are important human and animal pathogens (39, 53, 54, 56, 72, 204, 252, 264, 342). Members of this order form a strongly supported clade in phylogenetic trees based on 16S rRNA and other gene/protein sequences (Fig. 2) (3, 5, 192, 314, 343). The species of this order, similar to those of the *Pseudonocardiales*, have cell wall chemotype IV, defined by the presence of *meso*-diaminopimelic acid, arabinose, and galactose in their cell walls (111, 182, 295). However, unlike species of the order *Pseudonocardiales*, which lack mycolic acids, mycolic acids are an important component of the cell envelopes of all species (with the few exceptions noted below) of the order *Corynebacteriales* (111, 187). Although the presence of mycolic acids in the cell wall is considered to be a defining characteristic of members of the order *Corynebacteriales*, a number of genera (*viz.*, *Turicella* and *Amycolicicoccus*) as well as *Corynebacterium amycolatum* and *C. kroppenstedtii* lack mycolic acids (111, 178, 187, 191). Other than the presence of mycolic acids, very few reliable markers that are distinctive characteristics of various species of this order are known.

The order *Corynebacteriales* is currently divided into six families: *Corynebacteriaceae*, *Mycobacteriaceae*, *Nocardiaceae*, *Dietziaceae*, *Segniliparaceae*, and *Tsukamurellaceae* (77, 103, 191). Since genome sequences are now available for species of each of these families, a phylogenetic tree for species from the sequenced genomes was constructed based upon the concatenated sequences of three large and conserved proteins (RpoB, RpoC, and gyrase B) (Fig. 4). In this tree, and also in previous studies (111), species of the families *Corynebacteriaceae* and *Mycobacteriaceae* formed strongly supported clades and were clearly distinguished. The genera *Rhodococcus* and *Nocardia*, which until recently were the only two genera that constituted the family *Nocardiaceae* (103), also formed a well-supported clade in the tree. This clade branched distinctly from *Gordonia bronchialis*, which is now proposed to be a part of the family *Nocardiaceae* (191). A clade consisting of *Gordonia* and *Tsukamurella* was supported both in this phylogenetic tree as well as in the tree shown in Fig. 2.

CSIs and CSPs That Are Specific for the Order *Corynebacteriales*

Analyses of protein sequences from *Corynebacteriales* genomes have identified many CSIs and CSPs that are specific for members of this order. In a macrolide transporter ATP-binding protein, a 2-aa insert in a conserved region is specifically present in all of the *Corynebacteriales* but no other *Actinobacteria* (Fig. 5A). Likewise, in the enzyme alpha-ketoglutarate decarboxylase (KGD), which is a part of the tricarboxylic acid cycle (301), a 1-aa deletion in a conserved region is uniquely present in all available *Corynebacteriales* sequences (see File S8 in the supplemental material). Although sequence information is shown for only a limited number of *Corynebacteriales* and other *Actinobacteria*, these indels are highly specific characteristics of all *Corynebacteriales* and are not found in any other *Actinobacteria*. Another CSI consisting of a 1-aa insert that is largely specific for the order *Corynebacteriales* is found in the chromosome segregation DNA-binding protein (ParB) (see File S9 in the supplemental material), which binds to DNA at the origin of replication and is involved in chromosome partitioning (156). The conserved insert in ParB is again present in all of the sequenced genera of *Corynebacteriales*, and with the sole exception of *Leifsonia xyli*, it is not found in any other *Actinobacteria* or in other phyla of bacteria. The presence of this indel in *L. xyli* could be due to LGT or could result from other possibilities that we cannot distinguish at present. Interestingly, the insert in ParB, although it is present in most of the genome-sequenced *Corynebacterium* species, is not found in *C. aurimucosum* and a number of other *Corynebacterium* species (*viz.*, *C. ammoniagenes*, *C. pseudogenitalium*, *C. tuberculostearicum*, *C. accolens*, *C. striatum*, and *C. glucuronolyticum*), whose genomes are not sequenced and which are not shown in the phylogenetic tree in Fig. 4. In a phylogenetic tree based on ParB protein sequences, the *Corynebacterium* species lacking this insert formed a distinct clade (see File S10 in the supplemental material). Hence, the most plausible way to explain the species distribution of this indel is that the genetic change leading to this occurred in a common ancestor of the *Corynebacteriales*, followed by the loss of this CSI from this gene, or LGT of this gene, in this particular subclade of *Corynebacterium*.

In addition to these CSIs, our Blast analysis of various proteins from the genome of *Corynebacterium glutamicum* ATCC 13032 identified four CSPs (Table 4), for which homologs showing significant sequence similarity are restricted to all of the sequenced *Corynebacteriales* species but are not detected in other bacteria. Two of these proteins, *viz.*, arabinosyltransferase (EmbB) and AftA, are involved in the synthesis of cell wall arabinan (6, 24, 259), whereas the other proteins are of unknown functions.

Molecular Signatures of *Mycobacteriaceae*/*Mycobacterium*

The family *Mycobacteriaceae* contains a single genus, *Mycobacterium*, which harbors some of the most important human pathogens, including those responsible for tuberculosis and leprosy (103, 142, 191, 252, 264). Sequence information for large numbers of species of this genus is now available (*viz.*, *M. tuberculosis*, *M. abscessus*, *M. avium*, *M. bovis*, *M. gilvum*, *M. leprae*, *M. marinum*, *M. ulcerans*, and *M. vanbaalenii*) (Table 1) (1, 32, 55, 56, 88, 101, 186, 204, 242, 260, 288, 342). Multiple strains have been se-

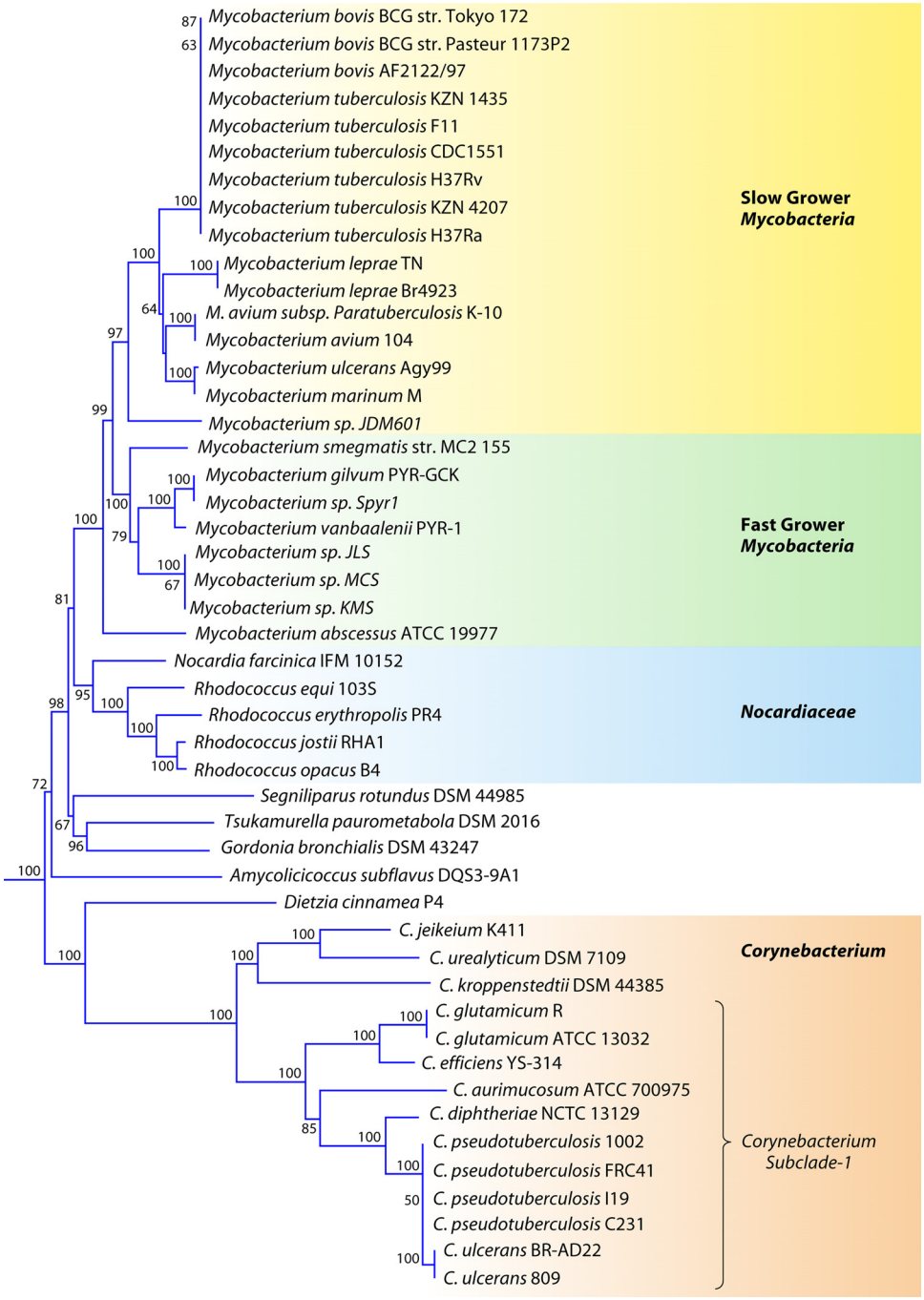

FIG 4 Bootstrapped neighbor-joining tree for *Corynebacteriales* species based upon concatenated sequences for the RpoB, RpoC, and gyrase B proteins. The distinctness of a number of clades seen in this tree is independently supported by many identified CSIs and CSPs.

quenced for a number of species. *Mycobacterium* species have been divided into two major groups (slow growers and fast growers) depending upon their growth rates (142). The species of these two groups generally branch distinctly in phylogenetic trees (232, 285). Their distinctness is also supported by the presence of a longer helix between positions 451 and 482 in the 16S rRNA gene in the slow growers than in the fast growers (232). Of these, the slow-growing *Mycobacterium* species/strains are clinically important, whereas the fast growers are ecologically important (142). In the phylogenetic tree shown in Fig. 4, all of the sequenced *Mycobacterium* species/strains formed a strongly supported clade, and within it, a cluster consisting of the slow-growing *Mycobacterium* species was also strongly supported. We have identified a number of CSIs and CSPs that are specific for either all sequenced *Mycobacterium* species or the slow-growing clade. Sequence information for one of the CSIs that is specific for the genus *Mycobacterium* is presented in Fig. 5B. In the enzyme pantoate-beta-alanine ligase, which is involved in the metabolism of beta-alanine (200),

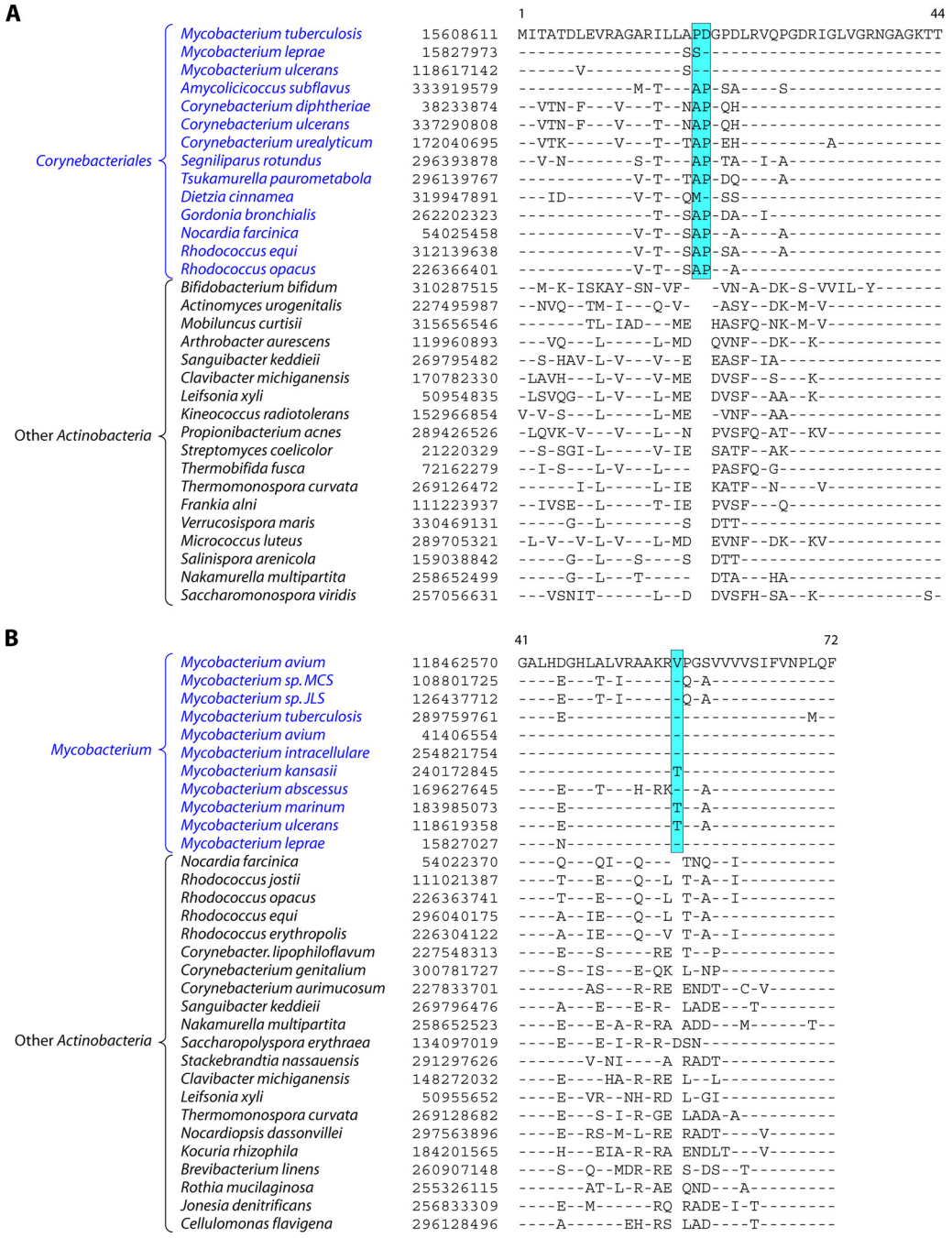

FIG 5 (A) Partial sequence alignment of a macrolide ABC transporter ATP-binding protein showing a 2-aa conserved indel that is uniquely present in various *Corynebacteriales* species. Information for two other CSIs that are specific for *Corynebacteriales* is provided in Files S8 and S9 in the supplemental material. Sequence information for most of the CSIs is shown for a limited number of species; however, unless otherwise indicated, they are specific for the indicated groups. (B) Excerpt from the sequence alignment of the pantoate beta-alanine ligase (PanC) protein showing a 1-aa conserved insert that is specific for *Mycobacterium* species but not found in any other *Actinobacteria*. Sequence information for another *Mycobacterium*-specific CSI in the protein OMP-decarboxylase is presented in File S11 in the supplemental material.

a 1-aa insert in a conserved region is uniquely present in all of the sequenced *Mycobacterium* species but is not found in any other bacteria (Fig. 5B). Similarly, in the enzyme orotidine-5′-phosphate-decarboxylase (OMP-decarboxylase), which catalyzes the last essential step in the *de novo* biosynthesis of pyrimidines (199), a 1-aa deletion is specifically present in all *Mycobacterium* species (see File S11 in the supplemental material). Both these signatures are highly specific for the sequenced *Mycobacterium* species and provide novel molecular markers for this genus.

In our earlier work, Blastp searches for various proteins from the genome of *M. leprae* TN led to the identification of 24 CSPs that were indicated to be specific for the genus *Mycobacterium*

TABLE 4 Signature proteins that are specific for the order *Corynebacteriales*[a]

Gene or protein	GenBank accession no.	Protein function (reference)	Length (aa)
ML0099	NP_301197	Hypothetical	336
Arabinosyl transferase (EmbB)	NP_301201	Mycobacterial cell wall arabinan synthesis protein (300)	1,083
AftA (ML0107)	NP_301204	Cell wall arabinan biosynthesis (6)	632
ML1270	NP_301915	Tryptophan-associated transmembrane protein	265

[a] These signature proteins were identified by Blastp searches for different proteins from the genome of *Mycobacterium leprae* TN. For these proteins, all significant Blast hits were observed for the order *Corynebacteriales*.

(100). A reevaluation of the specificity of these proteins by Blastp searches revealed that all of these proteins are still specific for the genus *Mycobacterium*. However, of these, the first 18 proteins listed in Table 5 are specifically present in all of the sequenced *Mycobacterium* genomes (with isolated exceptions as noted), whereas the last 6 proteins are limited to the subclade of slow-growing *Mycobacterium* species (*viz.*, *Mycobacterium bovis*, *M. tuberculosis*, *M. ulcerans*, *M. marinum*, *M. avium*, *M. paratuberculosis*, *M. leprae*, and *Mycobacterium* sp. strain JDM601), which are clinically important members of this genus. Although the exact cellular functions of most of these proteins remain to be determined, some of them are putative virulence factors belonging to the PE/PPE or Lpq family of proteins (158, 289).

