TAXONOMIC STUDIES ON CORYNEFORM BACTERIA

III. DNA BASE COMPOSITION OF CORYNEFORM BACTERIA

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The DNA base composition (GC content) of 114 strains belonging to the so-called coryneform bacteria was examined from the viewpoint of bacterial taxonomy. The GC content of these bacteria ranged widely from 46 to 78%. Although the GC content of *Cellulomonas* fell into a relatively narrow range near 72%, those of *Corynebacterium*, *Arthrobacter*, *Microbacterium*, and *Brevibacterium* did not exhibit the specific range to characterize the genus. This fact probably shows that the genera of coryneform bacteria, except *Cellulomonas*, are taxonomically heterogeneous. A new method for the isolation of DNA from lysozyme-insensitive or poorly sensitive strains was described.

In the taxonomic studies on the so-called coryneform bacteria, the present authors (1, 2) pointed out that this group of bacteria was clearly distinguished from the true bacteria on the basis of the mode of cell division and that the coryneform bacteria were divided into three groups by the principal amino acids of cell wall.

For the bacterial taxonomy, DNA base composition (GC content) has been considered to be one of the acceptable criteria (3). Following this concept, the present authors tried to seek inter-relationships among the coryneform bacteria. This paper deals with GC content of members of the genera *Corynebacterium*, *Arthrobacter*, *Microbacterium*, *Cellulomonas*, and *Brevibacterium*, and discusses the significance of GC content in the classification of the coryneform bacteria.

MATERIALS AND METHODS

Microorganisms. Tested bacteria comprised 114 strains which were obtained from the culture collections or freshly isolated by present authors. They belonged to 61 species of 6 genera. The names of strains from culture

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Table 1. Bacteria tested.

Genus	Number of species	Number of strains
<i>Arthrobacter</i>	12	19
<i>Brevibacterium</i>	24	47
<i>Cellulomonas</i>	5	7
<i>Corynebacterium</i>	17	32
<i>Microbacterium</i>	2	2
<i>Micrococcus</i>	1	1
Unidentified coryneform bacteria		6
Total	61	114

collections were used as designated. *Micrococcus glutamicus* was employed in this work because this bacterium had been considered to belong to the category of coryneform bacteria (1, 2, 4). All the bacteria tested were confirmed to be pure. Number of species and strains tested in each genus are shown in Table 1.

Isolation of DNA. The bacteria tested were cultured in a liquid medium consisting of peptone (Difco), 10 g; yeast extract (Difco), 5 g; malt extract (Difco), 5 g; Casamino acids (Difco), 5 g; Tween 80, 0.05 g; distilled water 1,000 ml, and adjusted to pH 7.2, for 20 to 48 hr at 30° or 37° with shaking. Only for the poorly growing strains, 2 g of brain heart infusion (Nissui Seiyaku, Tokyo) was added to the medium mentioned above. Bacterial cells in logarithmic phase were harvested by centrifugation at a low temperature and washed twice with 0.15 M NaCl plus 0.1 M EDTA, pH 8.0. The procedure for isolation of DNA from lysozyme-sensitive strains were essentially the same as that described by MARMUR (5). Isolation of DNA from the bacterial cells that hardly lysed by lysozyme was considerably difficult by this procedure, or even by other treatments such as sonication, mechanical disruption, etc. Therefore, a new procedure for the isolation of DNA from such cells was developed by the present authors. Lysozyme-insensitive or poorly sensitive strains were cultured in the liquid medium mentioned above containing glycine in a final concentrations of 0.25, 0.5, 1.0, or 2.0%, respectively. Cells grown in each medium were harvested by centrifugation and treated with lysozyme. Generally, yield of the cells was reduced and they became more and more lysozyme sensitive with increasing of concentration of glycine. When the cells from the medium containing 2% glycine were still lysozyme-insensitive, the concentration of glycine might be up to 3% or higher. The most appropriate concentration of glycine was experimentally decided from cell yield and from lysozyme-sensitivity. The procedure for DNA preparation from lysozyme-insensitive strains treated with glycine was the same as that from lysozyme-sensitive strains.

