Heterogeneity of Strains Assigned to *Gluconobacter frateurii*
Mason and Claus 1989 Based on Restriction Analysis of 16S-23S rDNA Internal Transcribed Spacer Regions

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Twenty-three strains, which were assigned to *Gluconobacter frateurii* and maintained at Culture Collection NBRC, were re-identified at the species level on the basis of restriction analysis of 16S-23S rDNA ITS regions by digestion with six restriction endonucleases: Bsp1286I, MboII, AvaII, TaqI, BsoBI, and BstNI. The strains examined were divided into six groups, Group III-1, Group III-2, Group III-3, Group III-4, Group III-5, and Group IV. Group III-1 and Group III-4 respectively were divided into two subgroups, Subgroup III-1a, Subgroup III-1b and Subgroup III-4a, Subgroup III-4b. *Gluconobacter frateurii* NBRC 3264T was included in Group III-2, along with strains NBRC 3265 and NBRC 3270, and *G. thailandicus* BCC 14116T was included in Group III-3, along with strains NBRC 3254, NBRC 3256, NBRC 3258, NBRC 3255, and NBRC 3257. These groupings were supported by a phylogenetic tree based on 16S-23S rDNA ITS sequences. Strains of group III-2 and Group IV were unequivocally re-identified as *G. frateurii*, but strains of Group III-3, Group III-4, and Group III-5 were not necessarily re-identified as *G. frateurii*. The results obtained indicate that the 23 strains have a taxonomically heterogeneous nature, and they are referred to as the *G. frateurii* complex.

Key words: 16S-23S rDNA ITS; acetic acid bacteria; *Gluconobacter frateurii*; restriction analysis; taxonomy

In the genus *Gluconobacter* Asai 1935, 689AL, three species were once recognized:1–5 *Gluconobacter oxydans* (Henneberg 1897) De Ley 1961, 47AL (the type species), *Gluconobacter cerinus* Yamada and Akita, 1984, 503VP,1,6 and *Gluconobacter frateurii* Mason and Claus 1989, 182VP,2) Recently, the additional two species, *Gluconobacter albidus* (ex Kondo and Ameyama 1958) Yukphan et al. 2005, 983VP and *Gluconobacter thailandicus* Tanasupawat et al. 2005, 983VP were described.2–10) Of the two new species, *G. albidus* was located phylogenetically in the sublineage of *G. oxydans/G. albidus*,3) but *G. thailandicus* was located in the sublineage of *G. cerinus/G. frateurii/G. thailandicus*7,8) and related especially to *G. frateurii* phenotypically and molecular-biologically.9)

For species-level identification and classification of *Gluconobacter* strains, phenotypic characteristics such as acid production from sugars and sugar alcohols and assimilation of carbon compounds are generally utilized,1–5,7,9,11) but data obtained by phenotypic characterization are sometimes unreliable. On the other hand, DNA–DNA hybridization is required as a decisive criterion for the species-level classification and identification, but this technique is laborious and it is impossible to construct databases.

In previous papers,7,12,13) we reported that restriction analysis of 16S-23S rDNA ITS regions is applicable to species-level identification and classification of *Gluco-
Among a total of 40 strains, 23 strains were identified as G. frateurii, which in fact accounted for 58% of all the strains examined, as contrasted with only 10% for the four strains that were identified as G. cerinus. In addition, the 23 strains were supposed to have a taxonomically heterogeneous nature, since a representative strain, NBRC 3271 had a relatively long branch in a phylogenetic tree based on 16S-23S rDNA ITS sequences.

This paper describes the heterogeneity of strains assigned to G. frateurii in the group of lower DNA G+C contents or in the sublineage of G. cerinus/G. frateurii/G. thailandicus of the lineage of the genus Gluconobacter on the basis of restriction analysis.

Materials and Methods

Bacterial strains. Twenty-three strains, which were once identified as G. frateurii and maintained at Culture Collection NBRC, Kisarazu, Chiba, Japan, were examined in this study (Table 1). Gluconobacter frateurii NBRC 3264T and G. thailandicus BCC 14116T were used as reference strains.

