Identification of *Clostridium butyricum* and *Clostridium beijerinckii* by Gas–Liquid Chromatography and Sugar Fermentation: Correlation with DNA Homologies and Electrophoretic Patterns

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Sixty-five strains of clostridia of the butyricum group were studied by DNA–DNA hybridization, electrophoresis of cell proteins, gas–liquid chromatography, and fermentation of glycerol, inositol and ribose. The DNA–DNA hybridization results confirmed that strains of this group belong to two main species, *Clostridium butyricum* and *C. beijerinckii*. Five strains did not hybridize with the reference strains of these two species. Most of the strains could be identified by quantitative gas–liquid chromatographic analysis combined with fermentation patterns. The other strains could be identified by their protein electrophoretic patterns.

INTRODUCTION

The butyric acid-producing strains of the genus *Clostridium* (hereafter referred to as the butyricum group) are difficult to identify at the species level, since they are phenotypically very similar. However, Cummins & Johnson (1971) reported that clostridia of the butyricum group fall mainly into two DNA homology groups, corresponding to two species: *Clostridium butyricum* (homology group I) and *Clostridium beijerinckii* (homology group II). These authors showed that the two species can also be differentiated by their nutritional requirements. In a recent study of β -lactamase-producing strains of the butyricum group (Magot, 1981*a, b*), we were unable to identify our strains using the semi-synthetic media described by Cummins & Johnson (1971).

Electrophoretic separation of cell proteins was shown recently to be a useful technique for identification of clostridia (Cato *et al.*, 1982), but polyacrylamide gel electrophoresis (PAGE) is not a routine technique in anaerobe laboratories. This lack of a means of performing routine identification of clostridia of the butyricum group led us to investigate the possibility of using gas–liquid chromatography (GLC), a widely available technique in anaerobe diagnostic laboratories.

Preliminary experiments suggested that quantitative GLC might be useful in achieving complete identification of bacteria of this group, especially when GLC results are combined with the fermentation patterns of glycerol, inositol and ribose. DNA–DNA hybridization, electrophoresis of cell proteins, GLC, and fermentation patterns were used to characterize 65 strains of the butyricum group, and the results are reported in the present paper.

METHODS

Bacterial strains. The 65 strains included in this study are listed in Table 1, together with their origins and some of their characteristics. These strains were assigned to the butyricum group on the basis of the following criteria, determined according to Sebald & Bréfort (1977): subterminal spore-forming strictly anaerobic rods; Gram-positive; motile; showing no lecithinase, lipase or protease activities; fermenting fructose, glucose, lactose, maltose, mannose and sucrose with production of gas; coagulating milk; hydrolysing aesculin; and producing acetic and butyric acids as major fermentation end-products in TYG broth. These strains were non-pathogenic to mice by intraperitoneal injection.

Table 1. *Designation, CF group, gel type and origin of organisms*

No.	CF group* Gel type†	Source (Reference)	No.	CF group* Gel type†	Source (Reference)
	Hybridization group I (<i>C. butyricum</i>):			Hybridization group I (<i>C. butyricum</i>)—cont.	
VPI 1536	A1 α	Virginia Polytechnic Institute (Cummins & Johnson, 1971)	71351	A1 α	H. Beerens' collection, Lille, France
VPI 1622	A1 α		3027	A1 α	
VPI 1718	A1 α		4P1	A1 α	Wetwood sample (Schink <i>et al.</i> , 1981)
VPI 2417	A1 α		CH2	A1 α	
VPI 2969	A1 α		Meb 125	A3 α	A. R. Prévot's collection, Paris, France
VPI 3266	A1 α				
CB 48	A1 α	Newborn faeces (Magot, 1981 <i>a</i>)	VPI 2408	B3 β	Hybridization group II (<i>C. beijerinckii</i>)
CB 49	A1 α		VPI 2681	B2 β	
CB 267	A1 α		VPI 2793	B3 β	
CB 269	A1 α		VPI 2965	A2 β	
CB 309	A1 α	Newborn faeces (this paper)	VPI 2966	B3 β	
CB 483	A1 α		VPI 2980	B2 β	
CB 756	A1 α		VPI 2983	B2 β	
CB 1001	A1 α		VPI 4420	A2 β	
CB 1002	A1 α		VPI 5481	B2 β	
CB 1003	A1 α		CB 90	B3 β	
CB 1004	A1 α		VA 4	B2 β	
CB 1005	A1 α		7423	B2 β	
CB 1020	A1 α		CNRZ 528	A2 β	
CB 21282	A1 α	Buttock abscess (this paper)	CNRZ 530	A3 β	J. L. Bergère: CNRZ, Jouy en Josas, France (Rousseau <i>et al.</i> , 1971)
CB 24681	A1 α		CNRZ 531	B2 β	
CB 27881	A1 α	Blood culture (this paper)	CNRZ 555	B3 β	Dairy products: J. L. Bergère, CNRZ, Jouy en Josas, France (this paper)
CB 108381	A1 α		CNRZ 529	A2 β	
CM 263	A1 α	Blood culture (Magot, 1981 <i>a</i>)	CNRZ 533	A2 β	
CM 474	A1 α		CNRZ 653	A3 β	
128	A1 α	Infected placenta (Magot, 1981 <i>a</i>)	CNRZ 654	B3 β	Hybridization group III
133/1	A1 α				
140/1	A1 α				
141/2	A1 α		1104	A3 γ	
143/1	A1 α		1106	A3 γ	
144/1	A1 α		103282	A3 γ	
144/3	A1 α		105082	A3 γ	
145/1	A1 α		105282	A3 γ	
147/2	A1 α				
153/2	A1 α				
		Newborn faeces: P. Raibaud, CNRZ, Jouy en Josas, France (this paper)			

