Deoxyribonucleic Acid Homologies Among Coryneform Bacteria

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The deoxyribonucleic acid homologies among 33 strains of coryneform bacteria with various cell wall types reflected the previous grouping of these organisms by Yamada and Komagata on the bases of the mode of cell division, principal amino acids in the cell wall, and the base composition of deoxyribonucleic acid. In addition, the following four homology clusters were found: (i) six strains of corynebacteria with a glycolyl type of cell wall, (ii) four strains of glutamic acid-producing bacteria, (iii) two strains of *Brevibacterium linens*, and (iv) three strains of coryneform bacteria containing LL-diaminopimelic acid in the cell wall.

The aerobic, nonsporeforming, irregularly shaped, gram-positive rods of the genera *Corynebacterium*, *Arthrobacter*, *Cellulomonas*, *Curtobacterium* (32), *Brevibacterium*, and *Microbacterium* are referred to as coryneform bacteria (19). Phenotypic characteristics seem to be of little value in the taxonomy of coryneform bacteria, but chemotaxonomic information on the chemical composition of cells has been useful in this regard. Yamada and Komagata (33) divided coryneform bacteria into seven groups, mainly on the bases of the mode of cell division (14), principal amino acids in the cell wall (30), and deoxyribonucleic acid (DNA) base composition (32). These groupings have subsequently been supported by the cell wall peptidoglycan types (21), the phospholipid compositions of the cells (16), and the menaquinone systems (4, 29) of these organisms. Studies on the DNA homologies of *Corynebacterium renale* and phenotypically related strains (10), cheese coryneforms and soil arthrobacters (6), plant pathogenic coryneform bacteria (25), arthrobacters and brevivacteria (23), and cellulomonads (24) have been reported. However, little information is available on the DNA homology of aerobic coryneform bacteria in general.

This report deals with DNA homologies among representative strains of coryneform bacteria selected from each of the groups described by Yamada and Komagata (33) and compares the results with the chemotaxonomic characteristics of these organisms.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains studied were selected from those previously studied by Komagata et al. (14) and Yamada and Komagata (30-33). A *Caseobacter polymorphus* strain, a species recently

established by Crombach (7), was also studied. The strains studied are listed in Table 1, together with their strain numbers, several corresponding culture collection designations, and their chemotaxonomic profiles. Names which are not on the Approved Lists of Bacterial Names, 1980 (22) are enclosed within quotation marks.

As references for the DNA homology experiments, generally one strain was chosen from each group. However, for group 1, which has a wide range of DNA base compositions, four strains—*Corynebacterium equi* CNF 002, *Corynebacterium vitrum* CNF 008 (17), *Brevibacterium ammoniagenes* CNF 012, and *Corynebacterium glutamicum* CNF 016—were selected as references. *Curtobacterium citreum* CNF 026 and *Curtobacterium testaceum* CNF 030 were chosen as references for group 5, in consideration of the fact that the latter is the only glycolate-positive species in group 5 (27).

Cultivation. The cultivation medium used was basically medium R (32), which contained 1% peptone, 0.5% yeast extract, 0.5% Casamino Acids, 0.2% beef extract, 0.5% malt extract (all from Difco Laboratories, Detroit, Mich.), 0.2% glycerol, 0.1% MgSO₄·7H₂O, and 0.005% Tween 80 (Wako Pure Chemical Industry Ltd., Osaka, Japan). When necessary, 0.25 to 4.0% of glycine was added to this medium to facilitate cell lysis by lysozyme (31). All organisms were grown in medium R with shaking for 24 h at 30°C, except for *Corynebacterium diphtheriae* CNF 017 which was grown for 48 h at 37°C.