PCR amplification of 16S-23S rDNA ITS regions and digestion with restriction endonucleases. PCR amplification of 16S-23S rDNA ITS regions for digestion with restriction endonucleases was performed by a modification of the method of Trček and Teuber. The two primers used were

$$5'$$-TGCGG(C/T)TGGATCCTCCT-3' (positions 1522–1540 on 16S rDNA by the Escherichia coli numbering system) and 5'-TGCGT(T/A)AGGCATCCACCG-3' (positions 38–22 on 23S rDNA). Single-banded and purified PCR products (about 715 bases) were digested separately with the following six restriction endonucleases: Bsp1286I (New

**Sequencing of 16S-23S rDNA ITS regions and sequence and restriction analyses.** Direct sequencing of the single-banded and purified PCR products obtained above (about 715 bases, from position 1 in the specified *G. oxydans* numbering system) was carried out as described previously. Except for the primers mentioned above, two additional primers were used for sequencing: 1 Tαlαf (5'-AGAGCACCTGC-TTG-GCA-3'), positions 285–302 on 16S-23S rDNA by the *Gluconacetobacter hansenii* numbering system (14) and Tαlαr (5'-ACCCCTGCTTGGAAA-3', positions 311–296 on 16S-23S rDNA). Alignment of the determined sequences was done with the program CLUSTAL X (version 1.81). 16) Distance matrices for the aligned sequences were calculated by the two-parameter method (version 1.81). 16) A computerized restriction analysis of 16S-23S rDNA ITS regions was made by means of the program NEBcutter (version 2.0, New England BioLabs). 12,13)

**Base sequence deposition numbers.** All the base sequences determined were deposited in the DDBJ databases. The base sequences of the 16S-23S rDNA ITS regions were filed under accession nos. AB206582 for strain NBRC 3254, AB206583 for strain NBRC 3255, AB206584 for strain NBRC 3260, AB206585 for strain NBRC 3263, AB206586 for strain NBRC 3268, AB206587 for strain NBRC 3269, and AB206588 for strain NBRC 3289.

**Results and Discussion**

The 23 strains examined were grouped into six groups, Group III-1, Group III-2, Group III-3, Group III-4, Group III-5, and Group IV, by digestion with the following six restriction endonucleases: *BspI*286I, *MboII*, *AvaiI*, *TaqI*, *BsoBI*, and *BstNI* (Table 1). Additionally, Group III-I and Group III-4 respectively were divided into two subgroups, Subgroup III-1a, Subgroup III-1b and Subgroup III-4a, Subgroup III-4b.

At the first step, including *BspI*286I digestion, all 23 strains showed six bands comprising about 244, 125, 121, 92, 82, and 51 bp (data not shown), designated the *G. frateurii* pattern (13) (Table 1), and were separated from strains of *G. oxydans*, *G. albidus*, and *G. cerinus* without any exception, along with *G. frateurii* NBRC 3264T and *G. thailandicus* BCC 14116T. 7,9,12,13)

*BspI*286I digestion was therefore one of the most effective molecular-biological methods to separate strains to be assigned to the two species, *G. frateurii* and *G. thailandicus* from strains of *G. oxydans*, *G. albidus*, and *G. cerinus*. 7,9,12,13)

At the second step, including *MboII* digestion, the strains that had an identical restriction pattern (data not shown), designated the *G. cerinus* pattern (13) with *G. cerinus* NBRC 3267T, which showed three bands comprising about 356, 186, and 171 bp, were found and grouped into Group IV (Table 1). 15)

At the third step, including *AvaiI* digestion, the 23 strains were divided into two groups (Fig. 1), as reported by Tanasupawat et al. 9) One was composed of strains accommodated in Group III-2, Group III-4, and Group IV, all of which formed two bands comprising about 610 and 105 bp (Table 2), and whose patterns were designated restriction pattern a (Table 1). The other was composed of strains accommodated in Group III-1, Group III-3, and Group III-5, all of which formed a single band comprising about 715 bp without any digestion, and whose patterns were designated restriction pattern b. *G. thailandicus* NBRC 3264T and *G. thailandicus* BCC 14116T were discriminated from each other at this step: the former was accommodated in Group III-2, and the latter was accommodated in Group III-3. The two groups were practically differentiated from each other by the presence or absence of two restriction fragments of about 610 and 105 bp.

At the fourth step, including *TaqI* digestion, the 23 strains were divided into two groups (Fig. 1). One was composed of strains accommodated in Group III-1, Group III-2, Group III-3, and Group IV, all of which formed three bands comprising about 399, 287, and 29 bp (Table 2), and whose patterns were designated restriction pattern c (Table 1). The other was composed of strains accommodated in Group III-4 and Group III-5, all of which formed two bands comprising about 427 and 287 bp, and whose patterns were designated restriction pattern d. The two groups were practically differentiated from each other by the presence or absence of a small restriction fragment of 29 bp.

At the fifth step, including *BsoBI* digestion, the 23 strains were divided into two groups (Fig. 1). One was composed of strains accommodated in Subgroup III-1a, Group III-2, Group III-4, Group III-5, and Group IV, all of which formed four bands comprising about 343, 147, 131, and 94 bp (Table 2), and whose patterns were designated restriction pattern e (Table 1). The other was composed of strains accommodated in Subgroup III-1b and Group III-3, all of which formed three bands comprising about 343, 224, and 147 bp, and whose patterns were designated restriction pattern f. Group III-I was divided into the two subgroups, Subgroup III-1a and Subgroup III-1b. The two groups were practically differentiated from each other by the presence or absence of a restriction fragment of about 224 bp.