Molecular Signatures of *Rhodococcus* and *Nocardia*

The species of the genera *Rhodococcus* and *Nocardia* form a strongly supported clade in various phylogenetic trees (Fig. 2) (3, 5, 111, 136, 178). The distinctness of species of these two genera from all other genera or families of the order *Corynebacteriales* is also strongly supported by several CSIs and CSPs that we have identified. A partial sequence alignment of one protein showing a CSI that is specific for *Rhodococcus* and *Nocardia* species is presented in Fig. 6. In a protein annotated as an ATP-binding protein, a 3-aa insert in a conserved region is specifically present in species of these two genera. Similarly, another CSI consisting of a 1-aa deletion in a conserved region is found in the alpha-subunit of the enzyme acetyl coenzyme A (acetyl-CoA) carboxylase (ACC), which catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA (51) (see File S12 in the supplemental material). Both these indels are not found in *Gordonia bronchialis* or any other *Actinobacteria*. Our Blastp searches of the genome of *Rhodococcus jostii* RHA1 have led to the identification of 14 CSPs whose homologs are specifically found in *Rhodococcus* and *Nocardia* species (Table 6), except for isolated exceptions. However, in our analyses, we have not come across any CSI or CSP that is commonly shared by *Rhodococcus* and *Nocardia* species as well as by *Gordonia bronchialis*, whose genome has been sequenced (153). These observations make a strong case that the family *Nocardiaceae* should be limited to the genera *Rhodococcus* and *Nocardia*, as it was in the past (103, 136), and that the genus *Gordonia*, which was part of the

TABLE 5 Signature proteins that are specific for the genus *Mycobacterium* or its subclade[a]

Gene or protein	GenBank accession no.	Function (references)	Length (aa)	Species specificity
PE family protein	YP_879413.1	Hypothetical	101	Genus *Mycobacterium*[b]
MAP0046c	NP_958980.1	Hypothetical	113	Genus *Mycobacterium*
PPE family protein	YP_879414.1	Hypothetical	557	Genus *Mycobacterium*
MAV_1008	YP_880267.1	Hypothetical	91	Genus *Mycobacterium*
Proline-rich 28-kDa antigen	YP_879354.1	Lipoprotein LpqN (55, 294)	366	Genus *Mycobacterium*
MAV_0378	YP_879665.1	Hypothetical	277	Genus *Mycobacterium*[b]
MAV_0398	YP_879683.1	Hypothetical	220	Genus *Mycobacterium*
MAV_1034	YP_880290.1	Hypothetical	129	Genus *Mycobacterium*[b]
34 kDa antigenic protein	YP_880332.1	Hypothetical	302	Genus *Mycobacterium*
MAV_1122	YP_880374.1	Hypothetical	220	Genus *Mycobacterium*[b]
LpqT protein	YP_880404.1	Lipoprotein LpqT (55, 293)	219	Genus *Mycobacterium*[b]
LprE protein	YP_880642.1	Hypothetical	195	Genus *Mycobacterium*
MAV_1668	YP_880900.1	Hypothetical	253	Genus *Mycobacterium*
MAV_1760	YP_880985.1	Hypothetical	376	Genus *Mycobacterium*
MAV_2294	YP_881498.1	Hypothetical	210	Genus *Mycobacterium*
MAV_2346	YP_881550.1	Hypothetical	131	Genus *Mycobacterium*
ModD protein	YP_882045.1	Fibronectin attachment protein	385	Genus *Mycobacterium*
MAV_3078	YP_882262.1	Hypothetical	61	Genus *Mycobacterium*
PPE family protein	YP_883994.1	Hypothetical	488	*Mycobacterium* subclade[c,d]
PE family protein	YP_882101.1	Hypothetical	99	*Mycobacterium* subclade[c]
MAV_1177	YP_880425.1	Hypothetical	94	*Mycobacterium* subclade[c]
PPE family protein	YP_880574.1	Hypothetical	555	*Mycobacterium* subclade[c]
PPE family protein	YP_883484.1	Hypothetical	529	*Mycobacterium* subclade[c]
PPE family protein	YP_884001.1	Hypothetical	527	*Mycobacterium* subclade[c]

[a] These CSPs were identified by Blastp searches for proteins from the genome of *M. leprae* TN as described previously (100).
[b] A significant Blast hit was also observed for 1 to 2 other species of the suborder *Corynebacteriales*.
[c] Specific for a subclade consisting of the slow-growing mycobacteria *Mycobacterium bovis*, *M. tuberculosis*, *M. ulcerans*, *M. marinum*, *M. avium*, *M. paratuberculosis*, *M. leprae*, and *Mycobacterium* sp. JDM601.
[d] Also found in *M. abscessus*.

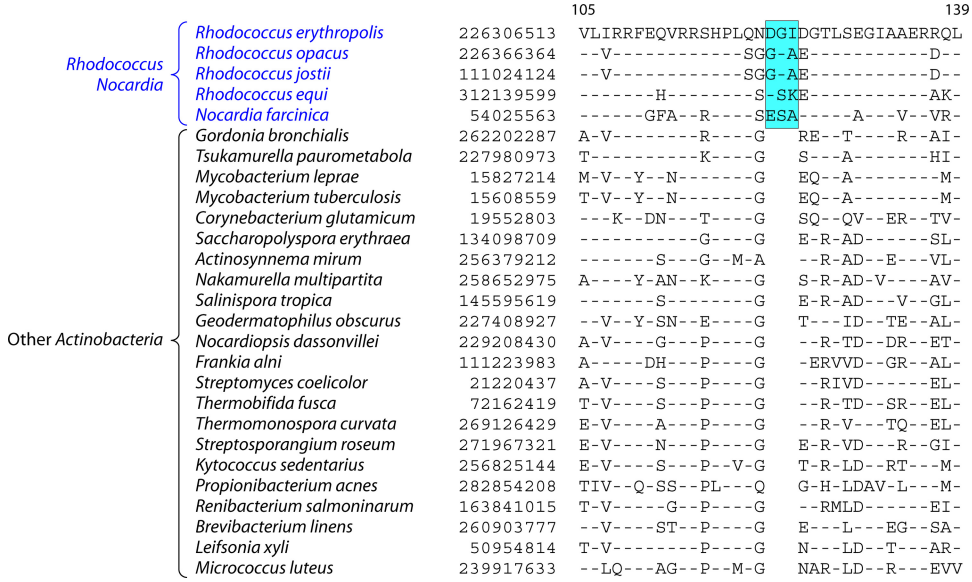

FIG 6 Partial sequence alignments of an ATP-binding protein showing a 3-aa CSI that is uniquely found in *Rhodococcus-Nocardia* species. Another CSI that is specific for *Rhodococcus-Nocardia* is shown in File S12 in the supplemental material.

family *Gordoniaceae*, should not be merged with it, as was recently proposed (191). There is no sequence information available at present for the genera *Skermania* and *Williamsia* to determine if they are specifically related to *Rhodococcus* and *Nocardia*.

In addition to these CSIs and CSPs that are shared by both *Rhodococcus* and *Nocardia* species, we have also identified a 3-aa insert in a hypothetical protein, BlinB_00480, that is specifically shared by all four sequenced *Rhodococcus* species (*viz.*, *R. jostii*, *R. opacus*, *R. equi*, and *R. erythropolis*), providing a molecular marker for this genus (see File S13 in the supplemental material).

Molecular Signatures of *Corynebacterium* and the *Corynebacteriaceae*

The genus *Corynebacterium* contains numerous species that are of much interest due to their involvement in human and animal diseases (*viz.*, *C. diphtheriae*, *C. striatum*, *C. jeikeium*, *C. urealyticum*, *C. ulcerans*, and *C. pseudotuberculosis*) and also for large numbers of industrial applications, including the production of amino acids, nucleotides, and other nutritional factors; hydrocarbon degradation; and the bioconversion of steroids, etc. (27, 37, 57, 94, 113, 149, 166, 224, 267, 327). As a result, large numbers of genomes of *Corynebacterium* species and strains, including *C. aurimucosum*, *C. diphtheriae*, *C. efficiens*, *C. glutamicum*, *C. jeikeium*, *C. kroppenstedtii*, *C. pseudotuberculosis*, *C. ulcerans*, and *C. urealyticum*, have been sequenced, and many are in the process of being sequenced (39, 159, 213, 267, 298, 299, 304, 339). The family *Corynebacteriaceae* contains two genera, *Corynebacterium* and *Turicella* (57, 103, 187, 191). However, currently, no sequences are available for the latter genus, which also lacks mycolic acids, which is a uniquely shared characteristic of most other members of the *Corynebacteriales* (58, 111, 187, 191). In phylogenetic trees based upon 16S rRNA (187, 227, 343) or concatenated protein sequences (Fig. 2 and 4), *Corynebacterium* species formed a strongly supported clade, and it was separated from other *Corynebacteriales* species by a long branch. In the tree shown in Fig. 4, *Dietzia* was

TABLE 6 Signature proteins that are specific for the family *Nocardiaceae*[a]

Gene	GenBank accession no.	Protein function	Length (aa)	Species specificity
RHA1_ro00267	YP_700261.1	Hypothetical	108	*Rhodococcus* and *Nocardia*
RHA1_ro00333	YP_700327.1	Hypothetical	172	*Rhodococcus* and *Nocardia*
RHA1_ro01075	YP_701060.1	Hypothetical	250	*Rhodococcus* and *Nocardia*[b]
RHA1_ro01170	YP_701155.1	Hypothetical	151	*Rhodococcus* and *Nocardia*
RHA1_ro02067	YP_702032.1	Hypothetical	111	*Rhodococcus* and *Nocardia*
RHA1_ro02254	YP_702219.1	Hypothetical	207	*Rhodococcus* and *Nocardia*
RHA1_ro02467	YP_702430.1	Hypothetical	109	*Rhodococcus* and *Nocardia*
RHA1_ro02848	YP_702811.1	Hypothetical	97	*Rhodococcus* and *Nocardia*[b]
RHA1_ro04046	YP_704001.1	Hypothetical	275	*Rhodococcus* and *Nocardia*[b]
RHA1_ro04254	YP_704203.1	Hypothetical	389	*Rhodococcus* and *Nocardia*
RHA1_ro05348	YP_705286.1	Hypothetical	201	*Rhodococcus* and *Nocardia*[b]
RHA1_ro05515	YP_705453.1	Hypothetical	311	*Rhodococcus* and *Nocardia*
RHA1_ro05936	YP_705871.1	Hypothetical	52	*Rhodococcus* and *Nocardia*
RHA1_ro05750	YP_705686.1	Hypothetical	141	*Rhodococcus* and *Nocardia*

[a] These CSPs were identified by Blastp searches of proteins from the genome of *Rhodococcus jostii* RHA1.
[b] Significant hits were also observed for other isolated actinobacterial species.

its closest relative, and a clade consisting of these two genera was also strongly supported. Within the clade consisting of *Corynebacterium* species, a number of distinct clusters or subclades were also well resolved (Fig. 4). The distinctness of *Corynebacterium* species from all other members of the *Corynebacteriales* and the existence of a number of discrete clades within this genus are also independently supported by many CSIs and CSPs that we have identified.

The presence of an arabinogalactan polymer in the cell wall is a unique characteristic of members of the orders *Corynebacteriales* and *Pseudonocardiales* (111, 182, 187). The enzyme phosphoribose diphosphate:decaprenyl-phosphate phosphoribosyltransferase (UbiA) plays an essential role in this process by catalyzing the transfer of ribose-5-phosphate from phosphoribose diphosphate to decaprenylphosphate to form decaprenylphosphoryl-5-phosphoribose (198). In this enzyme, we have identified a 2-aa insert in a conserved region that is uniquely present in all of the sequenced *Corynebacterium* species (Fig. 7A) but not in any other *Actinobacteria*. Similarly, in the enzyme acetate kinase, which carries out the phosphorylation of acetate to produce acetyl phosphate, a 3-aa insert in a conserved region is specifically present in all available sequences of *Corynebacterium* species (see File S14 in the supplemental material). Another CSI that is specific for *Corynebacterium* is present in the enzyme protoheme IX farnesyltransferase (CyoE), which is involved in the biosynthesis of heme A (250). Most *Corynebacterium* species have a 7-aa insert in this protein; however, *C. jeikeium* and *C. urealyticum*, which form a distinct clade, contain a longer insert (10 aa) in the same position (see File S15 in the supplemental material). Blast searches for various proteins from the genome of *Corynebacterium glutamicum* ATCC 13032 have also identified 20 CSPs that are uniquely present in all or most of the sequenced *Corynebacterium* species (Table 7). While 16 of these 20 CSPs are entirely specific for the genus *Corynebacterium*, the homologs of three of them are also present in *Dietzia cinnamea* (belonging to the family *Dietziaceae*), which forms the outgroup of the *Corynebacterium* clade in the phylogenetic tree (Fig. 4). The shared presence of these CSPs in *Corynebacterium* and *Dietzia cinnamea* supports the inference from the phylogenetic tree (Fig. 4) that species of these two families are distantly but specifically related to each other.

In the phylogenetic tree shown in Fig. 4, *C. diphtheriae*, *C. pseudotuberculosis*, *C. ulcerans*, *C. aurimucosum*, *C. glutamicum*, and *C. efficiens* formed a distinct cluster (marked as cluster I) within the genus *Corynebacterium*. The existence of this clade is also strongly supported by a number of identified CSIs and CSPs. One example of a CSI that is specific for cluster I *Corynebacterium* species is shown in Fig. 7B. In this case, in the β'-subunit of RNA polymerase (RpoC), which is highly conserved and universally distributed, a 7- to 8-aa insert in a conserved region is specifically present in all of the cluster I *Corynebacterium* species, but it is not found in other species, such as *C. jeikeium*, *C. urealyticum*, and *C. kroppenstedtii*, that are not part of this clade. Another 2-aa insert that is specific for cluster I species is present in a conserved region of the GTP-binding protein LepA (see File S16 in the supplemental material), which plays an important role in protein synthesis, particularly under stress conditions (236). For some species that contain the RpoC and LepA inserts (*viz.*, *C. matruchotii*, *C. striatum*, *C. ammoniagenes*, *C. accolens*, *C. lipophiloflavum*, *C. tuberculostearicum*, and *C. glucuronolyticum*), because their genomes were not sequenced, sequence information is not present in the phylogenetic tree (Fig. 4). However, based upon the shared presence of CSIs in both the RpoC and LepA proteins, it is predicted that these species will also group with cluster I *Corynebacterium* species. The genetic distinctness of cluster I is also strongly supported by 21 CSPs that are uniquely present in all of the sequenced species of this cluster (see the first 21 entries in File S17 in the supplemental material). Additionally, File S17 in the supplemental material lists 19 other CSPs that are uniquely found in *C. jeikeium* and *C. urealyticum*, which form another strongly supported cluster in the phylogenetic tree (Fig. 4) (187). These CSIs and CSPs provide novel molecular markers for the identification and circumscription of the genus *Corynebacterium* and two of its clades. It should be noted that the genetic distances between these subclades of the genus *Corynebacterium* are greater than those observed among or between species of the families *Mycobacteriaceae* and *Nocardiaceae*. Hence, it can be argued that species of these subclades should be recognized as distinct genera rather than being part of the same genus. It should also be noted that the various CSIs or CSPs that are specific for cluster I *Corynebacterium* species or for *C. jeikeium* and *C. urealyticum* are not found in *C. kroppenstedtii*, which is separated from both of these clusters by a long branch (Fig. 4). Unlike other *Corynebacterium* species, *C. kroppenstedtii* lacks mycolic acid (298), and its phylogenetic position and the absence of signatures for the other two clusters suggest that it forms a distinct subgroup of *Corynebacterium* species.

Molecular Signatures Supporting the Deeper Branching of *Corynebacterium* and *Dietzia* within the Order *Corynebacteriales*

Within the Order *Corynebacteriales*, a clade consisting of *Corynebacterium* species and *Dietzia* shows the deepest branching, and it is separated from other *Corynebacteriales* by a long branch (Fig. 4). Within this clade, the species belonging to the families *Mycobacteriaceae* and *Nocardiaceae* generally group together, and the other *Corynebacteriales* species branch in between these two clades. Our analyses of *Corynebacteriales* genomes have identified a number of CSPs that further strongly support these relationships. Table 8 lists a number of CSPs that are present in most other *Corynebacteriales* species except *Corynebacterium* species and *Dietzia*. The genes for these proteins likely originated from a common ancestor(s) of the other *Corynebacteriales* following the divergence of *Corynebacterium* and *Dietzia*. Of the proteins listed in Table 8, the first four are found mainly in *Mycobacteriaceae* and *Nocardiaceae* species, supporting a closer relationship between these two families. The homologs of the remainder of these CSPs are found in *Gordonia bronchialis* and also, in some cases, in *Segniliparus rotundus*, *Tsukamurella paurometabola*, and *Amycolicicoccus subflavus*, supporting their branching in between the *Corynebacterium-Dietzia* clade and the *Mycobacteriaceae-Nocardiaceae* clade.

MOLECULAR SIGNATURES SHOWING THAT *CORYNEBACTERIALES* AND *PSEUDONOCARDIALES* ARE CLOSELY RELATED

The orders *Pseudonocardiales* and *Corynebacteriales* are the only two orders within the phylum *Actinobacteria* that have cell walls containing *meso*-diaminopimelic acid, arabinose, and galactose (cell wall chemotype IV) (58, 111, 187). However, unlike the *Co-*

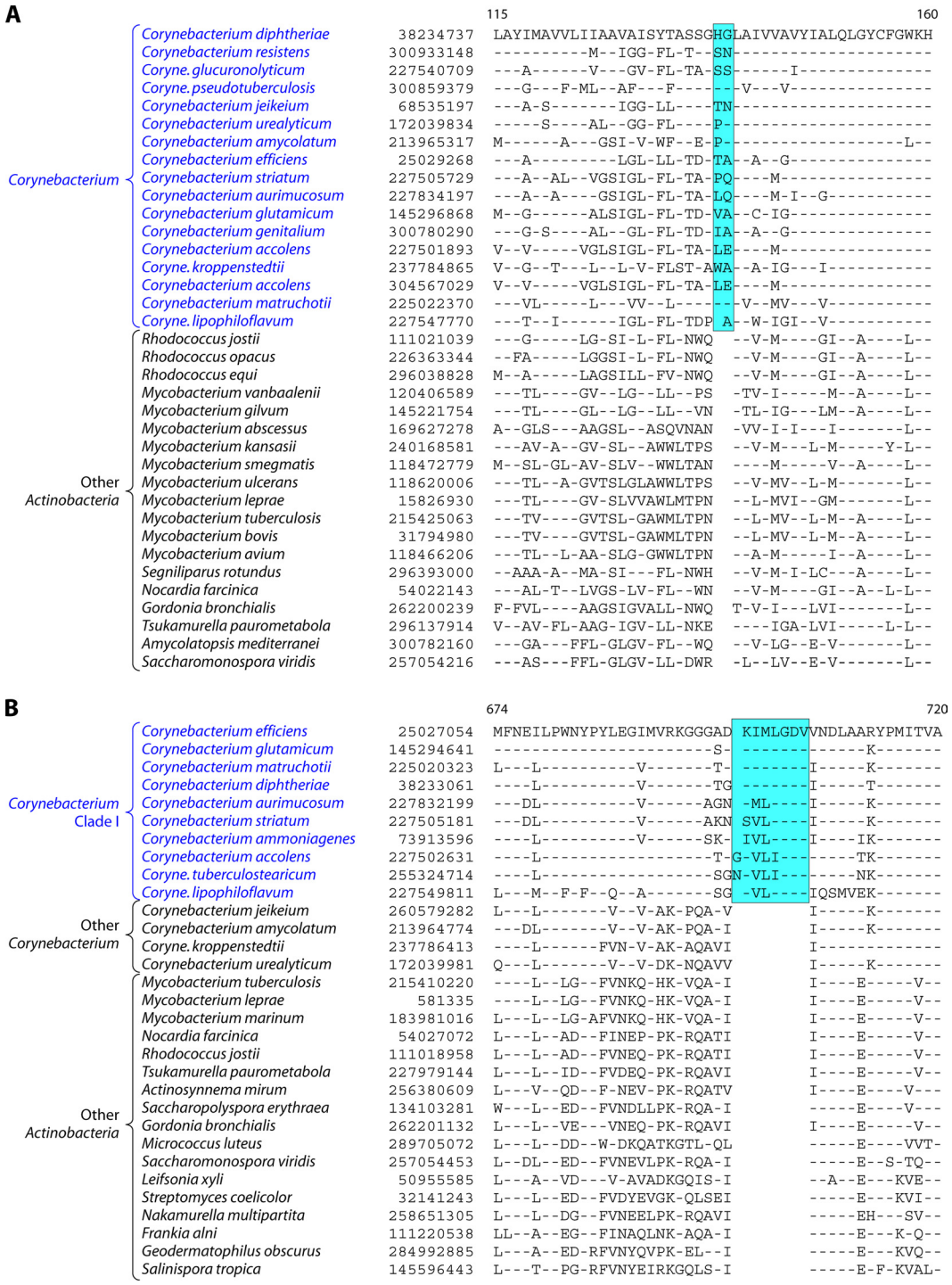

FIG 7 (A) Partial sequence alignments of the protein phosphoribose diphosphate:decaprenyl-phosphate phosphoribosyltransferase acyl-CoA carboxylase acetate kinase showing a 2-aa conserved insert that is uniquely found in various *Corynebacterium* species but not in any other bacteria. The acetate kinase and CyoE proteins also contain CSIs that are specific for the genus *Corynebacterium* (see Files S14 and S15 in the supplemental material). (B) Partial sequence alignments of the RNA polymerase β'-subunit (RpoC) showing a 7- to 8-aa conserved insert that is specifically found in clade I *Corynebacterium* species (Fig. 4). Another CSI that is specific for clade I *Corynebacterium* species is present in the GTP-binding protein LepA (see File S16 in the supplemental material).