Extraction of DNA. Cells collected by centrifugation were washed twice with saline-ethylenediamine-tetraacetic acid (0.15 M NaCl-0.1 M ethylenediamine-tetraacetic acid, pH 8.0), suspended in a volume of saline-ethylenediamine-tetraacetic acid equal to the cell mass, and stored in a freezer at -20°C. DNA was prepared by the phenol extraction method described by Saito and Miura (20), with some minor modifications. Similarly, labeled DNA was extracted from cells grown in medium R supplemented with 10 µCi of [*methyl*-³H]thymidine per ml. However, since only the four reference strains of group 1 took up little [*methyl*-

TABLE 1. *Bacterial strains studied*

Species ^a	Strain number	Strain designations ^b			Taxonomic status	Cell wall murein type ^c	G+C content ^d (mol%)	PE ^e	Menaquinone systems ^f	Glycolate test ^g
		AJ	ATCC	IAM						
Group 1: <i>Corynebacterium</i>										
<i>Corynebacterium equi</i>	CNF 002	1402	6939	12426	Type	<i>Meso</i> -DAP, A31 ^h	67.8	+	MK-8 (H ₂)	+
<i>Corynebacterium equi</i>	CNF 003	1403	10146	12444		<i>Meso</i> -DAP	67.1		MK-8 (H ₂)	
<i>Corynebacterium equi</i>	CNF 004	1372		12445		<i>Meso</i> -DAP	66.1		MK-8 (H ₂)	
<i>Corynebacterium equi</i>	CNF 005	1376		12446		<i>Meso</i> -DAP	65.9		MK-8 (H ₂)	
<i>Corynebacterium xerosis</i>	CNF 010	1396	7711	12431		<i>Meso</i> -DAP, A31	68.5			
<i>Corynebacterium fascians</i>	CNF 006	1398	12974	12427	Type	<i>Meso</i> -DAP	67.6	+	MK-8 (H ₂)	-
<i>Corynebacterium hoagii</i>	CNF 007	1408	7005	12428	Type	<i>Meso</i> -DAP, A31	64.6			+
<i>Corynebacterium vitarium</i>	CNF 008	1525	10234	12429	Type	<i>Meso</i> -DAP	64.4			+
<i>Corynebacterium renale</i>	CNF 009	1411	10848	12430	Type	<i>Meso</i> -DAP, A31	59.3			
<i>Corynebacterium flavesens</i>	CNF 011	1415	10340	1642		<i>Meso</i> -DAP	58.3	Trace	MK-8 (H ₂), MK-9 (H ₂)	-
<i>Brevibacterium ammoniagenes</i>	CNF 012	1444	6872	1645		<i>Meso</i> -DAP, A31	54.6	Trace	MK-9 (H ₂)	-
<i>Brevibacterium divaricatum</i>	CNF 013	1498	14020	12432	Type	<i>Meso</i> -DAP, A31	54.4			
" <i>Brevibacterium flavum</i> "	CNF 014	1510	14067	12433	Type	<i>Meso</i> -DAP	54.1		MK-9 (H ₂)	
<i>Corynebacterium glutamicum</i>	CNF 016	1502	13032	12435		<i>Meso</i> -DAP, A31	53.4			
<i>Corynebacterium lilium</i>	CNF 015	1517	15990	12434	Type	<i>Meso</i> -DAP, A31	52.9	Trace	MK-9 (H ₂)	-
<i>Corynebacterium diphtheriae</i>	CNF 017	1414	11913			<i>Meso</i> -DAP	52.4		MK-8 (H ₂)	-
Group 2: <i>Brevibacterium</i>										
<i>Brevibacterium linens</i>	CNF 019	1521	9172	12437	Type	<i>Meso</i> -DAP	63.4	-	MK-8 (H ₂)	
<i>Brevibacterium linens</i>	CNF 018	1520	8377	12436		<i>Meso</i> -DAP	62.7	-	MK-8 (H ₂)	
Group 3: <i>Arthrobacter</i>										
" <i>Brevibacterium helvolum</i> "	CNF 020	1445	11822	1637		L-Lysine	64.1	-	MK-9 (H ₂)	-
<i>Arthrobacter citreus</i>	CNF 021	1423	11624	12341	Type	L-Lysine, A11.27 ^h	62.9	-	MK-9 (H ₂)	-
<i>Arthrobacter globiformis</i>	CNF 022	1422	8010	12438	Type	L-Lysine, A11.6 ^h	62.0	-	MK-9 (H ₂)	-
<i>Arthrobacter ureofaciens</i>	CNF 023	1421	7562	1658	Type	L-Lysine, A11.17 ^h	61.7	-	MK-9 (H ₂)	-
Group 4: <i>Cellulomonas</i>										
<i>Cellulomonas biazotea</i>	CNF 024	1569	486	12107	Type	L-Ornithine, A21.5 ^h	72.2	-	MK-9 (H ₄)	-
<i>Cellulomonas fini</i>	CNF 025	1571	484	12106	Type	L-Ornithine, A21.5	71.7	-	MK-9 (H ₄)	-
Group 5: <i>Curtobacterium</i>										
<i>Curtobacterium citreum</i>	CNF 026	1469	15828	1614		Ornithine	70.5	-	MK-9	-
" <i>Curtobacterium poinsettiae</i> "	CNF 027	1999	9682	12439	Type	d-Ornithine, B5 ^h	70.0	-	MK-9	-
" <i>Curtobacterium flaccum-faciens</i> "	CNF 029	1400	6887	12440		d-Ornithine, B5	68.3	-	MK-9	-
<i>Curtobacterium testaceum</i>	CNF 030	1464	1537			Ornithine	65.4	-	MK-11	+