At the sixth step, including *BstNI* digestion, the 23
strains were divided into three groups (Fig. 1). The first was composed of strains accommodated in Subgroup III-1a, Group III-2, Group III-3, Subgroup III-4b, and Group IV (Table 1), all of which formed five bands comprising about 383, 116, 111, 98, and 7 bp (Table 2), and whose patterns were designated restriction pattern $h$ (Table 1). The second was composed of strains accommodated in Subgroup III-1b, which formed six bands comprising about 382, 116, 111, 81, 17, and 7 bp, and whose patterns were designated restriction pattern $g$.

The third was composed of strains accommodated in Subgroup III-4a and Group III-5, which formed four bands comprising about 498, 111, 98, and 7 bp, whose patterns were designated restriction pattern $i$. The three groups were practically differentiated from one another by the presence or absence of a restriction fragment of either about 498 or 81 bp. To sum up, all 23 strains examined were divided into six groups and four subgroups, along with $G. frateurii$ NBRC 3264$^T$ (Group III-2), $G. thailandicus$ BCC 14116$^T$ (Group III-3), $G. frateurii$ NBRC 3251 (Group IV), NBRC 3268 (Subgroup III-1a), NBRC 3289 (Group III-1b), $G. frateurii$ NBRC 3265 (Group III-2), NBRC 3254 (Group III-3), NBRC 3256 (Group III-3), NBRC 3260 (Subgroup III-4a), NBRC 3269 (Subgroup III-4b), NBRC 3271 (Group III-5), NBRC 3263 (Group III-5), M, 50-bp DNA marker.

The 16S-23S rDNA ITS regions of representative strains from all the groups and subgroups mentioned above were sequenced by means of the above-mentioned PCR products (about 715 bases, from position 1 on the 16S-23S rDNA ITS regions). The calculated positions were based on the specified $G. oxydans$ numbering system.$^{12)}$ The resulting restriction fragments and the number of restriction sites of the 16S-23S rDNA ITS PCR products formed by digestion with the six restriction endonucleases were identical in their sizes and numbers with the theoretical fragments and the number of restriction sites calculated by use of the program NEBcutter (Table 2).

A phylogenetic tree based on 16S-23S rDNA ITS sequences was constructed for 10 representative strains assigned to $G. frateurii$. It is to be noticed that the clusters and the subclusters in the phylogenetic tree coincided precisely with the groups and the subgroups based on the restriction patterns by digestion with the six restriction endonucleases (Fig. 2). $Gluconobacter frateurii$ NBRC 3264$^T$ constituted the first large cluster, together with strain NBRC 3265 of Group III-2, strain NBRC 3251 of Group IV, and strain NBRC 3268 of Subgroup III-1a. In contrast, $G. thailandicus$ BCC 14116$^T$ constituted the second large cluster, which included strains NBRC 3254 and NBRC 3255 of Group III-3 and strain NBRC 3289 of Subgroup III-1b. Strains NBRC 3271 and NBRC 3263 of Group III-5, strain NBRC 3269 of Subgroup III-4b, and strain NBRC 3260 of Subgroup III-4a constituted the third large cluster.

Fig. 1. Restriction of 16S-23S rDNA ITS Region PCR Products by Digestion with Restriction Endonucleases AvaII, TaqI, BsoBI, and BstNI in Strains Assigned to $Gluconobacter frateurii$.

For estimation of digestion fragments, 50-bp DNA markers were used in agarose gel electrophoresis. (a) Restriction patterns by digestion with restriction endonuclease AvaII. (b) Restriction patterns by digestion with restriction endonuclease TaqI. (c) Restriction patterns by digestion with restriction endonuclease BsoBI. (d) Restriction patterns by digestion with restriction endonuclease BstNI. Abbreviations: 1, $G. frateurii$ NBRC 3264 (Group III-2); 2, $G. thailandicus$ BCC 14116 (Group III-3); 3, $G. frateurii$ NBRC 3251 (Group IV); 4, NBRC 3268 (Subgroup III-1a); 5, NBRC 3289 (Group III-1b); 6, $G. frateurii$ NBRC 3265 (Group III-2); 7, NBRC 3254 (Group III-3); 8, NBRC 3256 (Group III-3); 9, NBRC 3260 (Subgroup III-4a); 10, NBRC 3269 (Subgroup III-4b); 11, NBRC 3271 (Group III-5); 12, NBRC 3263 (Group III-5); M, 50-bp DNA marker.
and their phylogenetic distance was relatively far from either G. frateurii NBRC 3264\textsuperscript{a} or G. thailandicus BCC 14116\textsuperscript{b}.