* See text.

† See Fig. 2 and text.

Growth. Media and methods of culture were as described by Magot (1981 *b*). TYG broth was supplemented with 100 mM-sodium acetate for TYGA broth (pH 7.2). SI broth of Sebald & Costilow (1975), where sodium thioglycolate was replaced by 0.1% (w/v) cysteine was supplemented with 0.5% (w/v) glucose and 100 mM-sodium acetate for SIGA broth (pH 7.0).

Sugar fermentation. Sugar fermentations were performed in TY broth (g l⁻¹): trypticase, 30; yeast extract, 20; cysteine, 1 (pH 7.2), supplemented with 1% (w/v) carbohydrate. Inoculum was 2% (v/v) of an overnight culture in TYG. The pH was measured after 18 h incubation at 35 °C. Fermentation was considered to be positive if a fall of 1 pH unit or more was observed, using as control a TY broth without sugar inoculated and incubated under the same conditions.

DNA-DNA hybridization. Previously described procedures were used to extract and purify unlabelled DNAs, to shear unlabelled DNAs, and to label DNA with tritium-labelled nucleotides (Grimont *et al.*, 1980). Labelled DNAs were from strains VPI 3266 and VPI 2983, the reference strains of *C. butyricum* and *C. beijerinckii* respectively (Cummins & Johnson, 1971).

In the hybridization experiments, the S1 nuclease method (Crosa *et al.*, 1973) was used. The DNA duplexes remaining after S1 nuclease treatment for 30 min at 45 °C were separated from free nucleotides by using diethylaminoethyl-cellulose filters (DE-81 filters; Whatman) (Popoff & Coynault, 1980). These DNA-relatedness studies were performed at 56 °C (Cummins & Johnson, 1971). The degree of polynucleotide sequence homology was calculated by determining the ratio between the average counts in the S1-treated and untreated samples. Results, expressed as relative binding ratios (Brenner *et al.*, 1976) were normalized to the homologous reaction after subtracting the percentage of S1-resistant material in the control tube (containing only denatured radioactive DNA).

PAGE of cell proteins. The phenol extraction procedure for cell proteins was derived from that of Westphal & Jann (1965). An 18 h culture in 30 ml TYG broth was washed in distilled water and resuspended in Tris buffer (50 mM-Tris, pH 8.0; 1 ml Tris buffer for 100 mg cells, wet weight). An equal volume of hot (60 °C) phenol was added and the mixture was incubated for 30 min at 60 °C in a water bath, and for 30 min more at room temperature. After centrifugation for 20 min at 4500 g, the phenol phase was extracted and then dialysed for 3 to 5 d against Tris buffer/25% (v/v) glycerol at 4 °C. After a tenfold concentration against 10% (w/v) polyethylene glycol 4000 in Tris buffer, the mixture was cleared by centrifugation, and one volume of a solution containing 6 mM-Tris, 2% (w/v) SDS, 0.004% bromophenol blue and 0.025% β -mercaptoethanol, pH 7.0, was added to the supernatant. Samples were heated for 5 min at 100 °C in a water bath, and electrophoresed in a 0.1% SDS polyacrylamide gel following the method of Laemmli (1970).

GLC. For GLC analysis, cells were grown in TYGA or SIGA broth for 3 d at 35 °C. After centrifugation, 80 μ mol isobutyric acid (internal standard), 0.4 ml 50% (v/v) H₂SO₄ and 4 ml diethyl ether were added to 4 ml of supernatant. After mixing, 1 μ l of the ether phase was injected into an Intersmat model IGC 131 gas chromatograph equipped with a flame-ionization detector and connected to a model ICR 1B integrator. A glass column (1.8 m $\times$ 6 mm) packed with 5% Resoflex LAC 1R 296 on acid-washed Chromosorb G DMCS (60–80 mesh) was used. The operating conditions were: injector temperature, 200 °C; detector temperature, 250 °C; oven temperature, 125 °C; carrier gas (nitrogen) flow rate, 30 ml min⁻¹; attenuation, 1; sensitivity, 10⁻⁹ A. Typical chromatograms of *C. butyricum* VPI 3266 and *C. beijerinckii* VPI 5481 grown in SIGA broth are shown in Fig. 1.