Fig. 1. Detail of the cell holder compartment.

A thermosensor is thrust through a sliding cover, rubber cushion and quartz stopper, and inserted directly into DNA solution. The thermometer, rubber cushion, and quartz stopper are compactly cemented by epoxy-adhesive to avoid evaporation of the solution.

Determination of DNA base composition. DNA base composition was calculated from its thermal denaturation temperature (T_m) according to the procedure of MARMUR and DOTY (6). Modified thermophotometer of DELEY and SCHELL (7) was used. It consists of a Perkin-Elmer Spectrophotometer (Hitachi Ltd., Tokyo) equipped with thermospacers for circulating hot water or ethylene glycol. T_m in the standard saline-citrate solution was determined with a thermister (Shibaura Denshi, Tokyo) of which thermosensor was inserted directly in DNA solution as shown in Fig. 1. This modification was sufficient for measuring T_m value more exactly than in air by DELEY and SCHELL's apparatus. The thermister employed in this work had been calibrated with a reference thermometer authorized by the National Research Laboratory of Metrology of Japan.

RESULTS

Isolation of DNA

Sufficient amount of DNA to determine T_m was isolated from all the bacteria tested. Among them, DNAs of 60 strains were isolated by glycine treatment. The cells treated with glycine were still gram-positive and the cell forms were unchanged from normal cells under microscopic observation. The appropriate concentration of glycine was 0.25 to 4.0% as shown in Table 2,

Table 2. DNA base composition of coryneform bacteria.

Organism	T _m (°C)	GC content (mole %)	Maximum hyper- chromi- city ^a	Concen- tration of glycine (%)	Source
<i>Corynebacterium</i>					
<i>C. aquaticum</i> AJ 1413	97.7	69.3	1.33	0.5	ATCC ^c 14665
<i>C. bovis</i> AJ 1407	97.3	68.3	1.31	—	ATCC 7715
<i>C. bovis</i> AJ 1377	97.0	67.6	1.35	—	Sanraku Co.
<i>C. callunae</i> AJ 1518	90.3	51.2	1.37	0.5	NRRL ^d B-2244
<i>C. diphtheriae</i> AJ 1414	90.8	52.4	1.38	4.0	ATCC 11913
<i>C. equi</i> AJ 1372	96.4	66.1	1.34	0.5	NI ^e
<i>C. equi</i> AJ 1376	96.3	65.9	1.35	0.5	Sanraku Co.
<i>C. equi</i> AJ 1378	97.8	69.5	1.32	—	Sanraku Co.
<i>C. equi</i> AJ 1402	97.1	67.8	1.34	2.0	ATCC 6939
<i>C. equi</i> AJ 1403	96.8	67.1	1.26	1.0	ATCC 10146
<i>C. fascians</i> AJ 1373	95.1	62.9	1.37	2.0	NI
<i>C. fascians</i> AJ 1398	96.9	67.3	1.32	3.0	ATCC 12974
<i>C. flaccumfaciens</i> AJ 1400	97.3	68.3	1.26	—	ATCC 6887
<i>C. flaccumfaciens</i> subsp. <i>aurantiaca</i> AJ 1412	97.4	68.5	1.33	—	ATCC 12813
<i>C. herculis</i> AJ 1543	90.8	52.4	1.36	0.5	ATCC 13868
<i>C. hoagii</i> AJ 1374	96.2	65.6	1.30	—	NI
<i>C. hoagii</i> AJ 1408	95.8	64.6	1.20	—	ATCC 7005
<i>C. hydrocarboclastus</i> AJ 1379	94.4	61.2	1.36	3.0	IAM ^f 1399
<i>C. hydrocarboclastus</i> AJ 1386	94.3	61.0	1.35	2.0	IAM 1484
<i>C. insidiosum</i> AJ 1409	101.3	78.1	1.33	2.0	ATCC 10253
<i>C. lilium</i> AJ 1517	91.0	52.9	1.35	0.5	NRRL B-2243
<i>C. michiganense</i> AJ 1390	97.7	69.3	1.23	—	ATCC 492
<i>C. michiganense</i> AJ 1391	98.0	70.0	1.25	0.25	ATCC 4450
<i>C. michiganense</i> AJ 1392	98.1	70.2	1.31	—	ATCC 7429
<i>C. michiganense</i> AJ 1393	97.5	68.8	1.31	—	ATCC 7430
<i>C. michiganense</i> AJ 1394	98.3	70.7	1.32	0.25	ATCC 10202
<i>C. poinsettiae</i> AJ 1999	98.0	70.0	1.24	—	CCM ^g 1587
<i>C. sepedonicum</i> AJ 1406	97.9	69.8	1.27	—	ATCC 9850
<i>C. renale</i> AJ 1481	93.6	59.3	1.35	0.5	ATCC 10848
<i>C. xerosis</i> AJ 1375	96.9	67.3	1.33	0.5	ATCC 373
<i>C. xerosis</i> AJ 1395	93.5	59.0	1.35	2.5	ATCC 7094
<i>C. xerosis</i> AJ 1396	97.4	68.5	1.32	2.0	ATCC 7711
<i>Brevibacterium</i>					
<i>B. acetyllicum</i> AJ 1441	88.4	46.6	1.30	—	ATCC 953