rynebacteriales, mycolic acids are absent from the cell walls of *Pseudonocardiales* species. In phylogenetic trees based upon 16S rRNA or other gene/protein sequences, *Pseudonocardiales* species generally cluster with species of the order *Corynebacteriales* (3, 5, 343), but this clade is not strongly supported. The order *Pseudonocardiales* was until recently comprised of two families, *Pseudonocardiaceae* and *Actinosynnemataceae* (103). However, both these families are now combined into the family *Pseudonocardiaceae*

TABLE 7 Signature proteins that are specific for the genus *Corynebacterium*[a]

Gene	GenBank accession no.	Protein function	Length (aa)	Species specificity
NCgl0188	NP_599444.1	Hypothetical	75	Genus *Corynebacterium*[b]
NCgl0238	NP_599494.1	Hypothetical	183	Genus *Corynebacterium*
NCgl0362	NP_599621.1	Hypothetical	109	Genus *Corynebacterium*
NCgl0481	NP_599742.1	Hypothetical	233	Genus *Corynebacterium*
NCgl0588	NP_599849.1	Hypothetical	147	Genus *Corynebacterium*
NCgl1056	NP_600329.1	Hypothetical	137	Genus *Corynebacterium*
NCgl1090	NP_600363.1	Hypothetical	267	Genus *Corynebacterium*
NCgl1456	NP_600729.1	Hypothetical	126	Genus *Corynebacterium*
NCgl1866	NP_601148.1	Hypothetical	252	Genus *Corynebacterium*
NCgl2043	NP_601325.1	Hypothetical	77	Genus *Corynebacterium*[b]
NCgl2214	NP_601494.1	Hypothetical	226	Genus *Corynebacterium*
NCgl2224	NP_601505.1	Hypothetical	585	Genus *Corynebacterium*
NCgl2534	NP_601824.1	Hypothetical	109	Genus *Corynebacterium*
NCgl2641	NP_601932.1	Hypothetical	221	Genus *Corynebacterium*[c]
NCgl2776	NP_602066.1	Hypothetical	166	Genus *Corynebacterium*
NCgl2836	NP_602124.1	Hypothetical	183	Genus *Corynebacterium*[b,c]
NCgl2882	NP_602180.1	Hypothetical	63	Genus *Corynebacterium*
NCgl2888	NP_602186.1	Hypothetical	165	Genus *Corynebacterium*
NCgl2197	NP_601477.1	Hypothetical	194	Genus *Corynebacterium*
NCgl0807	NP_600070.1	Hypothetical	89	Genus *Corynebacterium*

[a] These CSPs were identified by Blastp searches for proteins from the genome of *C. glutamicum* ATCC 13032.
[b] Also found in *Dietzia cinnamea.*
[c] Also present in 1 to 2 *Pseudonocardiales* species.

(191). Genome sequences are now available for a number of genera of this order, including *Saccharomonospora*, *Saccharopolyspora*, *Actinosynnema*, and *Amycolatopsis* (Table 1) (222, 228).

In the phylogenetic tree based upon concatenated protein sequences (Fig. 2), the sequenced *Pseudonocardiaceae* species formed a strongly supported clade. *Nakamurella multipartite*, which is currently a part of the order *Frankiales* (77, 103), formed an outgroup of the *Pseudonocardiaceae* clade, and a clade consisting of *N. multipartite* and *Pseudonocardiaceae* species was also strongly supported (100% bootstrap score). However, other *Frankiales* species did not branch with *N. multipartite*. We have also identified a CSI consisting of a 1-aa insert in the enzyme uridylate kinase, which catalyzes the reversible phosphorylation of UMP to UDP, which is uniquely shared by most of the *Pseudonocardiaceae* species (all except *Saccharomonospora*) and also by *N. multipartite* (Fig. 8A). This CSI, in addition to providing a molecular marker for most of the *Pseudonocardiales*, also provides evidence that *N. multipartite* is closely related to this group.

A number of additional identified CSIs and CSPs provide evidence that species of the orders *Corynebacteriales* and *Pseudonocardiales* are specifically related to each other. In the enzyme UDP-galactopyranose mutase (UGM), which catalyzes the interconversion of UDP-galactopyranose (UDP-Gal*p*) and UDP-galactofuranose (UDP-Gal*f*) (303), a 3-aa insert in a conserved region is uniquely present in various *Corynebacteriales* and *Pseudonocardiales* species (Fig. 9A). This insert is also present in *N. multipartite* and also *Geodermatophilus obscurus* (another member of the *Frankiales*), which forms an outgroup of the clade consisting of the above-described two orders, but it is not found in any other *Actinobacteria*. The enzyme UDP-galactopyranose mutase plays an important role in the biosynthesis of cell wall arabinogalactan, and inhibitors of this enzyme are growth inhibitory to *M.*

TABLE 8 CSPs that are present in most members of the *Corynebacteriales* except *Corynebacterium*

Gene or protein	GenBank accession no.	Protein function	Length (aa)	Species specificity[a]
MAV_0513	YP_879795.1	Hypothetical	328	*Mycobacterium* and *Nocardiaceae*
MAV_1758	YP_880983.1	Hypothetical	216	*Mycobacterium* and *Nocardiaceae*
MAV_3193	YP_882377.1	Hypothetical	225	*Mycobacterium* and *Nocardiaceae*
MAV_0614	YP_879894.1	Hypothetical	133	*Mycobacterium*, *Nocardiaceae*, and *Gordonia*
MAV_0754	YP_880029.1	Hypothetical	32	*Mycobacterium*, *Nocardiaceae*, and *Gordonia*
LysM domain-containing protein	YP_882790.1	Bacterial cell wall degradation	164	*Mycobacterium*, *Nocardiaceae*, and *Gordonia*
MAV_4251	YP_883392.1	Hypothetical	86	*Mycobacterium*, *Nocardiaceae*, and *Gordonia*
MAV_0454	YP_879736.1	Hypothetical	126	*Mycobacterium*, *Rhodococcus*, and *Gordonia*[b]
MAV_4261	YP_883402.1	Hypothetical	108	*Mycobacterium*, *Nocardiaceae*, and *Gordonia*[b]
MAV_5300	YP_884410.1	Hypothetical	254	*Mycobacterium*, *Nocardiaceae*, and *Gordonia*[b]
MAV_2940	YP_882126.1	Hypothetical	186	*Mycobacterium*, *Nocardiaceae*, and *Gordonia*[b]
MAV_4016	YP_883169.1	Hypothetical	117	*Mycobacterium*, *Nocardiaceae*, and *Gordonia*[b]

[a] These CSPs were identified by Blastp searches for proteins from the genome of *Mycobacterium avium* 104. Although these CSPs are found mainly in the indicated families of *Corynebacteriales*, isolated hits for a few of them may also be present in 1 to 2 other species.
[b] Also present in one or more of the following *Corynebacteriales* species: *Segniliparus rotundus*, *Tsukamurella paurometabola*, and *Amycolicicoccus subflavus.*

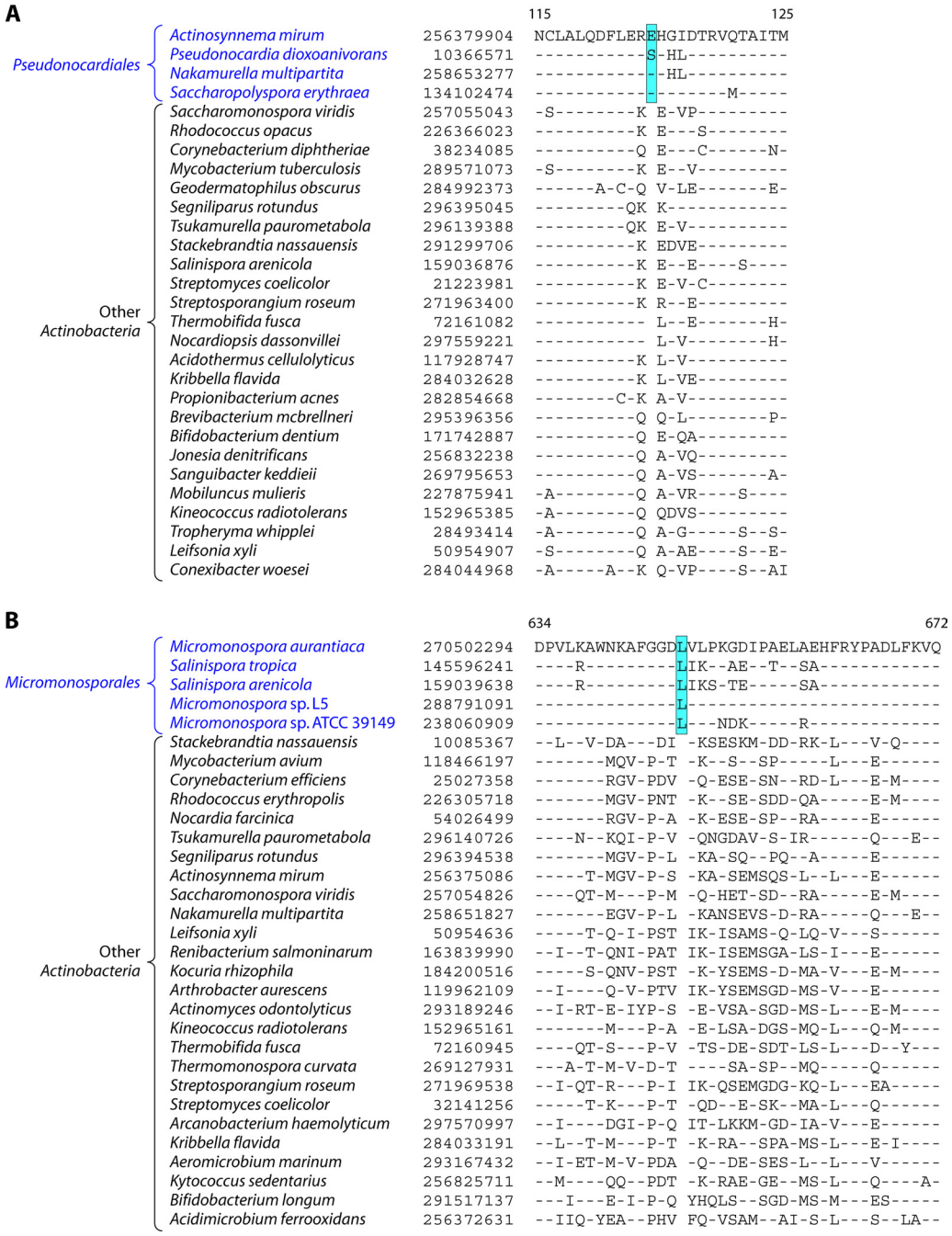

FIG 8 Excerpts from sequence alignments of the uridylate kinase protein (A) and the hypothetical protein Lxx093000 (B) showing two conserved inserts that are specific for species of the orders *Pseudonocardiales* and *Micromonosporales*, respectively.

tuberculosis (75, 275). Another CSI, consisting of a 2-aa deletion, that is uniquely shared by most of the species of these two orders is present in translation initiation factor 2 (IF-2) (see File S18 in the supplemental material), which plays an essential role in the process of protein biosynthesis (177). In this case, the identified CSI is commonly present in all of the *Corynebacteriales* as well as *Pseudonocardiales* species, but it is not found in any other bacteria except *G. obscurus*, which also contains the UGM insert (Fig. 9A). The shared presence of these CSIs in all of the *Corynebacteriales* as well as *Pseudonocardiales* species but not in any other *Actinobacteria* (except *G. obscurus* and *N. multipartite*, which branch with them or between them) strongly supports the inference from phylogenetic studies that these two orders are closely related and that they shared a common ancestor exclusive of other *Actinobacteria*. A number of studies indicated that species of the order *Frankiales* do not form a coherent phylogenetic lineage, and the taxonomy of this order needs to be emended (191, 215, 343). In this context, our observations that *N. multipartite* and *G. obscurus* consistently branch with *Pseudonocardiales* species in a phylogenetic tree based upon concatenated protein sequences and that they share several

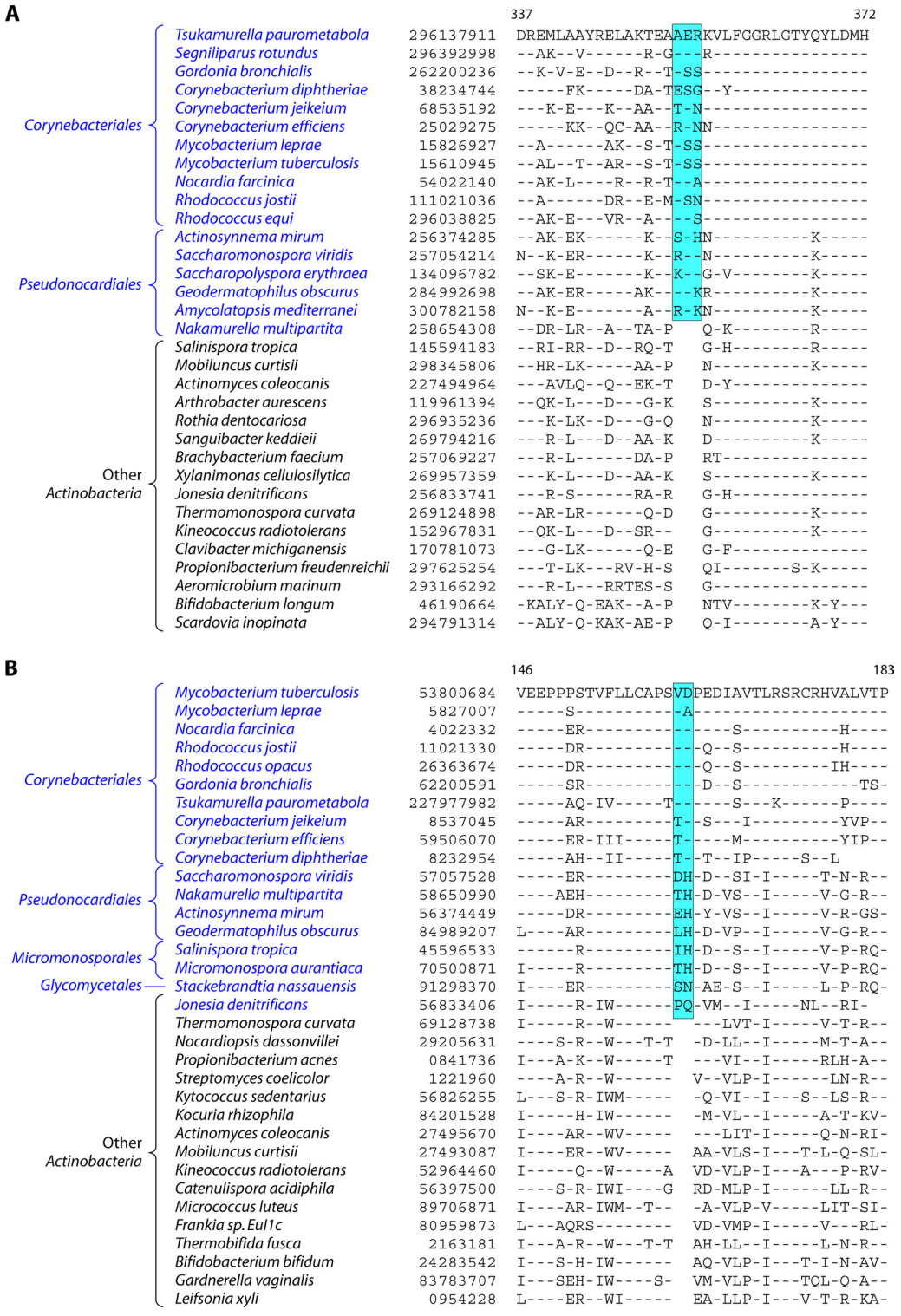

FIG 9 (A) Partial sequence alignments of the protein UDP-galactopyranose mutase showing a 3-aa CSI that is uniquely shared by various species of the orders *Corynebacteriales* and *Pseudonocardiales* but that is not found in other *Actinobacteria*. (B) Partial sequence alignments of DNA polymerase HolB showing a CSI that is uniquely shared by various species of the orders *Corynebacteriales*, *Pseudonocardiales*, *Micromonosporales*, and *Glycomycetales*, indicating that species from these groups shared a common ancestor exclusive of other *Actinobacteria*. Sequence information for other CSIs that are specific for these actinobacterial orders is presented in Files S18 to S20 and S22 in the supplemental material.

TABLE 9 Signature proteins that are specific for the orders *Corynebacteriales* and *Pseudonocardiales*[a]

Gene	GenBank accession no.	Protein function (references)	Length (aa)
ML0105	NP_301202	EmbA, arabinosyl transferase (7, 259)	1,111
ML0106	NP_301203	EmbC, arabinosyl transferase (112, 259)	1,070
ML0281	NP_301322	Hypothetical	229
ML0810	NP_301617	Hypothetical	407
ML0990	NP_301735	Hypothetical	209

[a] These CSPs were identified by Blastp searches of the genome of *M. leprae* TN (100). Significant Blast hits for some of these proteins have also been observed for *G. obscurus*.

CSIs in common with them support the placement of these species into this order of the *Actinobacteria*.

In addition to these CSIs, we have also identified 5 CSPs that are uniquely present in most of the species of the orders *Corynebacteriales* and *Pseudonocardiales* (Table 9). In addition to the species of these two orders, homologs of these proteins are also generally present in *G. obscurus* and *N. multipartite*, providing further evidence that they are closely related to species of these orders, particularly those of the *Pseudonocardiales*. Of these five CSPs, two (EmbA and EmbC) are involved in the synthesis of cell wall arabinan, which is a uniquely shared biochemical characteristic of the cell walls of these two orders of the *Actinobacteria* (58, 106, 178, 182, 263). In contrast to these two proteins, two other proteins involved in the synthesis of cell wall arabinan (*viz.*, EmbB and AftA) are limited to only various *Corynebacteriales* (Table 4). All four of these genes are part of the Emb operon, and they provide important targets for antitubercular drugs (24, 300). The antimycobacterial drug ethambutol inhibits the growth of *M. tuberculosis* through the inhibition of arabinofuranosyltransferases EmbA and EmbB (6, 24, 300). The other 3 CSPs listed in Table 9 are of unknown functions.