As described above, restriction analysis of 16S-23S rDNA ITS regions divided strains assigned to G. frateurii into six groups and four subgroups by digestion with six restriction endonucleases. This indicates that the combination of several restriction endonucleases can be widely utilized for classifying and identifying microorganisms at the species level. When restriction analysis is combined with sequence analysis in the 16S-23S rDNA ITS regions, some of the most valuable taxonomic data will be gained; the former will give "digitalized" phylogenetic information such as the grouping and the subgrouping that are supposed to represent the species, and the latter will evaluate phylogenetic relationships, which are "analogic" in determining the taxonomic circumscription of the Gluconobacter strains concerned.

Yamada and Akita\textsuperscript{1} divided strains of their Group II or the group of lower DNA G + C contents into three subgroups on the basis of calculated similarity values in electrophoretic enzyme patterns. Their first subgroup contained G. cerinus IFO 3267\textsuperscript{a}, and their second subgroup contained G. frateurii IFO 3264\textsuperscript{a}. It is interesting that their third subgroup was composed of strains (for example, strains IFO 3254 and IFO 3255) of Group III-3, which contained G. thailandicus BCC 14116\textsuperscript{b}, and strains (for example, strains IFO 3289 and IFO 3172) of Subgroup III-1b.

Katsura \textit{et al.}\textsuperscript{5} and Tanaka \textit{et al.}\textsuperscript{4} reported that strain IFO 3265 (= NBRC 3265 of Group III-2) gave very high DNA–DNA similarities respectively of 97 and 100% to G. frateurii IFO 3264\textsuperscript{a} (= NBRC 3264\textsuperscript{a} of Group III-2). In addition, Tanaka \textit{et al.}\textsuperscript{4} showed that strains IFO 3251, IFO 3286, and IFO 3262 of Group IV had high DNA–DNA similarities respectively of 81, 86, and 87% to G. frateurii IFO 3264\textsuperscript{a} (= NBRC 3264\textsuperscript{a} of Group III-2). The strains of Group III-2 and Group IV were therefore unequivocally re-identified as G. frateurii.

According to Tanaka \textit{et al.}\textsuperscript{4} strains IFO 3254 (= NBRC 3254 of Group III-3, in which G. thailandicus BCC 14116\textsuperscript{b} was contained), IFO 3260 (= NBRC 3260

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<th>Table 2. Six Restriction Endonucleases Discriminating Strains Assigned to \textit{Gluconobacter frateurii}</th>
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---, no restriction fragment.

\textsuperscript{a}The type strain of G. frateurii; \textsuperscript{b}the type strain of G. thailandicus.
of Subgroup III-4a), IFO 3269 (=NBRC 3269 of Subgroup III-4b), IFO 3271 (=NBRC 3271 of Group III-5), and IFO 3263 (=NBRC 3263 of Group III-5) showed relatively low DNA–DNA similarities of 45–66% to \(G. \text{frateurii} \) IFO 3264\(^T\). Tanasupawat et al.\(^9\) calculated 38% DNA–DNA similarity of \(G. \text{thailandicus} \) BCC14116\(^T\), which was grouped into Group III-3 along with strains NBRC 3254, NBRC 3256, and NBRC 3258 (Table 1), to \(G. \text{frateurii} \) NBRC 3264\(^T\), although unfortunately they did not make DNA–DNA hybridization between members of Group III-3 such as strains NBRC 3254, NBRC 3256, and NBRC 3258 (Table 1), to \(G. \text{frateurii} \) NBRC 3264\(^T\), although unfortunately they did not make DNA–DNA hybridization between members of Group III-3 such as strains NBRC 3254, NBRC 3256, and NBRC 3258 and \(G. \text{thailandicus} \) BCC14116\(^T\). These data indicate that strains of Group III-3, Group III-4, and Group III-5 are not necessarily to be classified into \(G. \text{frateurii} \).

As discussed above, it is obvious that the 23 strains examined in this study have a taxonomically heterogeneous nature, and they are referred to as the \(G. \text{frateurii} \) complex.

On considering grouping and clustering based on 16S-23S rDNA ITS sequences, the restriction analysis described above can be utilized for identifying and classifying \(Gluconobacter \) strains at the species level.\(^7,9,12,13\) Whether the strain of Subgroup III-1a of the first large cluster is identified as \(G. \text{frateurii} \), whether the strains of Group III-3 and Subgroup III-1b in the second large cluster are identified as \(G. \text{thailandicus} \), and whether the strains of Group III-4 and Group III-5 of the third large cluster are classified into a taxon separate from either the species of \(G. \text{frateurii} \) comprised of Group III-3, will be discussed elsewhere.

### Acknowledgment

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