Table 2. (continued)

Organism	T _m (°C)	GC content (mole %)	Maximum hyper- chromi- city ^a	Concen- tration of glycine (%) ^b	Source
<i>B. acetylicum</i> AJ 1442	88.5	46.8	1.38	—	ATCC 954
<i>B. albidum</i> AJ 1472	98.0	70.0	1.29	—	IAM 1631
<i>B. ammoniagenes</i> AJ 1443	91.5	54.1	1.36	3.0	ATCC 6871
<i>B. ammoniagenes</i> AJ 1444	91.7	54.6	1.38	3.0	ATCC 6872
<i>B. ammoniagenes</i> AJ 1483	91.3	53.7	1.37	1.5	IFM ^b AU-39
<i>B. citreum</i> AJ 1469	98.2	70.5	1.34	—	IAM 1514
<i>B. citreum</i> AJ 1471	98.2	70.5	1.37	—	IAM 1614
<i>B. divaricatum</i> AJ 1498	91.6	54.4	1.37	0.5	NRRL B-2312
<i>B. flavum</i> AJ 1510	91.5	54.1	1.35	0.5	Isolate (ATCC 14067)
<i>B. fulvum</i> AJ 1485	94.6	61.7	1.35	—	IFM A-34
<i>B. fuscum</i> AJ 1474	93.3	58.5	1.34	—	IFM AU-44
<i>B. fuscum</i> AJ 1486	94.2	60.7	1.35	—	Dr. Nakagawa (Osaka Univ.)
<i>B. fuscum</i> AJ 3124	93.2	58.3	1.31	—	CCEB 277
<i>B. helvolum</i> AJ 1445	95.6	64.1	1.29	0.5	ATCC 11822
<i>B. helvolum</i> AJ 1447	96.2	65.6	1.31	—	IAM 1478
<i>B. helvolum</i> AJ 1449	96.3	65.9	1.38	0.25	IAM 1498
<i>B. helvolum</i> AJ 1455	96.7	66.8	1.34	—	IAM 1391
<i>B. helvolum</i> AJ 1458	96.3	65.9	1.34	0.25	IAM 1434
<i>B. helvolum</i> AJ 1539	94.6	61.7	1.30	0.5	CCM 193
<i>B. imperiale</i> AJ 1446	97.9	69.8	1.44	0.5	ATCC 8365
<i>B. insectiphilum</i> AJ 1477	97.0	67.6	1.31	0.5	IFM AM-23
<i>B. lactofermentum</i> AJ 1511	90.9	52.7	1.36	0.5	Isolate (ATCC 13869)
<i>B. lactofermentum</i> AJ 1514	91.3	53.7	1.32	0.5	Isolate (ATCC 13655)
<i>B. linens</i> AJ 1473	97.0	67.6	1.32	1.0	IFM SC-84
<i>B. linens</i> AJ 1475	96.9	67.3	1.22	—	IFM AM-17
<i>B. linens</i> AJ 1484	96.7	66.8	1.32	—	IFM AM-27
<i>B. linens</i> AJ 1520	95.0	62.7	1.37	—	ATCC 8377
<i>B. linens</i> AJ 1521	95.3	63.4	1.31	—	ATCC 9172
<i>B. linens</i> AJ 1522	94.0	60.2	1.34	0.25	ATCC 9174
<i>B. linens</i> AJ 1523	94.0	60.2	1.32	1.0	ATCC 9175
<i>B. linens</i> AJ 1540	95.1	62.9	1.35	—	CCM 47
<i>B. luteum</i> AJ 1470	97.7	69.8	1.33	—	IAM 1623
<i>B. lipolyticum</i> AJ 1450	98.3	70.7	1.32	—	IAM 1398