Molecular Signatures of *Micromonosporales* and Identification of a Higher Clade Consisting of the Orders *Corynebacteriales*, *Pseudonocardiales*, *Glycomycetales*, and *Micromonosporales*

The order *Micromonosporales* contains a single family, *Micromonosporaceae*, that is made up of 20 genera (77, 103, 110, 283, 309). However, these genera do not form a distinct clade in the 16S rRNA trees, and species from other groups are interspersed within this order. Hence, this order is presently poorly defined in a phylogenetic or taxonomic sense (191). Genome sequences are now available for this order, from *Micromonospora aurantiaca*, *Micromonospora* sp. strain L5, *Salinispora tropica*, and *Salinispora arenicola* (Table 1) (229, 309). In the phylogenetic tree constructed based upon concatenated sequences for 35 broadly distributed proteins (Fig. 2), the sequenced *Micromonosporaceae* species formed a strongly supported clade branching in the neighborhood of *Pseudonocardiales*. *Stackebrandtia nassauensis*, which is the only species in our data set belonging to the order *Glycomycetales*, was most closely related to this group, and a clade consisting of the *Micromonosporaceae* species and *S. nassauensis* was strongly supported by the bootstrap score. A clade consisting of these species in turn was part of a larger clade that included all species of the orders *Corynebacteriales* and *Pseudonocardiales*.

A number of identified CSIs provide useful information regarding the *Micromonosporaceae* species and their relationships to other orders of the *Actinobacteria*. First, in a protein of unknown function, Lxx09300, we identified a 1-aa insert in a conserved region that is specifically present in all sequenced *Micromonosporaceae* species (Fig. 8B). This CSI provides a potential molecular marker for distinguishing species of this order from those of other *Actinobacteria*. Second, we have also identified 3 CSIs in important proteins that are uniquely shared by all sequenced species of the orders *Corynebacteriales*, *Pseudonocardiales*, *Micromonosporales*, and *Glycomycetales* (represented by *S. nassauensis*). The first of these CSIs consists of a 2-aa insert in the delta subunit of DNA polymerase III (HolB), which is involved in replicative DNA synthesis in bacteria (73) (Fig. 9B). This insert is uniquely shared by all species of these orders but is not found in any other *Actinobacteria* (except *Jonesia denitrificans*) or bacteria. Another CSI showing a similar species distribution is present in a highly conserved region of the ribosomal protein S3 (see File S19 in the supplemental material). Lastly, in the enzyme alpha-ketoglutarate decarboxylase (KGD), which is involved in the decarboxylation of alpha-ketoglutarate, a 1-aa insert in a conserved region is commonly present in all species of these orders, but except for *Acidothermus cellulolyticus*, it is not found in any other *Actinobacteria* (see File S20 in the supplemental material). The shared presence of these CSIs in these important housekeeping proteins by the above-described orders of *Actinobacteria*, which also cluster together in the phylogenetic tree, strongly indicates that these orders of *Actinobacteria* shared a common ancestor exclusive of all other *Actinobacteria*.

Molecular Signatures of *Frankia* and Identification of a Clade Consisting of the Orders *Corynebacteriales*, *Pseudonocardiales*, *Glycomycetales*, *Micromonosporales*, and *Frankiales*

The order *Frankiales* is presently comprised of six families: *Frankiaceae*, *Acidothermaceae*, *Nakamurellaceae*, *Cryptosporangiaceae*, *Geodermatophilaceae*, and *Sporichthyaceae* (77, 103, 216). Genome sequences are now available for a number of *Frankia* species (216) as well as a number of other genera (*viz.*, *Acidothermus*, *Nakamurella*, and *Geodermatophilus*) covering three other families (14, 154, 302) (Table 1). As noted above, species of the order *Frankiales* do not form a coherent phylogenetic lineage, and they branch in a number of independent positions in the 16S rRNA tree and other phylogenetic trees (283, 343). This is clearly seen from the branching positions of *G. obscurus*, *N. multipartite*, *Acidothermus cellulolyticus*, and *Frankia* species in the tree shown in Fig. 2. As discussed above, *G. obscurus* and *N. multipartite*, based upon their branching in the tree and a number of CSIs, are more closely related to the *Pseudonocardiales* than to the type genus of this order, *Frankia*, which contains the type species *F. alni*. Furthermore, although a clade consisting of different sequenced strains of *Frankia* branches in the proximity of *A. cellulolyticus* (5), a specific relationship between these species was not supported by our tree. Thus, the order *Frankiales*, as described currently, cannot be delimited by any means, and its taxonomy needs to be emended. However, we have identified a 7-aa insert in a highly conserved region of the DNA gyrase B protein that is uniquely present in various *Frankia* species (Fig. 10). In addition to the sequenced genomes, partial information for gyrase B covering this region is available for a large number of other *Frankia* species and strains (217), and this insert is present in all of them, thus providing a highly specific mo-

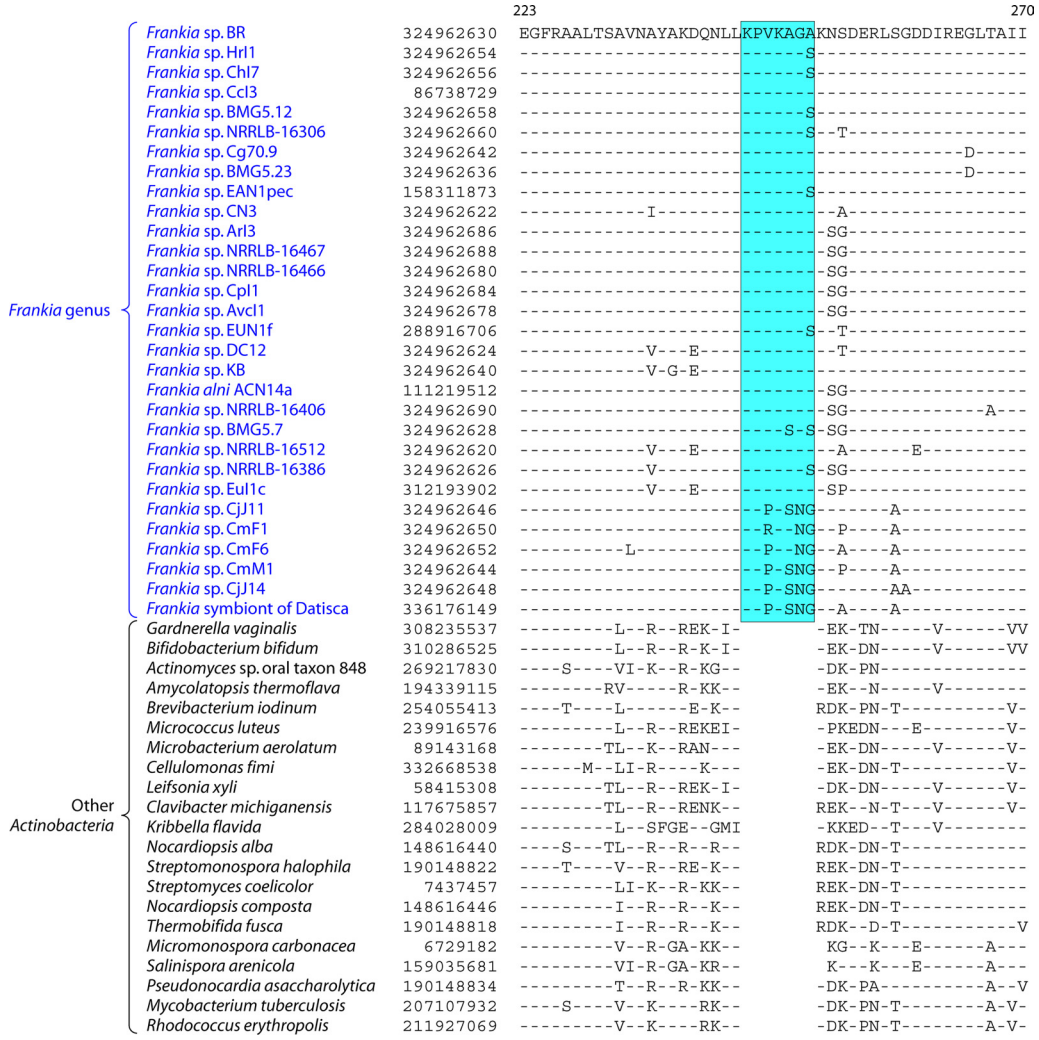

FIG 10 Excerpts from the sequence alignment of the gyrase B protein showing a 7-aa insert in a conserved region that is uniquely present in various *Frankia* species but is not found in other *Actinobacteria*. Two other CSIs that are also largely specific for the genus *Frankia* are shown in File S21 in the supplemental material.

lecular marker for this genus. In addition to the CSI in gyrase B, sequence information for two additional CSIs that are also specific mainly for *Frankia* species is provided in File S21 in the supplemental material. These CSIs include a 4- to 5-aa insert in the DNA repair protein RadA and a 3-aa insert in a hypothetical protein, Ncg1. However, besides *Frankia*, the latter CSIs are also present in a few other *Actinobacteria*, which could be due to LGTs.

In the phylogenetic tree based on concatenated protein sequences (Fig. 2), although the clade consisting of *Frankia* spp. branched in the proximity of *Micromonosporales*, it was not specifically related to this order or to the larger clade consisting of the orders *Corynebacteriales*, *Pseudonocardiales*, *Micromonosporales*, and *Glycomycetales* (Fig. 2). However, we have identified one CSI consisting of a 1-aa insert in a conserved region of the protein glutamine phosphoribosylpyrophosphate amidotransferase that is uniquely shared by all species of the orders *Corynebacteriales*, *Pseudonocardiales*, *Micromonosporales*, and *Glycomycetales* and also by various *Frankia* spp. (see File S22 in the supplemental material). Except for species of these orders, this insert is found only in *Propionibacterium acnes* and *Micrococcus luteus* and not in other *Propionibacteriales* or *Micrococcales* or other orders of *Actinobacteria*. This CSI provides suggestive evidence that *Frankia* spp. may also have shared a common ancestor with the clade consisting of *Corynebacteriales*, *Pseudonocardiales*, *Micromonosporales*, and *Glycomycetales*.

Based upon the species distribution patterns of various identified CSIs and CSPs that are discussed above, the evolutionary stages in which the genes for these CSPs, or the genetic changes responsible for the observed CSIs, are postulated to have evolved are depicted in Fig. 11. Most of the nodes in this diagram are supported by phylogenetic analysis and independently by many identified molecular markers indicating that these branching patterns are reliable.

MOLECULAR SIGNATURES OF THE *STREPTOMYCETALES* AND EVIDENCE FOR ITS RELATEDNESS TO THE *CATENULISPORALES*

The order *Streptomycetales* consists of a single family, *Streptomycetaceae*, that is comprised of three genera, *Streptomyces*, *Kitasatospora*, and *Streptacidiphilus* (77, 103, 160). This group of species,

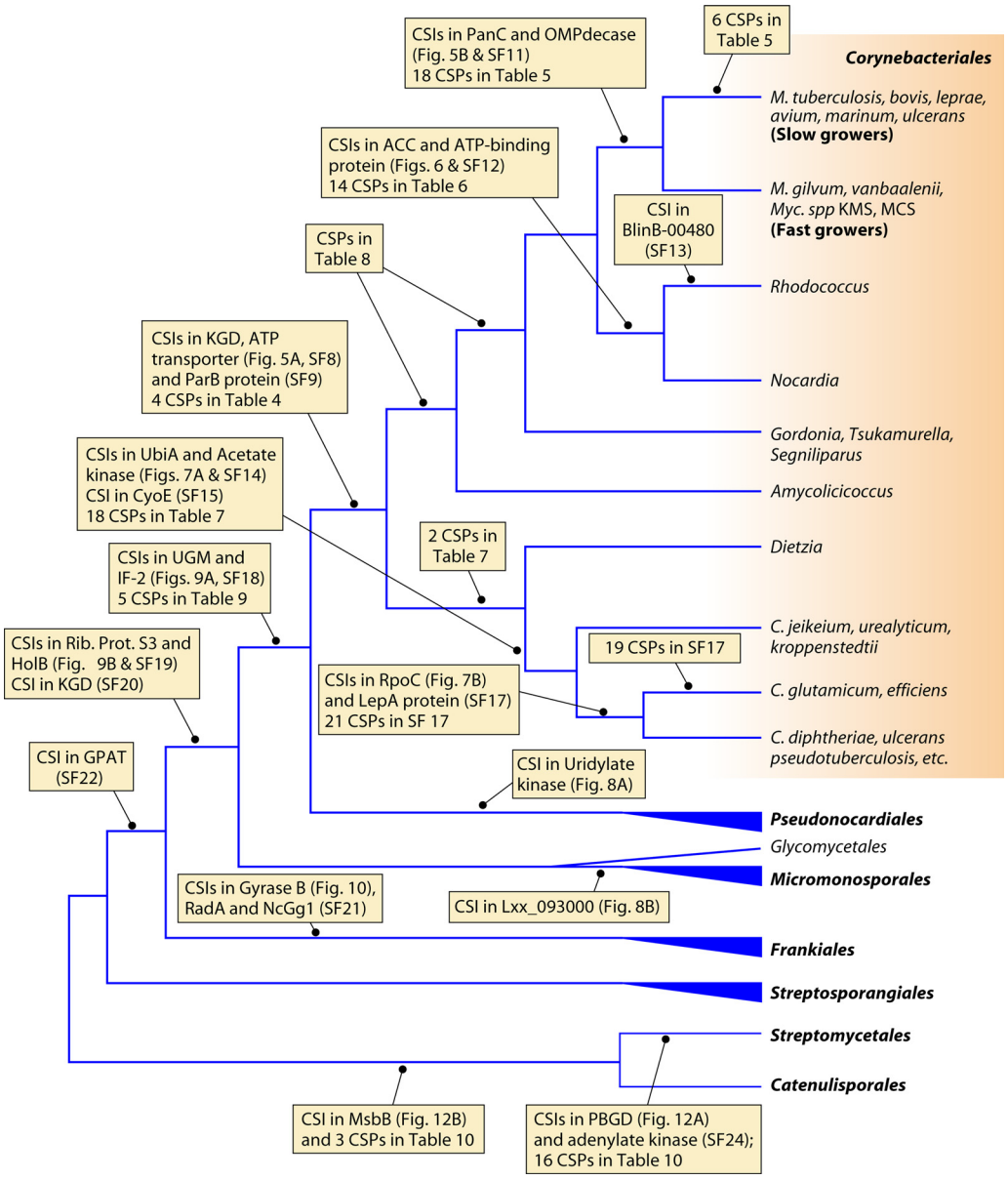

FIG 11 Summary diagram showing the evolutionary relationships among the orders *Corynebacteriales*, *Pseudonocardiales*, *Micromonosporales*, *Glycomycetales*, *Frankiales*, *Streptosporangiales*, *Streptomycetales*, and *Catenulisporales* based upon phylogenetic trees (Fig. 2 and 4, and see File S10 in the supplemental material) and various identified CSIs and CSPs. OMPdecase, OMP-decarboxylase; GPAT, glutamine phosphoribosyl amidotransferase; SF11, File S11 in the supplemental material.

particularly *Streptomyces*, has been extensively studied since the discovery of the earliest antibiotics from species of this genus in the 1940s (12, 21, 68, 197). *Streptomyces* spp. are now the source of nearly two-thirds of all known antibiotics, and they also produce numerous other biologically important compounds, including herbicides, antiparasitic agents, immunosuppressants, and other compounds that are of industrial interest (16, 21, 36, 41, 45, 86, 107). *Streptomyces* spp. in particular, and the *Actinobacteria* as a whole, are now recognized as the richest source of small-molecule diversity on the planet (12, 21, 36, 45, 86, 87, 220, 249). The genome sequences of these bacteria are among the largest of the prokaryotes (Table 1), and they contain the largest numbers of gene clusters involved in the synthesis of known or predicted novel small molecules (18, 36, 40, 45, 87, 219, 220, 222, 257, 314, 322, 332). *Streptomyces* species also possess a complex but well-studied developmental cycle, and of these species, *S. coelicolor* has provided a good model system for different types of studies (41–43, 137, 163, 257, 314). Methods for the genetic manipulation of *S. coelicolor* (*viz.*, gene expression and gene knockout and replacement, etc.) are also now well established (138, 163). Due to huge interest in the bioprospecting of *Streptomyces* and related bacteria for the discovery of novel biological compounds, >500 species of *Streptomyces* have now been identified (77, 160). The genomes of several *Streptomyces* species (*viz.*, *S. avermitilis*, *S. bingchenggensis*, *S. coelicolor*, *S. flavogriseus*, *S. griseus*, and *S. scabiei*) have been sequenced, and the sequencing of numerous other genomes is in

progress (18, 23, 144, 148, 322). These genomes provide a valuable resource for the identification of molecular signatures that are specific for the order *Streptomycetales* and provide information regarding its evolutionary relationship to other orders of *Actinobacteria*.

The order *Catenulisporales* contains a total of 5 species that are placed into two monogeneric families (*viz.*, *Catenulisporaceae* and *Actinospicaceae*) (77, 191). Very little work has been carried out on species of this order, and their phylogenetic relationships to other orders of *Actinobacteria* are presently unclear. The genome sequence of *Catenulispora acidiphila* of this order is now available (59). In the phylogenetic tree for *Actinobacteria* based upon concatenated protein sequences, the sequenced *Streptomyces* spp. formed a tight cluster, and *C. acidiphila* formed an outgroup of this cluster (Fig. 2). A clade consisting of *Streptomyces* species and *C. acidiphila* had a bootstrap score of 100%. No other actinobacterial groups showed a close or specific relationship to this cluster. A more detailed tree for *Streptomycetales* species based upon concatenated sequences for three large proteins (RpoB, RpoC, and gyrase B) that includes information for many additional *Streptomyces* species as well as *Kitasatospora setae* is presented in File S23 in the supplemental material. *K. setae* formed the immediate outgroup of the *Streptomyces* species in this tree.

CSIs and CSPs That Are Specific for the Order *Streptomycetales*

The sequence alignments of actinobacterial genomes have led to the identification of 3 CSIs that are of interest. In the enzyme porphobilinogen deaminase (PBGD), which converts porphobilinogen into hydroxymethylbilane and is the third enzyme in the heme biosynthetic pathway (190), a 4-aa insert in a conserved region is specifically present in all sequences of *Streptomyces* species and also *Kitasatospora setae*, but it is not found in any other *Actinobacteria* (Fig. 12A). Similarly, in the enzyme adenylate kinase, which catalyzes the interconversion of adenine nucleotides and plays an important role in cellular energy homeostasis, a 1-aa insert in a conserved region is specifically present in various *Streptomyces* species and *K. setae* but not in any other *Actinobacteria* (see File S24 in the supplemental material). Blastp searches for proteins of the genome of *S. coelicolor* A3(2) have also identified a number of CSPs, all significant hits of which are present in various sequenced *Streptomyces* species but not in any other bacteria (Table 10). For the first 5 proteins in Table 10, homologs showing significant similarity were detected in various *Streptomyces* species but not in *K. setae* or other bacteria. These proteins could be specific for the genus *Streptomyces*; however, as the complete genome of *K. setae* is not yet available, it is possible that homologs of these proteins will also be found in this species. For the next 11 entries of Table 10, homologs were detected in both *Streptomyces* species and *K. setae* but not in other *Actinobacteria*. These CSPs thus could be specific for the entire order *Streptomycetales*. Due to their specificity for species of the order *Streptomycetales*, they provide novel molecular markers for distinguishing this group of bacteria from all other *Actinobacteria*.