Group 6: LL-DAP-type coryne-
form bacteria

<i>Arthrobacter simplex</i>	CNF 035	1420	6946	1660	Type	LL-DAP, A41.1 ^h	71.7	–	MK-8 (H ₄)	–
“ <i>Brevibacterium lipolyticum</i> ”	CNF 036	1450		1398		LL-DAP, A41.1	70.7	–	MK-8 (H ₄)	–
“ <i>Brevibacterium lipolyticum</i> ”	CNF 037	1451		1413		LL-DAP	70.5			

Group 7: L-DAB-type cory-
neform bacteria

“ <i>Corynebacterium aquaticum</i> ”	CNF 038	1413	14665	12441		L-Diaminobutyric acid, B7 ^h	69.3	–	MK-11 [MK-10]	–
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Genus *Caseobacter*

<i>Caseobacter polymor- phus</i>	CNF 057			12549	LMD AC256	Type	Meso-DAP	66.1		
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^a Names in quotation marks are not on the Approved Lists of Bacterial Names (22).

^b Abbreviations: AJ, Central Research Laboratories, Ajinomoto Co., Inc., Kawasaki, Japan; ATCC, American Type Culture Collection, Rockville, Md.; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; NI, Nagao Institute, Tokyo, Japan; NRRL, ARS Culture Collection, Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Ill.; CCM, Czechoslovak Collection of Microorganisms, J. E. Purkyně University, Brno, Czechoslovakia; LMD, Laboratorium voor Microbiologie der Landbouwhogeschool, Wageningen, The Netherlands.

^c See references 3, 9, 21, 30, 33.

^d See references 7 and 31.

^e PE, Presence of phosphatidylethanolamine. See reference 16.

^f See reference 29.

^g +, Glycolyl-type cell wall; –, acetyl-type cell wall. See references 27 and 28.

^h See reference 3.

³H]thymidine, [6-³H]uracil was used instead. A similar phenomenon was observed by Honda and Yanagawa (10) with *Corynebacterium renale*.

DNA-DNA hybridization. DNA-DNA hybridization was carried out by the membrane filter method employed in previously reported studies (11, 13, 18). Single-stranded DNA (50 µg) was immobilized on a cellulose nitrate membrane filter (TM-2, 0.45-µm pore size, 24-mm diameter; Toyo Roshi Kaisha Ltd., Tokyo). It was incubated at 62°C for 38 h in 1 ml of 2× SSC (SSC = 0.15 M NaCl-0.015 M trisodium citrate)

containing 0.1% sodium dodecyl sulfate and approximately 5×10^3 cpm of labeled DNA which had been heat denatured and fragmented by sonication. Incubated filters were washed twice each with 2× SSC and 5 mM tris(hydroxymethyl)aminomethane (pH 9.5) to remove noise radioactivity (28).