Table 2. (continued)

Organism	T _m (°C)	GC content (mole %)	Maximum hyper- chromi- city ^a	Concen- tration of glycine (%) ^b	Source
<i>B. lipolyticum</i> AJ 1451	98.2	70.5	1.31	0.5	IAM 1413
<i>B. maris</i> AJ 1480	95.8	64.6	1.23	—	IFM S-30
<i>B. minutiferula</i> AJ 1482	97.3	68.3	1.34	0.5	IFM AU-27
<i>B. protophormiae</i> AJ 3125	95.2	63.2	1.23	0.25	CCEB 282
<i>B. pusillum</i> AJ 1462	97.6	69.0	1.30	—	IAM 1479
<i>B. pusillum</i> AJ 1463	97.7	69.3	1.32	0.25	IAM 1489
<i>B. saperdae</i> AJ 3126	97.4	68.5	1.28	—	CCEB 366
<i>B. stationis</i> AJ 1541	91.4	53.9	1.33	1.0	ATCC 14403
<i>B. sulfureum</i> AJ 1448	94.8	62.2	1.35	—	IAM 1488
<i>B. sulfureum</i> AJ 1478	95.7	64.4	1.34	—	IFM AU-38
<i>B. sulfureum</i> AJ 1481	96.3	65.9	1.33	—	IFM AU-28
<i>B. testaceum</i> AJ 1464	96.1	65.4	1.31	0.25	IAM 1537
<i>B. vitarumen</i> AJ 1525	95.7	64.4	1.29	—	ATCC 10234
<i>Arthrobacter</i>					
<i>A. atrocyaneus</i> AJ 1429	97.8	69.5	1.28	2.0	CCM 1645
<i>A. aureus</i> AJ 1430	94.5	61.5	1.24	2.0	CCM 1649
<i>A. citreus</i> AJ 1423	95.1	62.9	1.27	—	ATCC 11624
<i>A. citreus</i> AJ 1431	95.1	62.9	1.22	—	CCM 1647
<i>A. citreus</i> AJ 1435	95.1	62.9	1.36	—	Isolate
<i>A. citreus</i> AJ 1438	95.6	64.1	1.29	—	Isolate
<i>A. globiformis</i> AJ 1422	94.7	62.0	1.32	—	ATCC 8010
<i>A. nicotianae</i> AJ 1426	94.0	60.2	1.33	—	ATCC 15236
<i>A. oxydans</i> AJ 1425	95.7	64.4	1.27	—	ATCC 14359
<i>A. oxydans</i> AJ 1427	95.0	62.7	1.33	—	ATCC 14358
<i>A. pascens</i> AJ 1433	95.4	63.7	1.31	—	CCM 1653
<i>A. ramosus</i> AJ 1439	94.8	62.2	1.33	0.5	ATCC 13727
<i>A. simplex</i> AJ 1420	98.7	71.7	1.31	—	ATCC 6946
<i>A. tumescens</i> AJ 1424	97.9	69.8	1.30	—	ATCC 6947
<i>A. tumescens</i> AJ 1459	95.6	64.1	1.31	—	IAM 1447
<i>A. tumescens</i> AJ 1460	95.8	64.6	1.32	—	IAM 1458
<i>A. ureafaciens</i> AJ 1421	94.6	61.7	1.21	2.5	ATCC 7562
<i>A. ureafaciens</i> AJ 1436	94.7	62.0	1.35	1.0	IAM 1390
<i>A. variabilis</i> AJ 1434	98.0	70.0	1.29	—	CCM 1565
<i>Cellulomonas</i>					
<i>Cellul. biazotea</i> AJ 1569	98.9	72.2	1.26	0.25	ATCC 486
<i>Cellul. flavigena</i> AJ 1570	99.1	72.7	1.28	—	ATCC 482