CSIs and CSPs That Are Uniquely Shared by the Orders *Streptomycetales* and *Catenulisporales*

In the phylogenetic tree shown in Fig. 2, *C. acidiphila* formed an outgroup of the *Streptomyces* cluster, indicating that the orders *Streptomycetales* and *Catenulisporales* are closely related. This inference is also supported by a 1-aa CSI in the lipid A biosynthesis lauroyl acyltransferase (MsbB) protein, which is uniquely shared by all *Streptomycetaceae* species, including *K. setae*, as well as by *C. acidiphila* but not any other *Actinobacteria* (Fig. 12B). Further evidence that these two orders of *Actinobacteria* are closely related is provided by our identification of 3 CSPs listed in Table 10, whose homologs are specifically present in various *Streptomycetaceae* species as well as *C. acidiphila*. Based upon phylogenetic evidence as well as the identification of a number of molecular markers that are uniquely shared by species of the orders *Streptomycetales* and *Catenulisporales*, these observations make a strong case that the order *Catenulisporales*, which contains only a limited number of species, should be merged with the order *Streptomycetales.*

In the phylogenetic tree shown in Fig. 2, a superclade consisting of the orders *Corynebacteriales*, *Pseudonocardiales*, *Glycomycetales*, *Micromonosporales*, *Frankiales*, *Streptosporangiales*, *Streptomycetales*, and *Catenulisporales* is strongly supported by its observed bootstrap score (97%). The consensus tree reported previously by Alam et al. (5) also supported a clade consisting of these orders. Although we have not come across any CSI that is specifically shared by species of all of these orders, the placement of the orders *Streptosporangiales*, *Streptomycetales*, and *Catenulisporales* as the outer branches of the large clade shown in Fig. 11 is strongly supported by phylogenetic analyses.

MOLECULAR SIGNATURES OF THE ORDERS *BIFIDOBACTERIALES*, *ACTINOMYCETALES*, AND *MICROCOCCALES*

Molecular Signatures of the *Bifidobacteriales* and *Bifidobacteriaceae*

Species of the order *Bifidobacteriales* are generally found in the human gastrointestinal tract, and they are important for establishing and maintaining the homeostasis of the intestinal ecosystem to allow for normal digestion (61, 183, 307, 312, 318). The order *Bifidobacteriales* is comprised of a single family, the *Bifidobacteriaceae*, which in turn consists of seven genera, *Bifidobacterium*, *Gardnerella*, *Scardovia*, *Parascardovia*, *Alloscardovia*, *Metascardovia*, and *Aeriscardovia* (22, 191). Except for the genus *Bifidobacterium*, which contains 29 species, all other genera are monospecific and contain only a single species (77, 191). Due to the importance of bifidobacteria for human health and also due to their probiotic potential, the genomes of large numbers of *Bifidobacterium* species and strains as well as *Gardnerella vaginalis* have been sequenced (15, 93, 102, 141, 164, 183, 254, 262, 292, 306, 313, 314, 318, 326, 337, 344). The genetic, biochemical, and genomic characteristics of *Bifidobacterium* species were reviewed previously by Ventura and coworkers (28, 61, 307, 308, 313, 314). In addition to *Bifidobacterium*, sequence information for most of the genes and proteins from the genomes of *Scardovia inopinata* and *Parascardovia denticolens*, whose genomes are at assembly stages, is also now available in public databases.

In a phylogenetic tree for the *Bifidobacteriales*, *G. vaginalis* was found to branch in between different *Bifidobacterium* species, making this genus polyphyletic (see File S25 in the supplemental material). In particular, *Bifidobacterium animalis* was found to branch more deeply than *G. vaginalis.* Hence, the relationship of *G. vaginalis* to other *Bifidobacterium* species and its possible placement in this genus should be considered. Alignments of protein

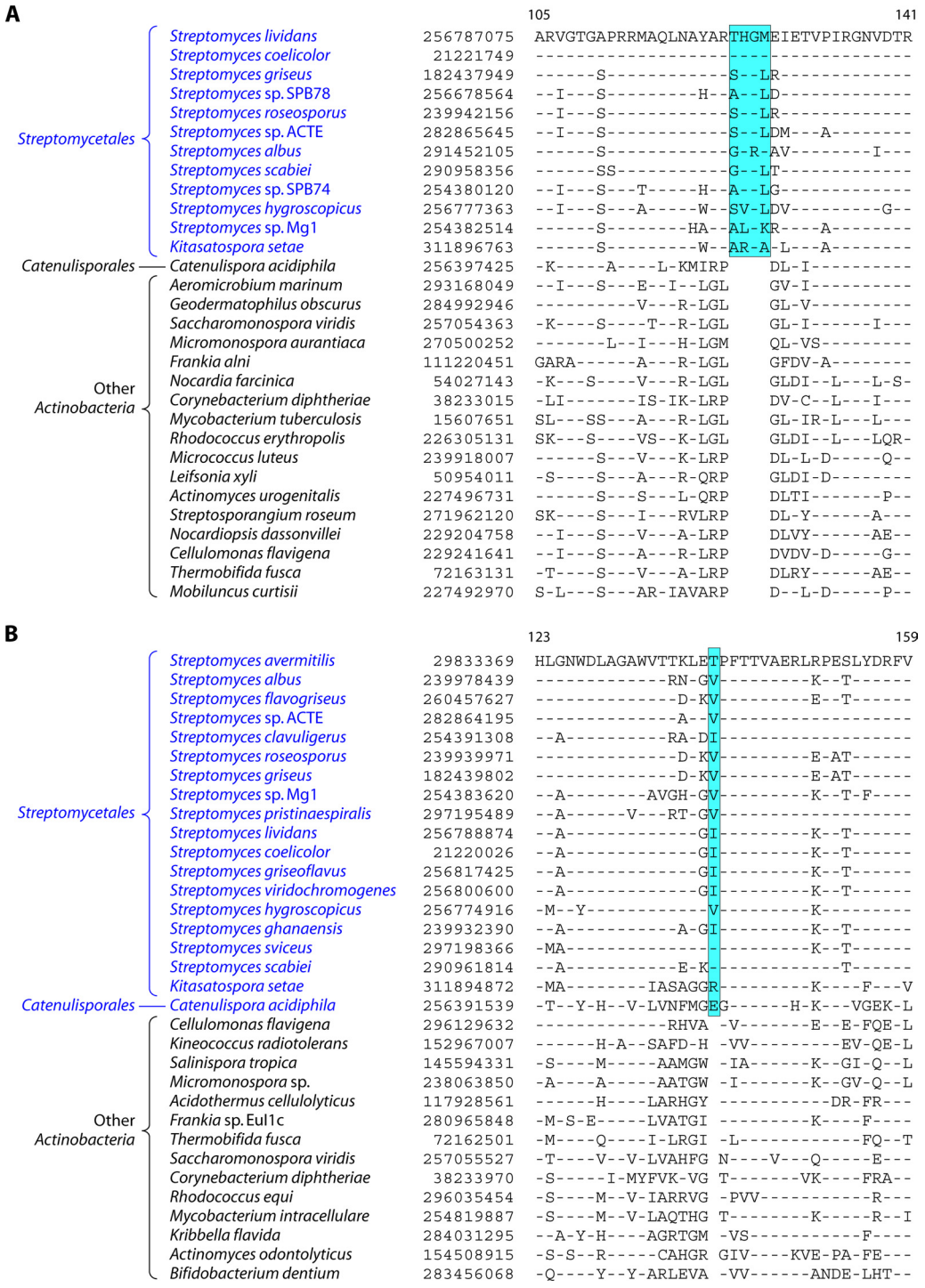

FIG 12 Excerpts from sequence alignments of two proteins showing CSIs that are specific for the order *Streptomycetales* or shared with *Catenulisporales*. (A) A 4-aa insert in the porphobilinogen deaminase (PBGD) protein that is specific for various *Streptomycetales* species, including *Kitasatospora setae*. The adenylate kinase protein also contains a CSI that is specific for the *Streptomycetales* (see File S24 in the supplemental material). (B) A 1-aa insert in the lipid A biosynthesis lauroyl acyltransferase (MsbB) protein that is uniquely shared by various *Streptomycetales* species and *Catenulispora acidiphila*.

sequences of *Actinobacteria* species have identified two CSIs that are specific for the *Bifidobacteriales*. One of these CSIs, consisting of a 1-aa deletion in the ribosomal protein L13, is present in all *Bifidobacteriales* species, including *S. inopinata* and *P. denticolens*, but it is not found in any other *Actinobacteria* (or other bacteria) (Fig. 13A). Thus, this CSI provides a potential molecular marker for the entire order *Bifidobacteriales*. Another 1-aa insert in the enzyme glucose-6-phosphate dehydrogenase, which is a part of the pentose phosphate pathway, is uniquely present in various *Bifidobacterium* species and also in *G. vaginalis*, but it is not found

TABLE 10 Signature proteins that are specific for *Streptomyces* (*Streptomycetales*)[a]

Gene or protein	GenBank accession no.	Protein function	Length (aa)	Species specificity
Small membrane protein	NP_625909.1	Small membrane protein	64	Genus *Streptomyces*
SCO2919	NP_627145.1	Hypothetical	114	Genus *Streptomyces*
SCO4335	NP_628506.1	Hypothetical	62	Genus *Streptomyces*
Secreted serine-rich protein	NP_627511.1	Secreted serine-rich protein	327	Genus *Streptomyces*
SCO3544	NP_627742.1	Hypothetical	132	Genus *Streptomyces*
SCO1392	NP_625675.1	Hypothetical	300	*Streptomycetaceae*
SCO1529	NP_625808.1	Hypothetical	551	*Streptomycetaceae*
Secreted protein	NP_626808.1	Secreted protein	258	*Streptomycetaceae*
Membrane protein	NP_626821.1	Membrane protein	356	*Streptomycetaceae*
SCO2621	NP_626857.1	Hypothetical	64	*Streptomycetaceae*
Lipoprotein	NP_627319.1	Lipoprotein	215	*Streptomycetaceae*
SCO3905	NP_628091.1	Hypothetical	101	*Streptomycetaceae*
Transmembrane protein	NP_628124.1	Transmembrane protein	290	*Streptomycetaceae*
Integral membrane protein	NP_628309.1	Integral membrane protein	102	*Streptomycetaceae*
Integral membrane protein	NP_627868.1	Integral membrane protein	350	*Streptomycetaceae*
SCO4669	NP_628829.1	Hypothetical	379	*Streptomycetaceae*
SCO3799	NP_627989.1	Hypothetical	156	*Streptomycetaceae* and *Catenulispora acidiphila*
Integral membrane protein	NP_628308.1	Integral membrane protein	266	*Streptomycetaceae* and *Catenulispora acidiphila*
SCO3624	NP_627818.1	Hypothetical	221	*Streptomycetaceae* and *Catenulispora acidiphila*[b]

[a] These CSPs were identified by Blastp searches of the genome of *Streptomyces coelicolor* A3(2).
[b] Also found in *Variovorax paradoxus* and *Cellulophaga lytica*.

in *Scardovia*, *Parascardovia*, or any other *Actinobacteria* (Fig. 13B). Thus, this CSI distinguishes the clade consisting of the genera *Bifidobacterium* and *Gardnerella* from other genera of this order. Blastp searches for various proteins of the genome of *Bifidobacterium dentium* Bd1 (318) also identified 16 proteins that are uniquely found in various *Bifidobacteriales* species as well as 6 CSPs for which all significant Blast hits are from the genera *Bifidobacterium* and *Gardnerella* (Table 11). Previously, many CSPs that were specific for *B. dentium* were also identified (316, 318). These CSPs provide additional markers for distinguishing *Bifidobacteriales* species from other *Actinobacteria*.

Molecular Signatures of the *Actinomycetales*

The order *Actinomycetales*, which corresponds to the suborder *Actinomycineae* in the earlier taxonomic scheme (103, 343), contains only one family, the *Actinomycetaceae*, which is comprised of several medically important genera, such as *Actinomyces*, *Arcanobacterium*, *Actinobaculum*, *Mobiluncus*, and *Varibaculum* (103, 191, 253, 343). Of these genera, the genus *Actinomyces* has been indicated to be quite diverse, and in a phylogenetic tree based upon 16S rRNA, it showed polyphyletic branching into a number of different clusters (253). The genome sequences of *Arcanobacterium haemolyticum* (336) and *Mobiluncus curtisii* are now available, and sequence information for most of the proteins from a number of other species (*viz.*, *Actinomyces odontolyticus*, *Actinomyces urogenitalis*, *Actinomyces coleocanis*, and *Mobiluncus mulleris*) is also available in the NCBI database. Our analyses have identified a number of CSIs that are specific for species of this order. The enzyme deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), which is a part of the nonmevalonate pathway of isoprenoid biosynthesis (245), contains a 12-aa insert in a highly conserved region that is uniquely present in all available sequences of *Actinomycetales* species, including those of the genera *Actinomyces*, *Arcanobacterium*, and *Mobiluncus* (Fig. 14A). Another CSI consisting of a 6-aa insert that is specific for all sequenced *Actinomycetales* species is present in the integral membrane protein Lxx09300 (see File S26 in the supplemental material). The high degrees of conservation and specificity of these signatures for species of this order indicate that they provide good and reliable molecular markers for this order of *Actinobacteria.* Isoleucine tRNA synthetase (IleRS), which is essential for protein synthesis, also contains a 3-aa insert in a conserved region that is specifically present in all available sequences of the genera *Actinomyces* and *Mobiluncus* but which is lacking in *Arcanobacterium haemolyticum* as well as all other *Actinobacteria* (Fig. 14B). In the phylogenetic tree for *Actinobacteria* based on protein sequences, *A. haemolyticum* showed the deepest branching of the three available genera, and a clade consisting of *Actinomyces* and *Mobiluncus* species was strongly supported (Fig. 2, and see File S25 in the supplemental material). Thus, it is likely that the genetic change responsible for this CSI took place in a common ancestor of these two genera after the divergence of *Arcanobacterium.* Lastly, we have also identified a 1-aa deletion in the excision endonuclease UvrC that is specifically present in the two *Mobiluncus* species (Fig. 14C), providing a molecular marker for this genus.

Molecular Signatures of the *Micrococcales* and Its Subclades

The order *Micrococcales* is the most diverse order within the phylum *Actinobacteria*, containing ecologically, morphologically, and chemotaxonomically divergent species (191, 283). The most updated taxonomic outline of this suborder encompasses 15 families and 86 genera, and information for some of these has been reviewed (38, 78, 157, 169, 191, 258, 282, 343, 343). In view of the importance of species of this order for bioremediation, industrial, and clinical purposes, large numbers of genomes of many species that are part of different genera and families of this order have been sequenced. The sequenced genera include *Arthrobacter* (202, 203), *Beutenbergia*, *Brachybacterium* (180), *Cellulomonas* (2), *Clavibacter* (105), *Intrasporangium* (66), *Jonesia* (233), *Kocuria* (296), *Leifsonia* (205), *Renibacterium* (328), *Rothia*, *Sanguibacter* (155), and *Tropheryma* (20, 239) (Table 1). In the phylogenetic

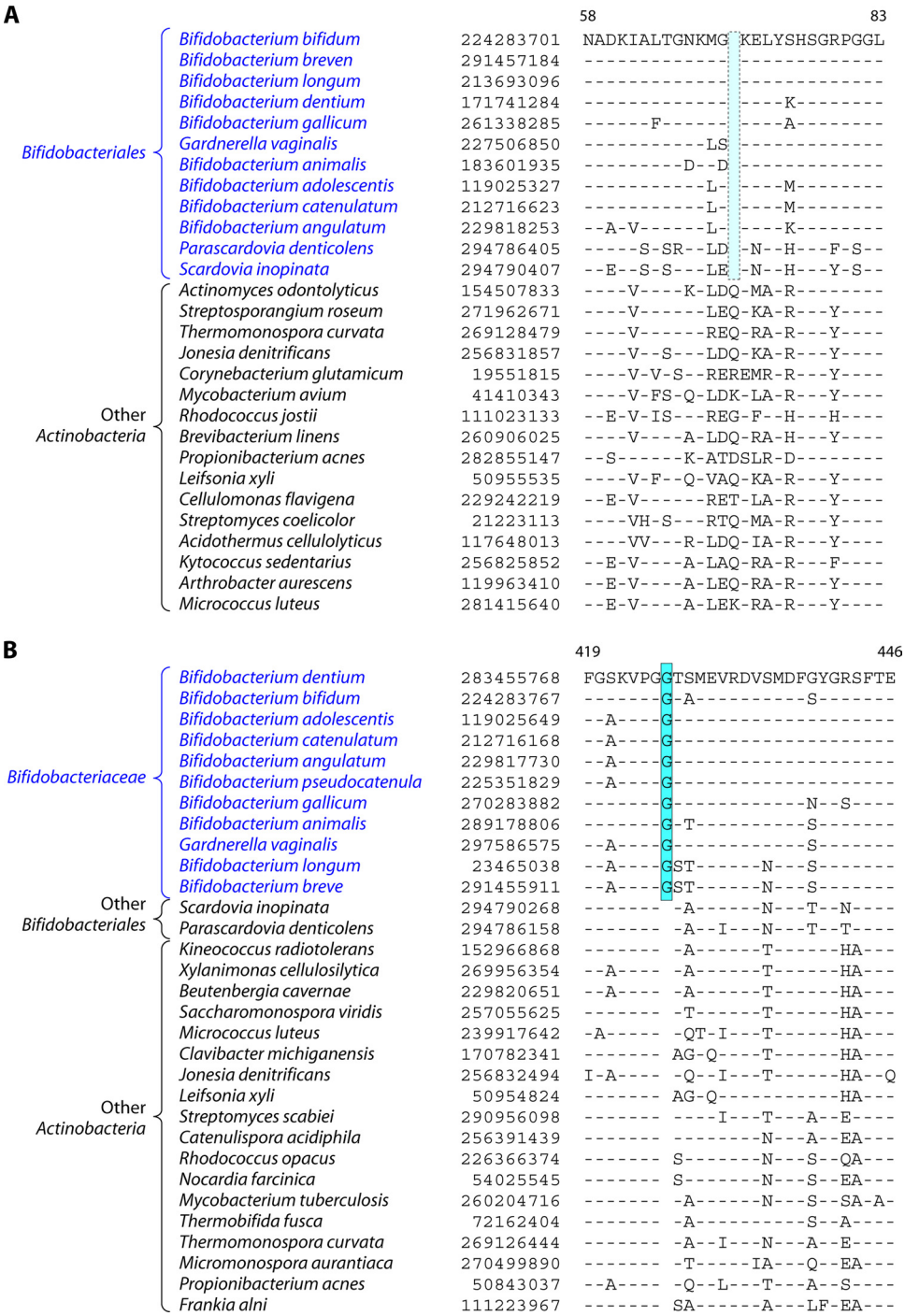

FIG 13 Partial sequence alignments of ribosomal protein L3 (A) and glucose-6-phosphate dehydrogenase (G6PDH) (B) showing two CSIs consisting of a 1-aa deletion and a 1-aa insert, respectively, that are specific for *Bifidobacteriales* species. The CSI in the ribosomal protein is present in all sequenced *Bifidobacteriales* species, whereas that in G6PDH is found only in *Bifidobacterium* and *Gardnerella* species.

tree based upon concatenated protein sequences (Fig. 2) or 16S rRNA (191, 343), species of the order *Micrococcales* are split into a number of clusters, with the orders *Actinomycetales* and *Bifidobacteriales* branching between them. Thus, the different families that are presently part of this order do not form a phylogenetically coherent group, and the taxonomy of this order needs to be emended. Hence, novel molecular markers that could serve to define and delimit different subclades of this order are of particular interest.