The optimum hybridization temperature depends not only on the DNA base composition but also on how much the parental DNAs differ from each other (unpublished data). Because the bacterial strains used in this study differed widely, we employed a lower

TABLE 2. DNA-DNA homologies

Species names ^a	Strain number	G+C content (mol%) ^b	Reference strain					
			<i>C. equi</i> CNF 002		<i>C. vitrumen</i> CNF 008		<i>B. ammoniagenes</i> CNF 012	
			A ^c	B ^c	A	B	A	B
Group 1: <i>Corynebacterium</i>								
<i>Corynebacterium equi</i>	CNF 002	67.8	100	100	43	11	5	3
<i>Corynebacterium equi</i>	CNF 003	67.1	95	91	44	12	6	3
<i>Corynebacterium equi</i>	CNF 004	66.1	95	85	33	9	6	3
<i>Corynebacterium equi</i>	CNF 005	65.9	91	88	31	11	5	3
<i>Corynebacterium fascians</i>	CNF 006	67.3	98	99	34	11	6	3
<i>Corynebacterium hoagii</i>	CNF 007	64.6	91	88	36	12	6	3
<i>Corynebacterium xerosis</i>	CNF 010	68.5	49	15	53	20	11	7
<i>Corynebacterium vitrumen</i>	CNF 008	64.4	34	14	100	100	13	8
<i>Brevibacterium ammoniagenes</i>	CNF 012	54.6	10	4	20	12	100	100
<i>Corynebacterium renale</i>	CNF 009	59.3	16	7	31	17	18	9
<i>Corynebacterium flavescens</i>	CNF 011	58.3	17	7	36	18	22	11
<i>Corynebacterium diphtheriae</i>	CNF 017	52.4	12	4	29	16		
<i>Corynebacterium divaricatum</i>	CNF 013	54.4	9	4	26	13	22	10
" <i>Brevibacterium flavum</i> "	CNF 014	54.1			24	15	21	12
<i>Corynebacterium lilium</i>	CNF 015	52.9	8	4	25	13	17	10
<i>Corynebacterium glutamicum</i>	CNF 016	53.4	6	3	19	12	21	11
Group 2: <i>Brevibacterium</i>								
<i>Brevibacterium linens</i>	CNF 018	62.7						
<i>Brevibacterium linens</i>	CNF 019	63.4	38	10	26	8	6	3
Group 3: <i>Arthrobacter</i>								
" <i>Brevibacterium helvolum</i> "	CNF 020	64.1						
<i>Arthrobacter citreus</i>	CNF 021	62.9						
<i>Arthrobacter globiformis</i>	CNF 022	62.0	26	8	24	8	6	3
<i>Arthrobacter ureafaciens</i>	CNF 023	61.7						
Group 4: <i>Cellulomonas</i>								
<i>Cellulomonas biasotea</i>	CNF 024	72.2						
<i>Cellulomonas fimi</i>	CNF 025	71.7						
Group 5: <i>Curtobacterium</i>								
<i>Curtobacterium citreum</i>	CNF 026	70.5	44	11	28	7	4	2
" <i>Curtobacterium poinsettiae</i> "	CNF 027	70.0						
" <i>Curtobacterium flaccumfaciens</i> "	CNF 029	68.3						
<i>Curtobacterium testaceum</i>	CNF 030	65.4	40	12	36	9	4	2
Group 6: LL-DAP type coryneform bacteria								
<i>Arthrobacter simplex</i>	CNF 035	71.7						
" <i>Brevibacterium lipolyticum</i> "	CNF 036	70.7						
" <i>Brevibacterium lipolyticum</i> "	CNF 037	70.5						
Group 7: L-DAB type coryneform bacteria								
" <i>Corynebacterium aquaticum</i> "	CNF 038	69.3	49	14	36	10	5	2
Genus <i>Caseobacter</i>								
<i>Caseobacter polymorphus</i>	CNF 057	66.1	37	14	40	18		

^a Names in quotation marks are not on the Approved Lists of Bacterial Names (22).

^b See references 7 and 31.

^c A, Homology index at 62°C; B, homology index at 75°C.

[illegible]

cantly higher than they were with the reference strains of the other groups. In other words, no groups shared high homology indices against the reference strains from other groups, and the groups were thus clearly separated from each other by DNA homology indices. However, some groups were fairly homogeneous with respect to DNA homology, whereas others were not. The results obtained for each group are discussed below.