Table 2. (continued)

Organism	T _m (°C)	GC content (mole %)	Maximum hyper- chromi- city ^a	Concen- tration of glycine (%) ^b	Source
<i>Cellul. gelida</i> AJ 1567	99.0	72.4	1.24	—	ATCC 488
<i>Cellul. fimi</i> AJ 1566	95.3	63.4	1.25	0.5	ATCC 8183 ⁱ
<i>Cellul. fimi</i> AJ 1571	98.7	71.7	1.30	0.25	ATCC 484
<i>Cellul. fimi</i> AJ 1572	98.8	72.0	1.30	0.25	ATCC 15724
<i>Cellul. uda</i> AJ 1568	98.8	72.0	1.24	—	ATCC 491
<i>Microbacterium</i>					
<i>Microb. flavum</i> AJ 1415	93.2	58.3	1.36	2.0	ATCC 10340
<i>Microb. lacticum</i> AJ 1416	97.7	69.3	1.33	0.25	ATCC 8180
<i>Micrococcus</i>					
<i>M. glutamicus</i> AJ 1502	91.2	53.4	1.33	0.5	ATCC 13032
Unidentified coryneform bacteria					
AJ 1488	94.9	62.4	1.33	1.0	Dr. Yano (Univ. of Tokyo)
AJ 1490	91.5	54.1	1.35	0.5	Isolate
AJ 1548	91.4	53.9	1.36	0.5	Isolate
AJ 1549	91.2	53.4	1.37	0.5	Isolate
AJ 1562	90.3	51.2	1.36	0.5	Isolate
AJ 1958	91.3	53.7	1.38	0.5	Isolate

^a Max. absorbance at 260 m μ /min. absorbance at 260 m μ .

^b Concentration of glycine added in medium for isolation of DNA; (—) indicates no glycine treatment.

^c ATCC: American Type Culture Collection, Rockville, Maryland, U.S.A.

^d NRRL: Northern Utilization Research and Development Division, Peoria, Illinois, U.S.A.

^e NI: Nagao Institute, Tokyo, Japan.

^f IAM: The Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan.

^g CCM: Czechoslovak Collection of Microorganisms, J.E. Purkyne University, Brno, Czechoslovakia.

^h IFM: Institute of Food Microbiology, Chiba University, Chiba, Japan.

ⁱ CCEB: Culture Collection of Entomogenous Bacteria, Department of Pathology, Institute of Entomology, Prague, Czechoslovakia.

and the highest concentration was in the case of *Corynebacterium diphtheriae* ATCC 11913. From lysozyme-insensitive cells, DNAs were also isolated by penicillin treatment, but lysis of the cells took place less satisfactorily than that by glycine treatment. Lysozyme-insensitive cells grown in the liquid medium for 24 to 48 hr at 30 or 37° were harvested by centrifugation and

Table 3. T_m and GC content of DNA obtained by various treatments.

Organism	T _m , GC content and hyperchromicity of DNA obtained by									
	Glycine treatment ^a				Penicillin treatment ^a			Mechanical disruption ^b		
	T _m (°C)	GC (%)	Hyper- chro- micity	Concn. of ^c glycine	T _m (°C)	GC (%)	Hyper- chro- micity	T _m (°C)	GC (%)	Hyper- chro- micity
<i>C. xerosis</i> AJ 1375	96.9	67.3	1.33	0.5	96.5	66.3	1.27	97.0	69.6	1.27
<i>B. ammoniagenes</i> AJ 1443	91.5	54.1	1.36	3.0	91.4	53.9	1.22	91.5	54.1	1.20
<i>B. flavum</i> AJ 1510	91.5	54.1	1.35	0.5	91.8	54.9	1.28	— ^d	—	—
<i>A. globiformis</i> AJ 1422	94.7	62.0	1.32	0	—	—	—	95.3	63.4	1.28

^a See the text.