Our analyses of protein sequences from *Actinobacteria* have identified some CSIs that are specific for some of the subclades of *Micrococcales.* In the universally distributed and highly conserved β-subunit of the RNA polymerase (RpoB), a 2-aa insert is present in a conserved region that is specific for clade I *Micrococcales* (Fig.

TABLE 11 Signature proteins that are specific for the *Bifidobacteriaceae*[a]

Gene	GenBank accession no.	Protein function	Length (aa)	Species specificity
BIFDEN_00796	ZP_02917515.1	Hypothetical	124	*Bifidobacteriaceae*
BIFDEN_00793	ZP_02917512.1	Hypothetical	73	*Bifidobacteriaceae*
BIFDEN_00600	ZP_02917322.1	Hypothetical	275	*Bifidobacteriaceae*
BIFDEN_00594	ZP_02917316.1	Hypothetical	119	*Bifidobacteriaceae*
BIFDEN_00539	ZP_02917261.1	Hypothetical	336	*Bifidobacteriaceae*
BIFDEN_00419	ZP_02917147.1	Hypothetical	228	*Bifidobacteriaceae*
BIFDEN_00378	ZP_02917106.1	Hypothetical	399	*Bifidobacteriaceae*
BIFDEN_00301	ZP_02917034.1	Hypothetical	204	*Bifidobacteriaceae*[b]
BIFDEN_02476	ZP_02919152.1	Hypothetical	201	*Bifidobacteriaceae*
BIFDEN_02473	ZP_02919149.1	Hypothetical	174	*Bifidobacteriaceae*
BIFDEN_02131	ZP_02918813.1	Hypothetical	121	*Bifidobacteriaceae*
BIFDEN_00191	ZP_02916931.1	Hypothetical	84	*Bifidobacteriaceae*
BIFDEN_01066	ZP_02917770.1	Hypothetical	76	*Bifidobacteriaceae*
BIFDEN_02253	ZP_02918933.1	Hypothetical	321	*Bifidobacteriaceae*
BIFDEN_00382	ZP_02917110.1	Hypothetical	213	*Bifidobacterium* and *Gardnerella*
BIFDEN_00315	ZP_02917048.1	Hypothetical	222	*Bifidobacterium* and *Gardnerella*
BIFDEN_02465	ZP_02919141.1	Hypothetical	299	*Bifidobacterium* and *Gardnerella*
BIFDEN_02410	ZP_02919088.1	Hypothetical	260	*Bifidobacterium* and *Gardnerella*
BIFDEN_01330	ZP_02918031.1	Hypothetical	283	*Bifidobacterium* and *Gardnerella*
BIFDEN_02361	ZP_02919040.1	Hypothetical	189	*Bifidobacterium* and *Gardnerella*

[a] These CSPs were identified by Blastp searches of the genome of *B. dentium* Bd1.
[b] Also found in *Isoptericola variabilis* and *Xylanimonas cellulosilytica.*

15A). This clade, which is comprised of species of the families *Micrococcaceae* (*Arthrobacter*, *Renibacterium*, *Micrococcus*, *Kocuria*, and *Rothia*) and *Brevibacteriaceae* (*Brevibacterium*), is also strongly supported in the phylogenetic tree (Fig. 2). Another CSI that is specific for the *Micrococcales* has been identified in the ribose-5-phosphate isomerase (RPI) protein, which is a key enzyme of the pentose phosphate pathway that catalyzes the conversion of ribose-5-phosphate into ribulose-5-phosphate (340). In this highly conserved protein, a 4-aa insert in a conserved region is specifically present in all of the sequenced *Micrococcales* that are part of clusters I and III, but this insert is not found in cluster II *Micrococcales* or any other *Actinobacteria* (Fig. 15B). It should be noted that although clusters I and II branch in proximity of each other in the tree, they are phylogenetically quite distinct from each other. For cluster III *Micrococcales* species, although they branch deeply in the tree, their deep branching could be due to a long-branch length effect (82, 97), as *Tropheryma*, which is a part of this cluster, has a long branch length. Thus, although a clade consisting of cluster I and cluster III *Micrococcales* is not observed in the phylogenetic tree (Fig. 2), the CSI in the RPI protein suggests that these two subclades of *Micrococcales* might be more closely related to each other than the cluster II species. We have also identified one additional CSI consisting of a 4-aa insert in the pyruvate carboxylase protein that is uniquely present in various *Micrococcales* except *Tropheryma* (see File S27 in the supplemental material). Although homologs of this protein were not detected in all sequenced *Micrococcales*, this CSI suggests that despite their divergent branching in the phylogenetic trees, all of the *Micrococcales* might be derived from a common ancestor exclusive of other *Actinobacteria*.

As noted above, in phylogenetic trees, species of the orders *Actinomycetales* and *Bifidobacteriales* branch between the different clusters of *Micrococcales*, indicating that these orders are closely related. One additional CSI that we have identified supports this inference. In the highly conserved DnaK or Hsp70 family of proteins, a 5-aa insert in a conserved region is present in all of the *Bifidobacteriales*, *Actinomycetales*, and *Micrococcales* (clusters I, II, and III), but with a few exceptions, this insert is not present in most other *Actinobacteria* (see File S28 in the supplemental material). The presence of this CSI in a few other *Actinobacteria* could be due to LGTs. The shared presence of this CSI in all species of these actinobacterial orders suggests that they likely shared a common ancestor exclusive of other *Actinobacteria*.

Molecular Signatures of the *Propionibacteriales*

The order *Propionibacteriales* contains the families *Propionibacteriaceae* and *Nocardioidaceae* (103, 343). Members of the *Propionibacteriaceae* thrive in diverse habitats, covering human epidermal surfaces, dairy products, silage, soil, water, Antarctic sandstone, and sewage treatment plants (279). They are either aerobic or facultative anaerobes, have different morphologies, and exhibit different peptidoglycan type variations (279, 281, 343). The genome sequences of several species of this order covering both families are now available. These include sequences of several strains of *Propionibacterium acnes* as well as those of *Propionibacterium freudenreichii*, *Kribbella flavida*, and *Nocardioides* sp. strain JS614 (34, 79, 235). Additionally, sequence information for large numbers of genes and proteins from *Aeromicrobium marinum* is also available in the NCBI database. Sequence alignments of actinobacteria have identified two CSIs that are specific for this order. In the helicase DinG, which is involved in DNA repair and replication, a 3-aa insert in a conserved region is specifically present in all available sequences from this order of bacteria but not in any other *Actinobacteria* or other bacteria (Fig. 16). Another CSI that is specific for this order is present in the cytochrome *c* oxidase subunit 1 (Cox1) protein (see File S29 in the supplemental material), which also contains a CSI that is specific for most *Actinobacteria* (97). In this case, all *Propionibacteriales* homologs contain a 1-aa deletion that is not present in other *Actinobacteria* (see File

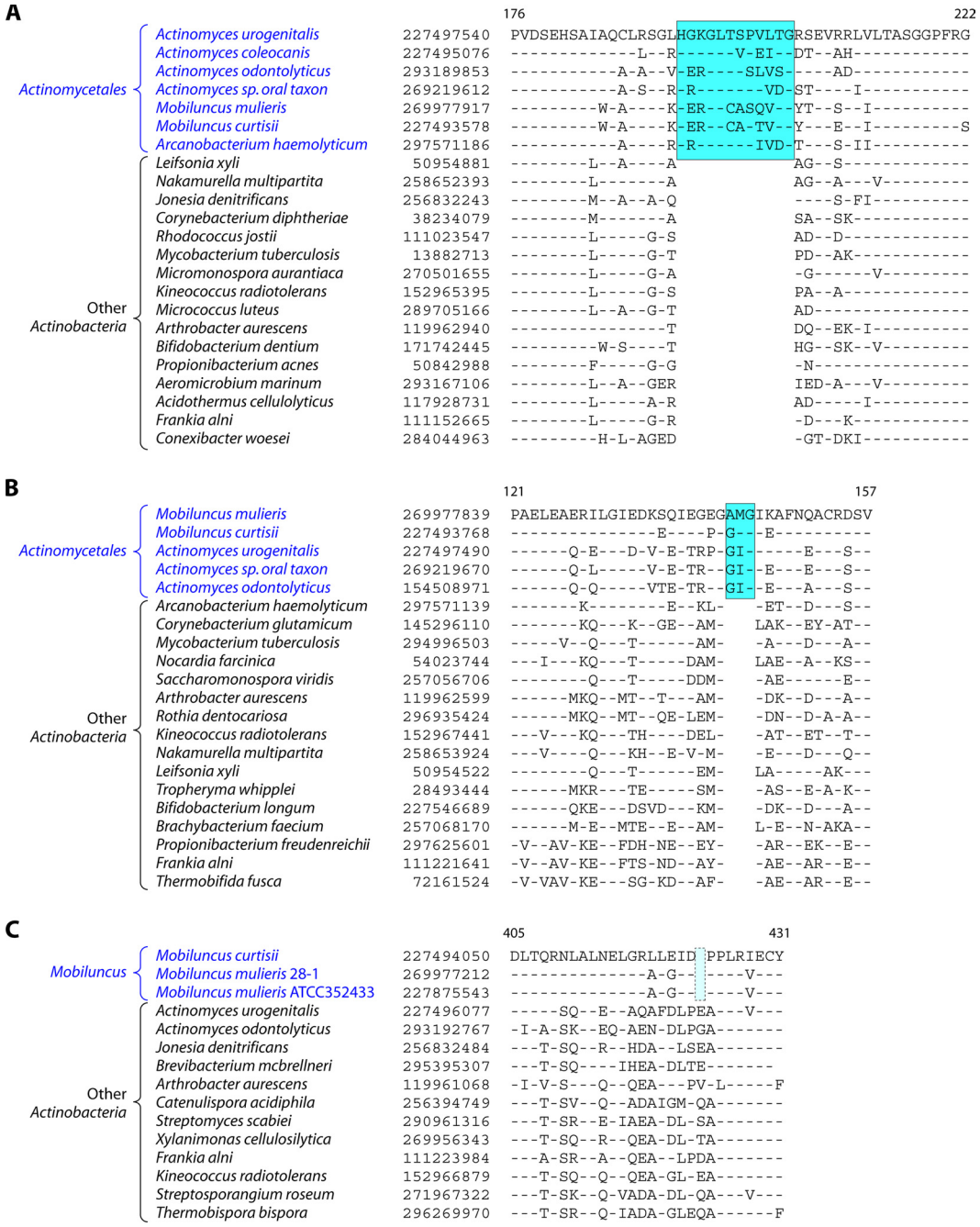

FIG 14 (A and B) Partial sequence alignments of the deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) (A) and isoleucine tRNA synthetase (IleRS) (B) proteins depicting 12-aa and 3-aa inserts, respectively, in highly conserved regions that are uniquely present in various sequenced *Actinomycetales* species. (C) Sequence alignment of the excision endonuclease UvrC showing a 1-aa deletion that is specific for the genus *Mobiluncus*. Information for another CSI that is specific for *Actinomycetales* is provided in File S26 in the supplemental material.

S29 in the supplemental material). Both these CSIs provide molecular markers that distinguish species of the order *Propionibacteriales* from all other *Actinobacteria*.

Molecular Signatures Identifying Larger Clades Consisting of the Orders *Bifidobacteriales*, *Actinomycetales*, *Micrococcales*, *Kineosporiales*, and *Propionibacteriales*

Due to the compact clustering of most actinobacterial orders in the 16S rRNA and protein trees, their branching orders are generally not resolved (Fig. 2). As indicated above, rare genetic changes such as CSIs, due to their rare and highly specific nature, are capable of resolving deep-branching relationships that are not resolved by phylogenetic trees (11, 119, 131, 243, 246). Our analyses have identified a number of CSIs that clarify the evolutionary relationships and branching orders of the actinobacterial orders *Bifidobacteriales*, *Actinomycetales*, *Micrococcales*, *Kineosporiales*, and *Propionibacteriales*.

In the phylogenetic tree shown in Fig. 2, the order *Kineosporia-*

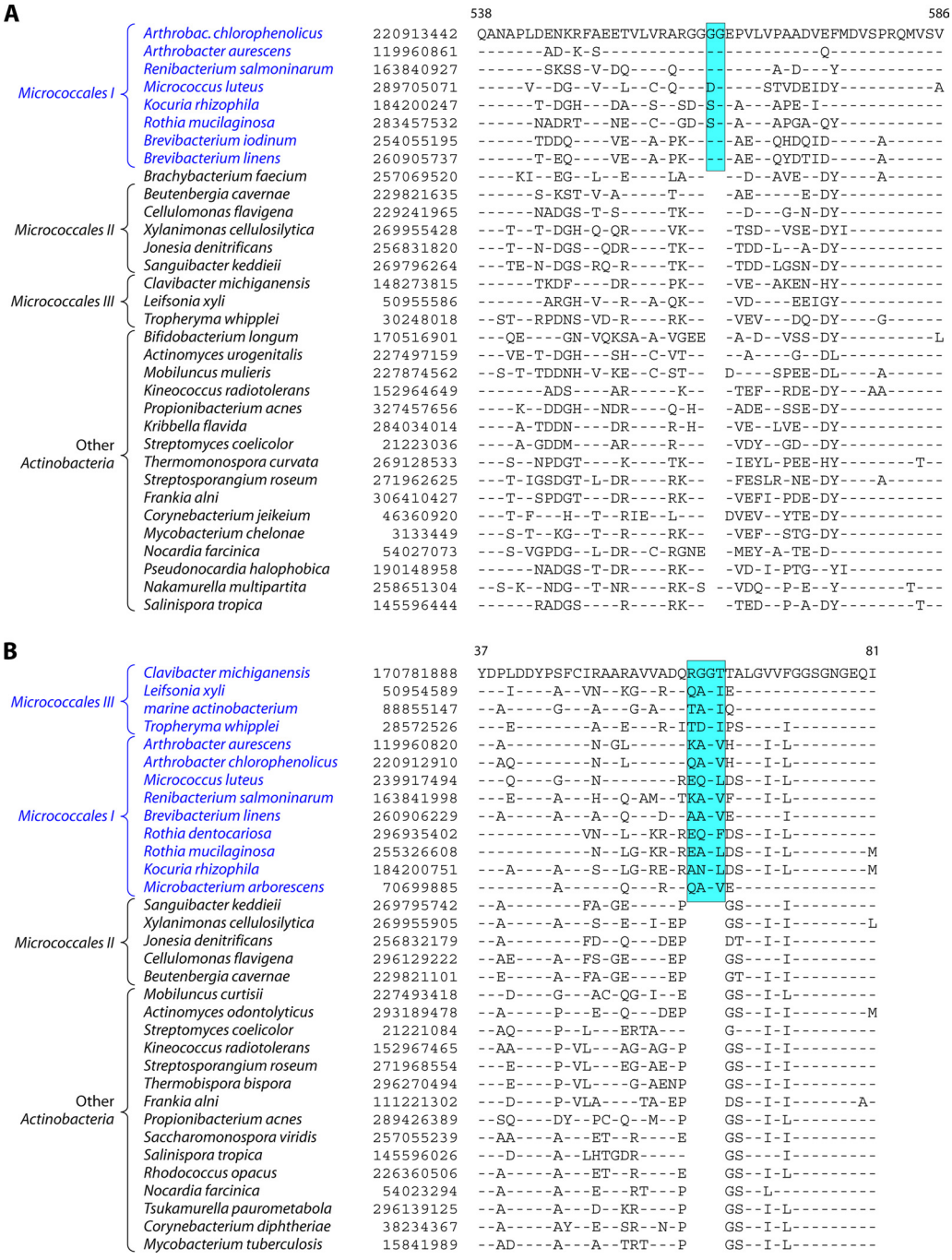

FIG 15 Partial sequence alignments of the RNA polymerase β-subunit showing a 2-aa insert in a conserved region that is specific for cluster I *Micrococcales* species (A) and a 4-aa insert in ribose-5-phosphate isomerase that is uniquely shared by both cluster I and cluster II *Micrococcales* species (B). Another CSI that is specific for *Micrococcales* can be found in File S27 in the supplemental material.

les branches in the proximity of the order *Micrococcales*, which is interspersed by the orders *Bifidobacteriales* and *Actinomycetales*. Although a clade consisting of these orders is not supported by the 16S rRNA or protein trees (Fig. 2) (5, 343), a number of CSIs provide evidence that species of these orders are specifically related and that they shared a common ancestor exclusive of other *Actinobacteria*. In the highly conserved and universally distributed ribosomal protein S3, a 5-aa insert in a conserved region is uniquely present in all species of these 4 orders of actinobacteria, but this insert is not found in any other *Actinobacteria* (Fig. 17). The shared presence of this insert in this important protein involved in the information transfer process strongly suggests that the genetic change leading to this insert was introduced in a common ancestor of these 4 orders of *Actinobacteria*. Two other CSIs that also support that these orders are specifically related are found in the CgR_2975 and Cox1 proteins. In the CgR_2975 pro-

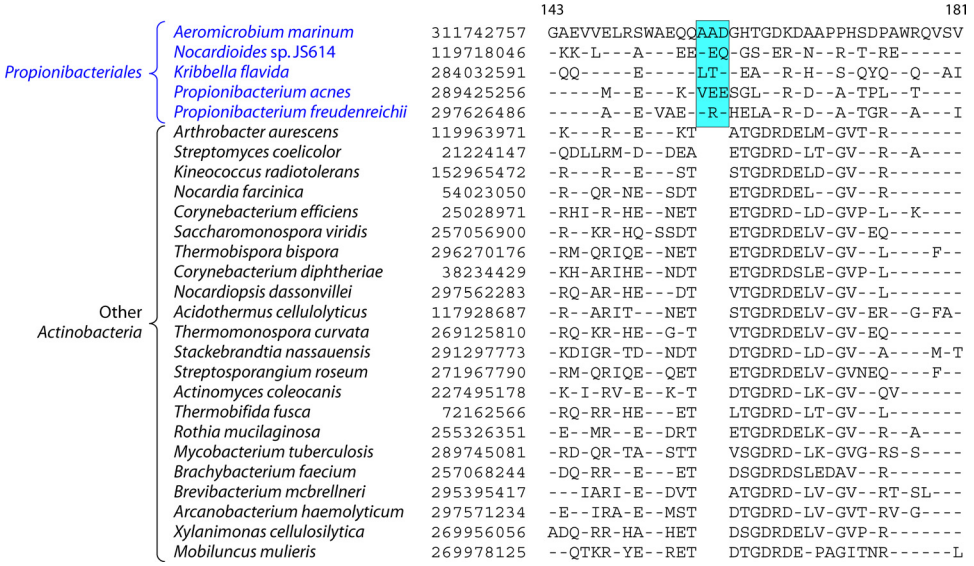

FIG 16 Excerpts from sequence alignments for the helicase DinG showing a CSI consisting of a 3-aa insert that is uniquely present in various sequenced *Propionibacteriales* species. Sequence information for another CSI that is specific for *Propionibacteriales* can be found in File S29 in the supplemental material.

tein, whose cellular function is not known, a 3-aa insert in a conserved region is present in all species of the orders *Bifidobacteriales*, *Micrococcales*, and *Kineosporiales* but not in any other bacteria (see File S30 in the supplemental material). Because homologs of this protein were not detected in *Actinomycetales*, it is likely that this CSI was introduced in a common ancestor of the orders *Bifidobacteriales*, *Actinomycetales*, *Micrococcales*, and *Kineosporiales*, followed by the loss of this gene from the order *Actinomycetales*. Similarly, in the Cox1 protein, which contains two other CSIs, one specific for most *Actinobacteria* (97) and the other specific for *Propionibacteriales* (see File S29 in the supplemental material), one additional CSI that is uniquely shared by all species of the orders *Micrococcales* and *Kineosporiales* has been identified (see File S31 in the supplemental material). Because Cox1 homologs were not detected in the orders *Actinomycetales* and *Bifidobacteriales*, it is likely that this CSI was also introduced in a common ancestor of the above-described 4 orders, followed by the loss of this gene from the orders *Bifidobacteriales* and *Actinomycetales*. Recently, the existence of a clade consisting of these 4 actinobacterial orders based upon 16S rRNA trees was also suggested by Ludwig et al. (191). However, in the phylogenetic trees of 16S rRNA genes reported by Zhi et al. (343) and Adekambi et al. (3), this clade was not observed.