Group 1. In group 1, which corresponds to the genus *Corynebacterium*, two DNA homology clusters, as well as strains independent of these clusters, were found. One cluster comprises four *Corynebacterium equi* strains (CNF 002, CNF 003, CNF 004, and CNF 005), *Corynebacterium fascians* CNF 006, and *Corynebacterium hoagii* CNF 007. Except for *C. fascians* CNF 006, which is a plant pathogen, these strains originated from animal sources (19). The members show extremely high homology (at least 88%) at 75°C to the reference strain *C. equi* CNF 002. They are also chemotaxonomically homogeneous; they all have 64.6 to 67.8 mol% of guanine plus cytosine (G+C) in their DNA (31), an MK-8 (H₂) type of menaquinone (29), phosphatidylethanolamine as cellular phospholipid (16), and a glycolyl type of cell wall (27).

Corynebacterium vitarumen CNF 008 and *Corynebacterium xerosis* CNF 010, which have almost the same DNA base composition as the members of the above-mentioned homology cluster, showed low indices not only to *C. equi* CNF 002 but also to *C. xerosis* CNF 010. These two strains have been reported to differ from the strains in the *C. equi* homology cluster with respect to the results of the glycolate test (26, 27).

The other homology cluster in group 1 consists of four strains of glutamic acid-producing bacteria. *Brevibacterium divaricatum* CNF 013, *Brevibacterium flavum* CNF 014, and *Corynebacterium lilium* CNF 015 showed high homology values (more than 80%) to *Corynebacterium glutamicum* CNF 016. This finding suggests that on the basis of DNA homology the four strains belong to the same species. Glutamic acid-producing bacteria have been reported to resemble each other phenotypically (1, 32, 33). It is interesting to note that *Brevibacterium ammoniagenes* CNF 012, which is not a glutamic acid-producing bacterium, showed a rather low homology index to *C. glutamicum* CNF 016, although they resemble each other phenotypically and chemotaxonomically (16, 27, 29, 32, 33). Komatsu and Kaneko (15) also reported that nine strains of glutamic acid-producing bacteria fell into a single group, and that *B. ammoniagenes* ATCC 13745 was closely related to *C. glutamicum* ATCC 13032 (=CNF 016). How-

ever, *B. ammoniagenes* ATCC 13745, studied by Komatsu and Kaneko, is a glutamic acid-producing bacterium and differs from CNF 012 (=ATCC 6872), which is one of the original strains of *B. ammoniagenes* on which Cooke and Keith established the species (5).

Corynebacterium diphtheriae CNF 017, *Corynebacterium flavesces* CNF 011, and *Corynebacterium renale* CNF 009 were not related to glutamic acid-producing bacteria or to *B. ammoniagenes* CNF 012. In the eighth edition of *Bergey's Manual of Determinative Bacteriology* (19), these three species are placed in "Section I. Human and animal parasites and pathogens," corresponding to "*Corynebacterium sensu stricto*" (12). Glutamic acid-producing bacteria, on the other hand, are accommodated by "Section III. Non-pathogenic corynebacteria." In this study, we did not determine the similarities among the three strains mentioned above, but their indices of DNA homology suggest that the organisms are rather closely related to each other. Recently, Barksdale et al. (2) transferred *Microbacterium flavum* to the genus *Corynebacterium*, recognized a new species, *C. flavesces*, and reported a high homology value (64%) for *C. flavesces* and *C. diphtheriae*.

Corynebacteria, in a broad sense, generally have cell walls containing meso-diaminopimelic acid (meso-DAP) and arabinogalactan (21), but their DNA base compositions vary widely from 52 to 70 mol% G+C (31). Crombach pointed out that this range is too wide to consider the corynebacteria as belonging to one genus (8). The present data also suggest that the species of the genus *Corynebacterium* be divided into more than four subgroups.

Group 2. Group 2 corresponds to the genus *Brevibacterium*. *Brevibacterium linens* CNF 018 had 70% DNA homology to the type strain, *B. linens* CNF 019. Although these strains have meso-DAP in their cell walls (21, 30), they did not relate any more closely to the members of group 1 than to those of the other groups whose members do not contain meso-DAP.