^b DNA from cells disrupted with glass beads by Mechanical Cell Homogenizer (Braun, Melsungen, Germany).

^c Concentration of glycine added in medium for isolation of DNA.

^d Not tested.

then suspended heavily (10 g wet cells/1000 ml medium) in the same fresh medium. Penicillin G was added into this suspension to give the final concentration of 100 units/ml. After shaking for 3 or 5 hr at 30°, cells in the suspension were harvested by centrifugation. In some cases, the lysozyme-insensitive cells were changed by penicillin treatment to lysozyme-sensitive cells. The method for isolation of DNA was the same as in the case of glycine treatment. Distinct difference was not found among values of GC content of the same strain obtained by glycine treatment, penicillin treatment and mechanical disruption as shown in Table 3. The DNAs giving different values of hyperchromicity actually did not differ in their T_m as shown in Table 3. The same results were reported in the determination of T_m of yeast DNA by NAKASE and KOMAGATA (8).

DNA Base Composition of Coryneform Bacteria

The DNA base composition of the coryneform bacteria ranged from 46 to 78% as summarized in Table 2. The genera of *Corynebacterium*, *Arthrobacter*, *Microbacterium*, and *Brevibacterium* did not exhibit the specific range of GC content to discriminate the genus. However, the GC content of *Cellulomonas* fell into a relatively narrow range, except *Cellulomonas fimi* ATCC 8183, as shown in Table 2 and Fig. 2. From these facts, it seemed that the genera of the coryneform bacteria except *Cellulomonas* were taxonomically heterogeneous. Especially, *Brevibacterium* showed the widest diversity. GC contents of two strains of *Microbacterium* differed considerably from each

Fig. 2. Distribution of GC content of coryneform bacteria.

other. The GC content of *Arthrobacter* species was distributed in a relatively wide range from 60 to 72%. The GC contents of the type strains of *Brevibacterium acetylicum* ATCC 953 and 954 (9) were 46.6 and 46.8%, respectively. These values differed from those of the coryneform bacteria and agreed with that of the genus *Bacillus* (3, 10). Furthermore, cells of these two strains were long rods and showed long chains, and the mode of cell division of these strains was the simple type which differs from those of the coryneform bacteria (1). From these facts, these strains were regarded as asporogenous strains of *Bacillus*. The GC content of the so-called glutamic acid-producing bacteria such as *Brevibacterium divaricatum* NRRL B-2312, *Brevibacterium flavum* ATCC 14067, *Brevibacterium lactofermentum* ATCC 13869 and ATCC 13655, *Corynebacterium callunae* NRRL B-2244, *Corynebacterium herculis* ATCC 13868, *Corynebacterium lilium* NRRL B-2243, and *M. glutamicus* ATCC 13032 fell into a narrow range from 51.2 to 54.4%. This fact probably shows that these bacteria are closely related to one another.

DISCUSSION

As shown in Fig. 2, the strains belonging to each genus did not show a specific range of GC content. On the other hand, the strains having almost the same GC content were not discriminated owing to their taxonomic versatility in morphological, physiological, and biochemical characteristics. The genera included in the category of the coryneform bacteria except *Cellulomonas* seemed to be heterogeneous, and each genus could not be defined only

on the basis of GC content. The genus *Corynebacterium* includes animal and plant habitants according to Bergey's Manual (11) and the GC content of this genus ranged widely as illustrated in Fig. 2. The heterogeneity of the genus *Brevibacterium* that SKERMAN (12) pointed out was also demonstrated on the basis of the wide range of GC content as shown in Fig. 2. Further studies from other viewpoints are required to establish the clear generic concepts of this group of bacteria.

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