Lastly, the triosephosphate isomerase protein, which plays a key role in glycolysis, contains a 2-aa insert in a conserved region that is commonly shared by all species of the orders *Bifidobacteriales*, *Actinomycetales*, *Micrococcales*, *Kineosporiales*, and *Propionibacteriales* but which is not found in any other *Actinobacteria* or other phyla of bacteria (Fig. 18). The genetic changes responsible for this CSI were likely introduced in a common ancestor of these orders, providing evidence that they are specifically related. The evolutionary relationships among various taxa belonging to these actinobacterial orders that emerge based upon various identified CSIs and CSPs are summarized in Fig. 19.

CONCLUSIONS AND FUTURE DIRECTIONS

The phylum *Actinobacteria* is very large and diverse in terms of its biology, ecology, and genetics, and it contains numerous organisms that are of great interest from medical, industrial, biotechnological, and environmental perspectives. The main focus of this review has been on the identification of molecular markers that are specific for either all *Actinobacteria* or their different constituent groups. Although this review describes a large number of signatures that are specific for *Actinobacteria* or their various subgroups, systematic studies to identify different CSIs or CSPs that are specific for various actinobacterial groups at different phylogenetic depths have not yet been carried out. As genome sequences of more actinobacteria become available, further studies in this regard should lead to the identification of many other signatures that are specific for various actinobacterial groups at different taxonomic levels. Nonetheless, the molecular markers thus far identified provide powerful new tools for a variety of studies that are briefly discussed below.

Usefulness of CSIs and CSPs for an Understanding of the Phylogeny and Taxonomy of *Actinobacteria*

One of the immediate applications of these signatures is that they provide potentially more definitive means for understanding or clarifying actinobacterial phylogeny and taxonomy. Our understanding of the phylogeny and taxonomy of *Actinobacteria* currently relies solely on phylogenetic trees based on 16S rRNA (103, 191, 283, 343). Although such trees have been and will remain some of the primary means for understanding microbial phylogeny and taxonomy, some of the limitations of these trees should be recognized (50, 171, 225, 278). While phylogenetic trees in general are most effective in resolving evolutionary relationships at intermediate taxonomic levels (*viz.*, genus, family, and order), their resolving power at either higher (among orders, classes, or phyla) or lower (i.e., among species or different strains of a species) phy-

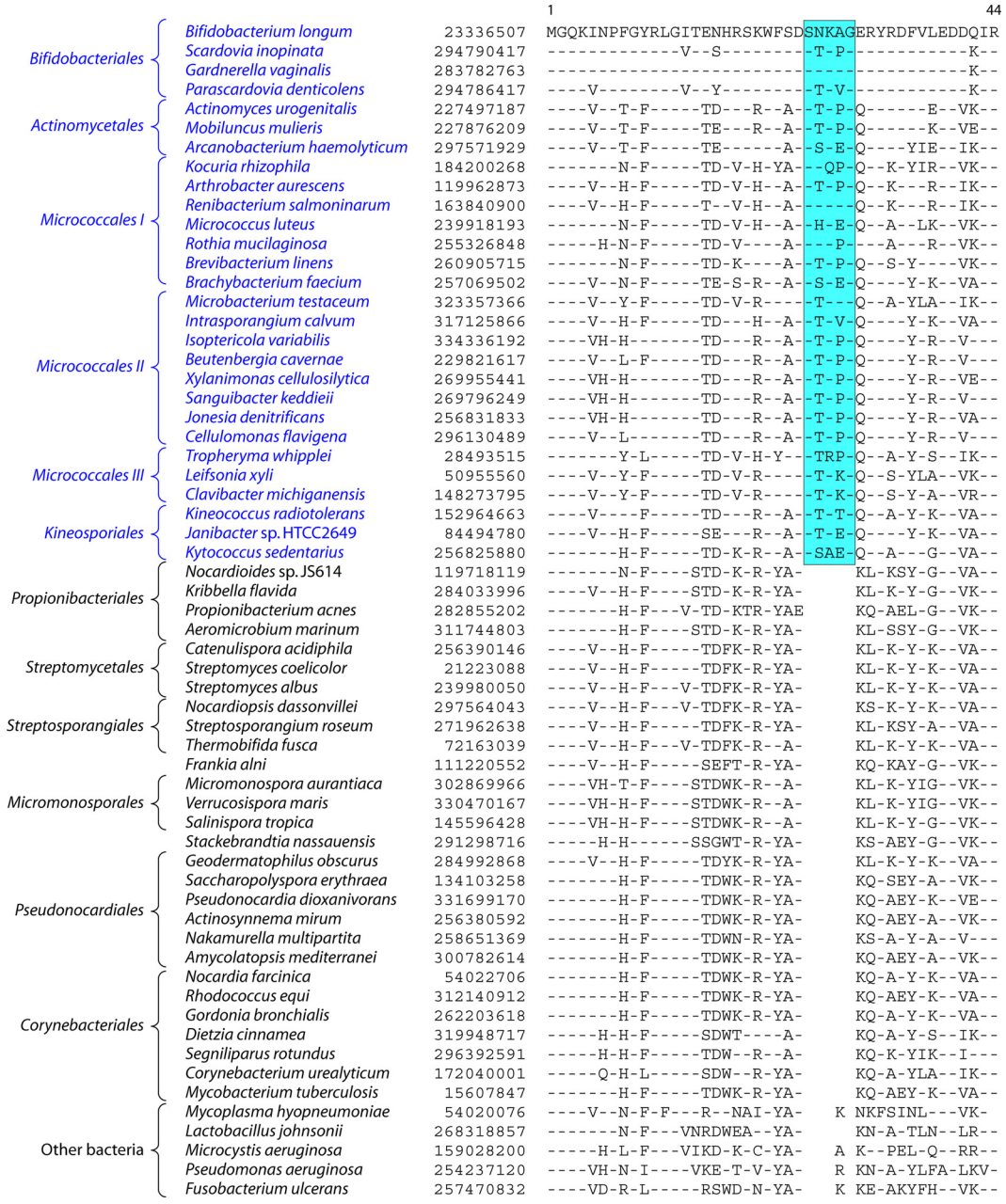

FIG 17 Partial sequence alignment of ribosomal protein S3 showing a 5-aa conserved insert that is commonly shared by various sequenced species of the orders *Bifidobacteriales*, *Actinomycetales*, *Micrococcales*, and *Kineosporiales* but which is not found in any other *Actinobacteria* or in other phyla of bacteria. Information for two other CSIs showing similar specificities is provided in Files S30 and S31 in the supplemental material.

logenetic depths is quite limited (192, 278, 286, 324, 343). Additionally, the phylogenetic trees are a continuum, with no fixed boundaries. Hence, on the basis of the branching in these trees, it is often difficult to delimit a phylum or any other taxa reliably, unless all members of the proposed clade or taxon share at least some unique and reliable molecular, biochemical, or physiological characteristics. The phylum *Actinobacteria* represented such a case, where no unique characteristic of any kind was known that was commonly shared by all species that have been assigned to this phylum. In this context, our identification of a number of CSIs and CSPs that are commonly and uniquely shared by most members of all other classes of the phylum *Actinobacteria*, except *Coriobacteriia*, argues strongly that the bacteria belonging to the class *Coriobacteriia*, which branches more deeply than all other *Actinobacteria*, should at present be excluded from the phylum *Actinobacteria*. If, in the future, some unique biochemical and/or molecular properties that are specifically shared by *Coriobacteriia* and *Actinobacteria* are discovered, the inclusion of *Coriobacteriia* in the phylum *Actinobacteria* could be reconsidered.

On the basis of the identified CSIs and CSPs, it is also now possible to identify and delimit most of the main orders within the phylum *Actinobacteria* in molecular terms (Fig. 11 and 19). For

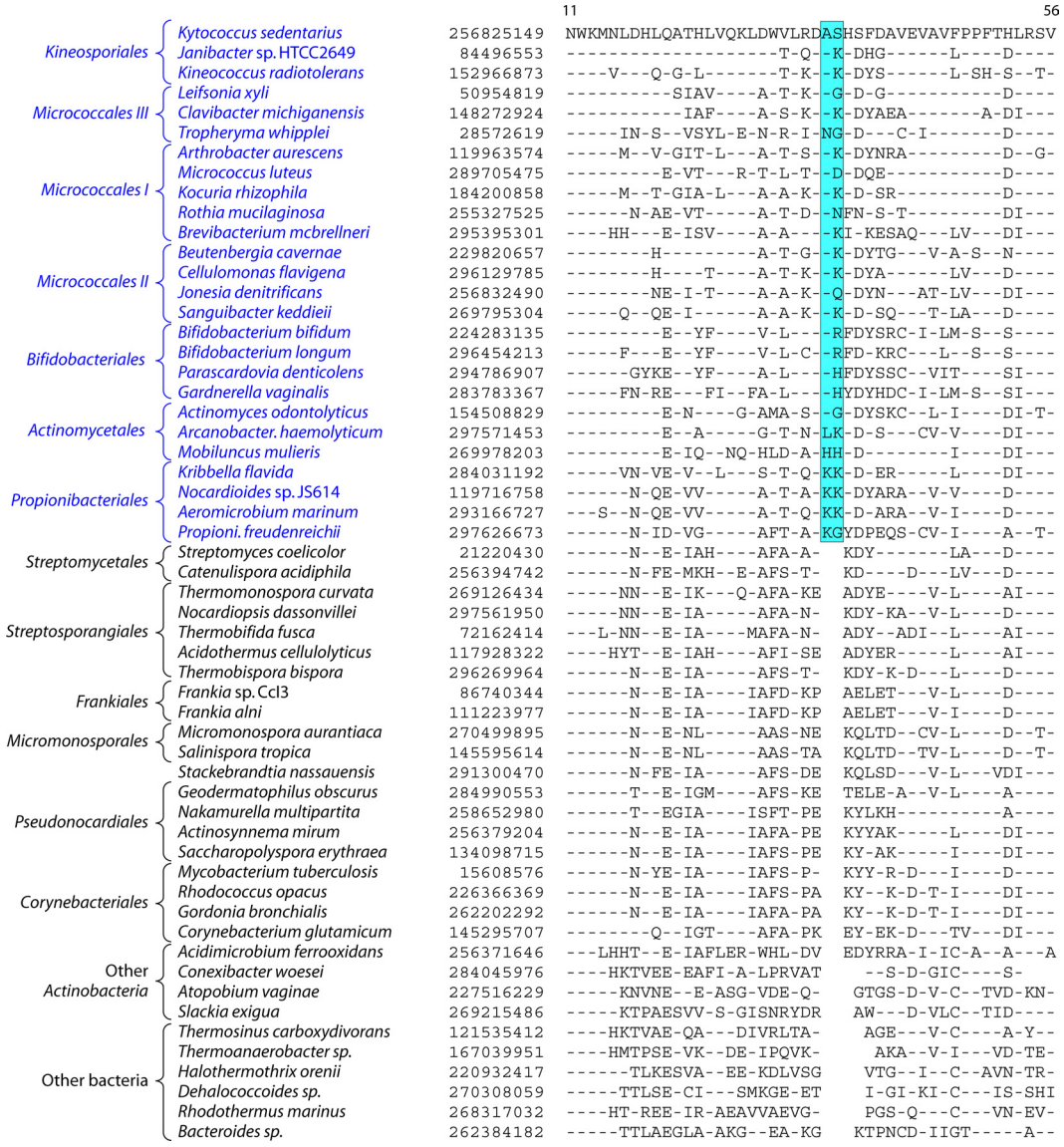

FIG 18 Partial sequence alignment of the triosephosphate isomerase protein showing a 2-aa conserved insert that is commonly shared by various sequenced species of the orders *Bifidobacteriales*, *Actinomycetales*, *Micrococcales*, *Kineosporiales*, and *Propionibacteriales* but which is not found in any other *Actinobacteria*.

some orders that have been studied in detail (*viz.*, *Corynebacteriales*, *Bifidobacteriales*, and *Streptomycetales*), individual families and genera as well as subclades of some genera (e.g., *Corynebacterium* and *Mycobacterium*) can now also be identified in clear molecular terms based upon multiple signatures. Additionally, based upon these molecular signatures, it is also possible to delineate the interrelationships among different orders of *Actinobacteria*, and several higher levels of clades can be identified (Fig. 11 and 19). These clades include those consisting of (i) the orders *Corynebacteriales* and *Pseudonocardiales*; (ii) the orders *Corynebacteriales*, *Pseudonocardiales*, *Glycomycetales*, and *Micromonosporales*; and (iii) the orders *Corynebacteriales*, *Pseudonocardiales*, *Glycomycetales*, and *Micromonosporales* and the genus *Frankia* (Fig. 11). Although the *Frankiales* species do not form a coherent clade, all sequenced species are part of this larger clade, indicating that they are related to this group of species. Phylogenetic studies also support a larger clade consisting of the orders *Corynebacteriales*, *Pseudonocardiales*, *Glycomycetales*, *Micromonosporales*, *Frankiales*, *Streptosporangiales*, and *Streptomycetales*, although no molecular signature that is specific for this large clade has thus far been identified. The other higher levels of clades within the phylum *Actinobacteria* that can be identified on the basis of identified molecular signatures include those consisting of the orders (iv) *Bifidobacteriales*, *Actinomycetales*, and *Micrococcales*; (v) *Bifidobacteriales*, *Actinomycetales*, *Micrococcales*, and *Kineosporiales*; and (vi) *Bifidobacteriales*, *Actinomycetales*, *Micrococcales*, *Kineosporiales*, and *Propionibacteriales* (Fig. 19). Several of these phylogenetic clades were also observed in a consensus phylogenetic tree for a limited number of actinobacteria constructed by using different approaches (5). Additionally, the phylogenetic analysis and molecular signatures reported here provide strong evidence that species of the order *Streptomycetales* are closely related to *Catenulisporales*, and a strong case can be made for the merger of *Catenulisporales* into the order *Streptomycetales*.

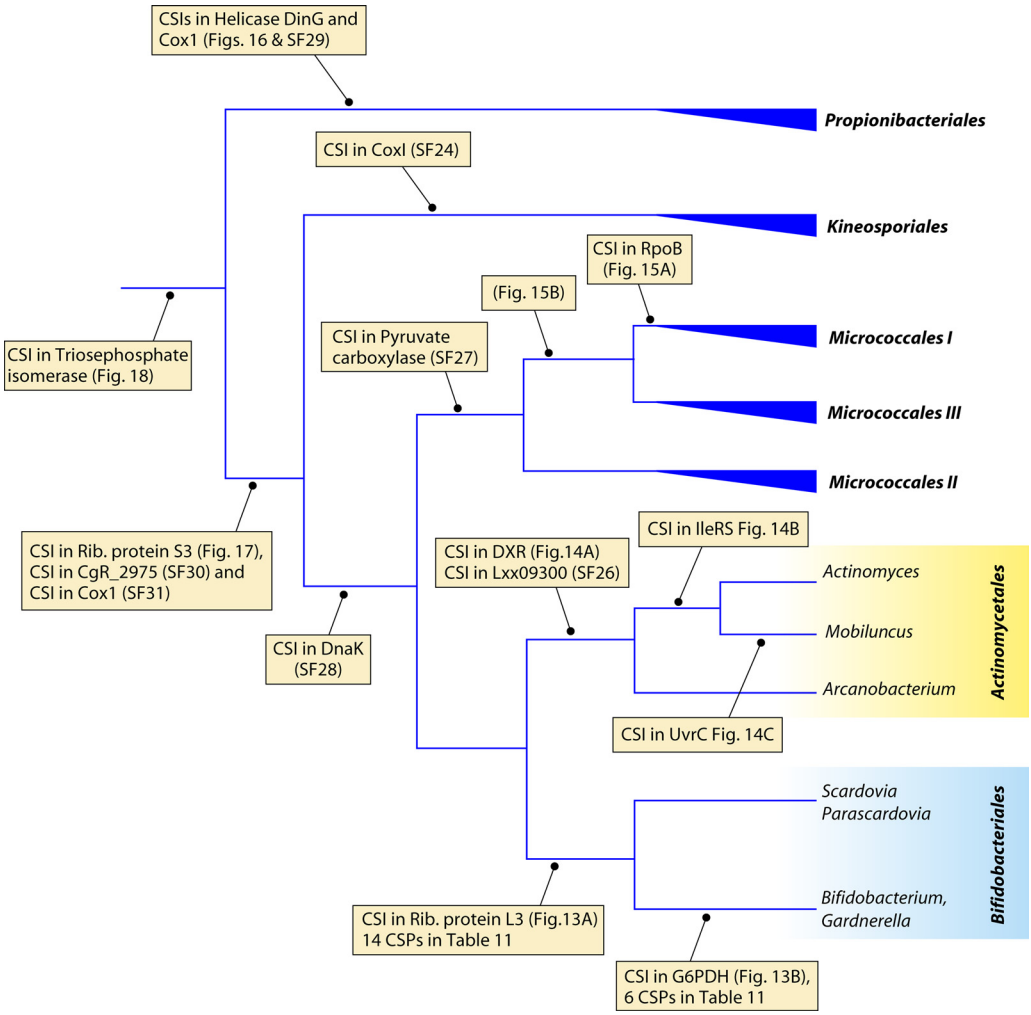

FIG 19 Summary diagram showing evolutionary relationships among the orders *Bifidobacteriales*, *Actinomycetales*, *Micrococcales*, *Kineosporiales*, and *Propionibacteriales* based upon phylogenetic trees (Fig. 2, and see File S25 in the supplemental material) and various identified CSIs and CSPs. Rib., ribosomal.