Group 3. Group 3 contains arthrobacters which have L-lysine in their cell walls (30). This group was found to have DNA heterogeneous with that of *Arthrobacter globiformis* CNF 022, the type species of the genus. Recently, Stackebrandt and Fiedler (23) studied the relationship between DNA homology and peptidoglycan in arthrobacters; they reported that arthrobacters are rather diverse even when they share the same peptidoglycan type in their cell walls.

Group 4. Group 4 contains the cellulomonads. *Cellulomonas fimi* CNF 025 was used as a reference strain. This strain has such a high G+C content that the homology indices with some strains with high G+C contents were themselves

quite high. *Cellulomonas biazotea* CNF 024 showed 54% homology to the reference strain, whereas the strains of the other groups studied showed approximately 30% or lower homology values. These homology values distinguished cellulomonads from other coryneform bacteria, and the moderate similarity between *C. fimi* CNF 025 and *C. biazotea* CNF 024 revealed that they belong to the same genus.

Group 5. This group contains the curtobacteria. The genus *Curtobacterium*, which was established by Yamada and Komagata (33), is composed mainly of motile brevibacteria from plants and seems to be rather homogeneous morphologically and physiologically (32, 33). However, high homology values were not found among the strains in this group. *Curtobacterium testaceum* CNF 030 showed exceptionally low relatedness to the other members of this group. The distinctiveness of this species was indicated not only by the results of the glycolate test (26, 27) and menaquinone system (29) but also by DNA homology results.

Group 6. The members of group 6 contain LL-DAP and glycine in their cell walls (21, 30), and their DNA base compositions are concentrated in a narrow range (70 to 72 mol% G+C). The result of the hybridization test at 75°C with *Arthrobacter simplex* CNF 035 as a reference strain showed 100% homology to *Brevibacterium lipolyticum* CNF 036 and 60% to *B. lipolyticum* CNF 037. These indices are high enough to separate them from others which showed low values (not more than 35%) and suggest that these three strains belong to one species.

Group 7. *Corynebacterium aquaticum* CNF 038, a member of group 7, was found to contain L-diaminobutyric acid in its cell wall and to have low homology indices with strains of the other groups, thus revealing that group 7 is unique.

Crombach (7) proposed a new genus, *Caseobacter*, for non-orange cheese coryneforms based on the fact that they are not "parasitic or pathogenic diphtheroids of human and animal origin" and that their G+C contents are 10% higher than that of *Corynebacterium diphtheriae*, the type species of the genus *Corynebacterium*. He reported the low DNA relatedness of caseobacters to *B. linens* and *A. globiformis*, but the DNA homology between *Caseobacter polymorphus* and other corynebacteria had not then been determined. In the present study, *C. polymorphus* CNF 057 appeared to be similar to *C. vitarumen* CNF 008 and *C. xerosis* CNF 010 with respect to their homology patterns with some reference strains of corynebacteria. The homology index between *C. polymorphus* CNF 057 and *C. vitarumen* CNF 008 was rather low, although these organisms have almost the same

DNA base composition. Further chemotaxonomy and DNA homology studies will help to clarify the relationships of these strains.

In this study, we found good agreement between the DNA homologies and the chemotaxonomic profiles reported to date for the coryneform bacteria; we also confirmed that chemotaxonomic characters are useful in the classification of this group of bacteria at the generic level. The type of peptidoglycan in the cell wall is one of the most important criteria for defining the genera of coryneform bacteria, but Stackebrandt and Fiedler (23) showed that there is a problem in defining species of *Arthrobacter* either by interpeptide bridge types or by DNA homologies. Moreover, Stackebrandt and Kandler (24) proposed neotype strains for seven *Cellulomonas* species on the basis of DNA homology and some phenotypic characteristics, thus dividing the genus still further.

In a previous paper (11), we pointed out the practical application of DNA homology in the identification of biovars of *Pseudomonas maltophilia*. The result of the study reported here should be helpful in defining species of coryneform bacteria, although we feel that additional systematic studies are required for this purpose.

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REPRINT REQUESTS

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