Recently, on the basis of 16S rRNA trees, Ludwig et al. (191) also indicated the identification of two large clades within the phylum *Actinobacteria*. One of these clades consists of the orders *Actinopolysporales*, *Corynebacteriales*, *Glycomycetales*, *Jiangellales*, *Micromonosporales*, *Pseudonocardiales*, and *Propionibacteriales*. This clade is similar to one of the large clades identified here, except that our results suggest that the genus *Frankia* and other species that are currently part of the order *Frankiales* are also affiliated with this clade, whereas those of the order *Propionibacteriales* are not part of this clade. There are no genome sequences available at present for the orders *Actinopolysporales* and *Jiangellales*. Hence, we are unable to determine the placement of these orders within this clade. The other large clade identified by Ludwig et al. (191), consisting of the orders *Bifidobacteriales*, *Actinomycetales*, *Micrococcales*, and *Kineosporiales*, is also supported by various identified signatures (Fig. 19). Although the identification of these large clades based upon different CSIs as well as the 16S rRNA trees strongly indicates that these clades are meaningful, it should be recognized that phylogenetic trees are dynamic constructs and that the branching of species within them is dependent upon large numbers of variables and assumptions, including the different species that are part of the data set and the models used to create the sequence alignment and phylogenetic trees (81, 83, 192, 206, 330). This is illustrated by the fact that the two large clades proposed by Ludwig et al. (191) were not observed in the phylogenetic trees for 16S rRNA reported by Zhi et al. (343) and Adekambi et al. (3). In contrast to the highly dynamic (and variable) nature of phylogenetic trees, the inferences derived from CSIs are based upon minimal assumptions, and their interpretation is generally straightforward (119, 126, 132). Based upon these CSIs, all of the identified clades are defined simply based upon the presence or absence of given indels in highly conserved regions of proteins (119, 126, 130, 132). Furthermore, these CSIs provide highly stable molecular markers with strong predictive abilities. This is evidenced by the fact that many of the *Actinobacteria*-specific CSIs and CSPs, which were identified when the number of sequenced genomes was very limited (97, 100), are still reliable characteristics of this phylum despite the nearly 10-fold increase in the number of sequenced genomes. Additionally, the investigated CSIs are also present in many other actinobacterial species whose genomes have not been sequenced, providing further strong evidence of their reliability and predictive power (97).

The specificity of various identified signatures for actinobacterial species or groups is presently based mainly upon the species and/or strains whose genomes have been sequenced (Table 1). Although these genomes represent only a small fraction of the actinobacterial species (52, 103), they cover most of the major orders and families of *Actinobacteria*. However, it is of much importance to obtain sequence information for these genes/proteins from other actinobacterial species to further validate and more precisely determine the boundaries of the clades that are defined by these signatures. These signatures are also very appealing for taxonomic studies, as the assignment of various species (or new isolates) to different clades can be readily done based upon the presence or absence of certain diagnostic signatures, without the need for the construction of detailed phylogenetic trees.

Interesting Cases of Lateral Gene Transfers Identified by CSIs and CSPs

Although most of the CSIs and CSPs described in this review are specific for particular clades of *Actinobacteria*, the shared presence of CSIs or CSPs in unrelated groups of bacteria also provides a novel means for the identification of lateral gene transfers. Two interesting cases of LGTs between *Actinobacteria* and *Chlamydiae* that have been identified by these means include those for the genes encoding the enzymes serine hydroxymethyltransferase (SHMT) (or the GlyA protein) and UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) (115, 117). In the enzyme SHMT, which links amino acid and nucleotide metabolisms by generating the key intermediate for one-carbon transfer reactions (238), two CSIs (3 and 31 aa long) are uniquely shared by all *Chlamydiae* species, *Treponema* species, as well as a subset of *Actinobacteria* (117). Interestingly, the actinobacterial species which contain these CSIs have multiple homologs of the *glyA* gene, and only one of them harbors the indicated CSIs (117). Similarly, in the MurA protein, which plays an important role in the synthesis of cell wall peptidoglycan, a 16-aa CSI was commonly shared by all *Chlamydiae* and a subset of *Actinobacteria* (115). In the phylogenetic trees based upon GlyA or MurA protein sequences, the *Chlamydiae* homologs branched with the various insert-containing *Actinobacteria* within a clade of other *Actinobacteria*. These results provide strong evidence that the shared presence of these CSIs in these two groups of bacteria is due to the lateral transfer of genes for these proteins from certain groups of *Actinobacteria* to a common ancestor of the *Chlamydiae* (117, 133). It is of much interest to understand the functional significances of the identified CSIs in these proteins and to determine why their genes were laterally transferred from *Actinobacteria* to the common ancestor of the *Chlamydiae*. Our work on actinobacterial CSPs has also revealed that homologs of some of them are also found in *Magnetospirillum magnetotacticum* (100), which is unrelated to the *Actinobacteria*. The CSI in the glycyl-tRNA synthetase, which is mainly a distinctive characteristic of *Actinobacteria*, is also found in this bacterium as well as in a few *Planctomycetes* (Table 2). The shared presence of these CSPs and CSI is again due to LGTs from *Actinobacteria* to *M. magnetotacticum*, and it is of much interest to determine what unique properties are shared by these two groups of bacteria.

Application of the Identified Molecular Signatures for Identification of *Actinobacteria* and Exploring Their Diversity

The phylum *Actinobacteria* is extremely diverse. In addition to containing many bacteria that are major human, animal, or plant pathogens (e.g., *Mycobacterium*, *Actinomyces*, *Renibacterium*, *Atopobium*, *Gordonia*, *Gardnerella*, *Leifsonia*, and *Clavibacter*), other actinobacterial taxa arguably provide the richest source for discovering diverse natural products that have proven to be of seminal importance in clinical and biotechnological applications (12, 21, 36, 45, 86, 87, 220, 249). Thus, it is of much interest and importance to discover novel means by which both known as well as novel species belonging to different actinobacterial groups can be readily and accurately identified in different settings (*viz.*, clinical or environmental). Because some taxa of *Actinobacteria* (e.g., *Streptomyces*, *Salinispora*, *Saccharopolyspora*, *Cellulomonas*, *Verrucosispora*, *Pseudonocardia*, *Micromonospora*, *Bifidobacterium*, and *Arthrobacter*, etc.) have proven to be particularly important sources for the discovery of novel compounds such as antibiotics and probiotics and compounds useful in bioremediation (42, 110, 162, 197, 220, 221, 308, 325), there is enormous interest in the discovery of novel actinobacterial species belonging to these taxa, which could lead to the discovery of either novel antibiotics or other natural products that can be gainfully employed for various applications (35, 36, 84, 110, 197). As emphasized by Goodfellow and coworkers (110, 323), a sound knowledge of actinobacterial systematics is of particular importance in this regard. A reliable phylogenetic framework for *Actinobacteria* in conjunction with specific probes for identifying different groups of *Actinobacteria* can greatly facilitate the discovery of novel actinobacterial species in different environments. In this context, molecular markers (CSIs and CSPs) that are specific for different major clades of *Actinobacteria* are of particular importance, since probes based on them can serve as novel and specific tools for the identification and discovery of novel actinobacterial species belonging to these taxa. The primary sequences of many of the CSPs and most of the proteins that contain these CSIs are highly conserved. Based upon conserved regions in these genes/proteins, degenerate PCR primers for these genes/proteins can be readily designed, which should specifically amplify gene sequences from these clades (95, 97, 115) and should provide novel means for the identification of new as well as existing actinobacterial species belonging to these clades from different environments. Using these molecular signatures, it should also be possible to readily and more accurately determine the presence or absence of different families and orders of *Actinobacteria* in metagenomic samples obtained from various environments (35, 109, 110, 147, 201, 241, 276, 277). Likewise, CSIs and CSPs that are specific for the pathogenic *Actinobacteria* (*viz.*, *Mycobacterium*, *Corynebacterium*, *Propionibacterium*, and *Actinomyces*) provide novel means for their diagnostics. Some of the CSPs and the proteins containing CSIs that are specific for these groups should also provide potential means for developing vaccines for these bacteria or potential targets for developing drugs that are specific for these bacteria.

Functional Significance of Actinobacterial CSIs and CSPs

An important area for future research is to understand the functional significance of various CSIs and CSPs that are specific for either all *Actinobacteria* or their various clades. For the phylum

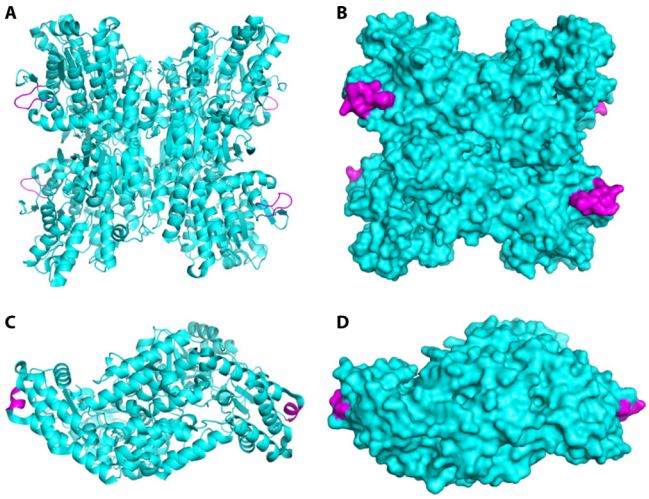

FIG 20 Structures of the *S*-adenosyl-L-homocysteine hydrolase (PDB accession number 3CE6) (240) (A and B) and serine hydroxymethyltransferase (PDB accession number 3H7F) (C and D) proteins from *M. tuberculosis* showing the locations in protein structures of the 9-aa and 5-aa actinobacterium-specific inserts that are found in these proteins (see Files S5 and S6 in the supplemental material). While panels A and C show ribbon representations, panels B and D depict the surface representations of these protein structures. The inserts in these proteins are shown in magenta.

Actinobacteria or most of its major clades, no biochemical or physiological characteristics that are unique to them are presently known. Hence, the identified CSIs and CSPs that are specific for different clades of *Actinobacteria* provide novel means for discovering biochemical and/or other characteristics that are unique to these groups. Most of the identified CSIs are located in widely distributed proteins (e.g., ribosomal proteins, RNA polymerase, gyrase, DNA polymerase, and various enzymes in key metabolic pathways) that are responsible for carrying out essential cellular functions. The primary functions of these proteins are vital for cell survival, and they are expected to remain the same in all organisms. Hence, the question arises, What is the functional significance of these evolutionarily conserved indels that are specific for different actinobacterial lineages?

Recent work on a number of conserved indels in the Hsp60 (GroEL) and Hsp70 (DnaK) proteins showed that the identified CSIs are essential for the groups of species where they are found and that deletions or most changes in them led to a failure of cell growth (269). Based upon this finding, we expect that the CSIs that are specific for *Actinobacteria* will also be essential for the particular lineages where they are found. An important observation in this regard is that most of these CSIs are generally present in the surface loops of various proteins (4, 118, 269). This is also true for most of the *Actinobacteria*-specific CSIs described in this work, and it is illustrated by the structures of two of the proteins, *viz.*, *S*-adenosyl homocysteine hydrolase (240) and serine hydroxymethyltransferase (Fig. 20), which contain 5-aa and 9-aa CSIs, respectively, that are specific for most *Actinobacteria* (see Files S4 and S6 in the supplemental material for sequence alignments of these proteins). The structures shown in Fig. 20 are from *M. tuberculosis*, which contains these inserts, and the regions corresponding to the inserts are colored magenta. As shown in Fig. 20, the inserts in both proteins are present in surface loops, and they are seen as patches or knobs on the surfaces of these proteins. The surface loops in protein sequences are known to play an important role in mediating protein-protein interactions, and they can either facilitate or disrupt certain interactions (4, 152). In view of the predicted essential nature of these CSIs and their locations on protein surfaces (generally away from the active sites), we have postulated that these CSIs are involved in conferring novel functional capabilities (i.e., ancillary functions) on these essential proteins through protein-protein or other forms of interactions (269). These ancillary functions are expected to be important for the lineages in which these CSIs are found, and they could include the ability of the protein(s) to interact with other cellular proteins or ligands (with the CSI serving as a docking site) that either modulate the activity of these proteins or confer some new function(s) on them. Recent studies of two large CSIs in the gyrase B and RpoC proteins that are specific for a number of bacterial phyla support this hypothesis (46, 116, 255). Hence, further studies toward an understanding of the cellular functions of these *Actinobacteria*-specific CSIs should lead to the discovery of novel aspect of many important proteins that contain these CSIs.

Unlike CSIs, which are commonly found in essential proteins of known functions, the cellular functions of most of the CSPs that are limited to particular lineages of *Actinobacteria* are generally not known. The evolutionary conservation and retention of genes for these proteins by different lineages strongly suggest that they perform important functions (62, 96, 133, 244) that are specific for these lineages and which distinguish them from other *Actinobacteria*. Hence, an understanding of the cellular functions of these CSPs should provide valuable insights into the biochemical and physiological characteristics that are unique to different taxa of *Actinobacteria*. The significance of such proteins for particular lineages is illustrated by the examples of the well-studied EmbA, EmbB, EmbC, and AftA proteins, which are CSPs that are limited to either the order *Corynebacteriales* or the orders *Corynebacteriales* and *Pseudonocardiales* (Tables 3 and 9). The species of these two orders have cell wall chemotype IV, defined by the presence of *meso*-diaminopimelic acid, arabinose, and galactose in their cell walls, and these proteins play key roles in the biosynthesis of arabinan, which is a unique component of their cell walls (7, 24, 106, 259, 263, 300). Thus, the lineage specificity of these proteins correlates with a unique and essential biochemical property of these orders of *Actinobacteria*.

Of the four CSPs that are distinguishing characteristics of nearly all *Actinobacteria*, the structures of two of them, *viz.*, SCO1997 and SCO1662 [gene identification from *S. coelicolor* A3(2), which corresponds to the ML1009 and ML1306 proteins from *M. leprae* TN], were recently solved (Protein Data Bank [PDB] accession number 3E35) (100). Although the structures of these two related proteins show limited structural similarity to purine nucleoside phosphorylase and the PAC2 family of proteins from the *Archaea* (PDB accession number 3GAA), at the sequence level, they exhibit no significant similarity to these proteins. Thus, the functions of these *Actinobacteria*-specific proteins are predicted to be novel. This inference is strongly supported by recent work showing that SCO1662 specifically interacts with the ParA protein, and it likely corresponds to the ParJ protein, which negatively regulates ParA polymerization *in vitro*, which is important for efficient chromosome segregation in sporulating aerial hyphae (71). However, further studies are needed to understand the roles of the two homologs of this protein (*viz.*, SCO1662 and SCO1997) and why they are uniquely found in *Actinobacteria*. Similarly, the WhiB

protein family, which has several gene copies in all *Actinobacteria* except the deepest-branching lineages, is indicated to play an essential role in controlling developmental transition in *Streptomyces* (43, 90). In nonsporulating actinobacterial species such as *Mycobacterium* species, WhiB proteins are differentially expressed, and they are important in regulating virulence, cell division, antibiotic resistance, and other stress responses (32, 208). These examples indicate that the CSPs that are specific for different actinobacterial lineages likely play important roles in different unique aspects of these bacteria, including their niche adaptation, pathogenic mechanisms, and other genetic, biochemical, and morphological characteristics that are unique to these bacteria. Hence, concerted efforts to understand their cellular functions should provide important insights into the unique biological aspects of these bacteria.

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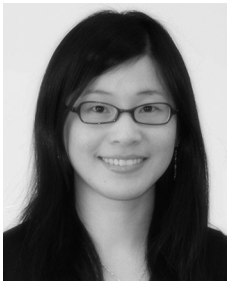
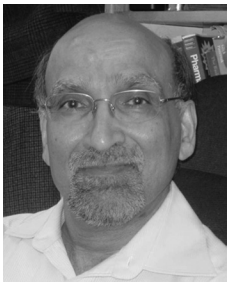


Beile Gao completed her Ph.D. under the supervision of Radhey Gupta in December 2009. Her thesis work was on identifying molecular signatures that are specific for *Actinobacteria*, and a number of CSIs and CSPs specific for actinobacteria were identified. She also solved the structure for the first *Actinobacteria*-specific protein. After the completion of her Ph.D. thesis work, Dr. Gao worked for another 6 to 8 months on the identification of additional CSIs and CSPs that were specific for *Actinobacteria*. Since March 2010, Dr. Gao has been working as a postdoctoral fellow in the Section of Microbial Pathogenesis at Yale University School of Medicine.



Radhey S. Gupta is a Professor of Biochemistry at McMaster University in Canada. He joined McMaster in 1978, and his research interests have covered many areas, including studies on drug-resistant mutants of mammalian cells and heat shock proteins, structure-function studies on enzymes, and studies on novel aspects of mitochondria. Since the 1990s, his primary research interests have been in using molecular sequence data to understand the early evolutionary history of life. His earlier work provided evidence that the eukaryotic cell nucleus is a chimera formed by fusion between an archaeon and a bacterium. For the past 10 to 12 years, the main focus of his work has been on using genomic sequence data to understand microbial systematics and evolution. The aim of these studies is to discover novel molecular markers for identifying different groups of *Bacteria* in more definitive molecular terms and to understand their branching order from a common ancestor. His laboratory has pioneered the discovery of conserved signature indels and whole proteins that are specific for different phyla of bacteria at various phylogenetic depths. Large numbers of such signatures for different bacterial groups have been identified. In addition to providing more reliable means for identifying different groups of bacteria, these signatures also provide novel tools for microbial diagnostics, as potential targets for drug and vaccine development, and powerful means for genetic, biochemical, and evolutionary studies. Professor Gupta has published >260 articles in peer-reviewed articles, and the current "h" score of his publications is 51. Further information on his work can be found at his website, http://www.science.mcmaster.ca/biochem/faculty/gupta/index.htm.