RESULTS

DNA-DNA hybridization

Results of DNA-DNA hybridization experiments are summarized in Table 2. The S1 nuclease-resistant core (in the incubated control tubes containing only labelled DNA) was calculated to be $1.33 \pm 0.30\%$ (mean $\pm$ s.d.). The 65 strains fell into three hybridization groups. The 40 strains of hybridization group I were 56 to 114% related to strain VPI 3266 (the reference strain of *C. butyricum*). The relative binding ratios between all other strains and strain VPI 3266 did not exceed 13%. The 20 strains of hybridization group II were 58 to 92% related to strain VPI 2983 (the reference strain of *C. beijerinckii*). The relative binding ratios between all other strains and VPI 2983 did not exceed 9%. None of the five strains (*Clostridium* spp. of the butyricum group) included in hybridization group III was more than 13% related to the reference strains of *C. butyricum* or *C. beijerinckii*.

PAGE patterns

Four PAGE patterns were obtained with phenol-extracted proteins of the butyricum group strains after SDS-PAGE (Fig. 2, Table 1). These patterns can be correlated with the DNA

Fig. 1. Typical GLC chromatograms in SIGA broth of (a) *C. beijerinckii* VPI 5481 and (b) *C. butyricum* VPI 3266. A, acetic acid; iB, isobutyric acid (internal standard); B, butyric acid. The arrows indicate the injection of the sample.

Table 2. Hybridization of DNA from the 65 strains with DNAs from *C. butyricum* VPI 3266 and *C. beijerinckii* VPI 2983

Hybridization group	Relative binding ratio (%) with labelled DNA from:			
	<i>C. butyricum</i> VPI 3266		<i>C. beijerinckii</i> VPI 2983	
	Mean $\pm$ S.D.	Range	Mean $\pm$ S.D.	Range
Group I (40 strains)	81.7 $\pm$ 15.0	56.2–114.2	5.3 $\pm$ 1.9	1.0–8.6
Group II (20 strains)	8.9 $\pm$ 1.6	6.2–11.2	74.2 $\pm$ 11.2	58.2–91.7
Group III (5 strains)	9.7 $\pm$ 2.7	7.0–12.4	5.3 $\pm$ 3.0	1.8–8.9

hybridization results. All strains of hybridization group I yielded a type α gel pattern. Types β and β' patterns were obtained with strains of hybridization group II; these differed mainly by the substitution of two bands in the fastest-running area of gel type β by a unique faint band in gel type β' . Other differences could be seen in the middle and even in the upper parts of the gels, but in the latter case differences were not always obvious, depending on the amount of proteins loaded on the gel. Strains of hybridization group III showed a different PAGE pattern, type γ .

GLC analysis of fermentation products

In preliminary experiments, we observed that *C. butyricum* and *C. beijerinckii* strains produced different amounts of butyric acid and acetic acid as end-products of glucose fermentation. When 100 mM-acetate was added to the growth medium, it appeared that most strains of *C. beijerinckii* produced butyric acid and metabolized small amounts of acetate, whereas most strains of *C. butyricum* produced acetate and also butyrate although in a lower amount than *C. beijerinckii* (data not shown). Taken independently, these data did not permit easy identification of all the strains tested, but differences between the two species were clearer when the butyric acid/ acetic acid ratio was determined after 3 d growth in a defined synthetic broth, SIGA. The results are diagrammatically shown in Fig. 3(a). All the *C. butyricum* strains

Fig. 2. PAGE patterns of cell proteins. Type α gels were obtained with hybridization group I strains (*C. butyricum*), type β or β' with hybridization group II strains (*C. beijerinckii*), and type γ with hybridization group III strains (*Clostridium* spp.). The stacking and resolving gels were respectively 4.7% and 12% polyacrylamide. The voltage was set at 12 V cm^{-1} for 4 h. The gel was stained for 15 min in Coomassie blue and decolorized for several hours as described by Laemmli (1970).

had a low butyric acid/acetic acid ratio, whereas most of the *C. beijerinckii* strains had higher values (>0.25). When the same experiments were done in TYG supplemented with acetate (TYGA), similar results were obtained (Fig. 3b). From these data, we defined two 'chromatography groups': group A includes strains with a low butyric acid/acetic acid ratio (<0.25 in SIGA or <0.29 in TYGA); group B includes strains with a high butyric acid/acetic acid ratio (≥ 0.25 in SIGA or ≥ 0.29 in TYGA). Figure 3 clearly shows that all strains of chromatography group B are representative of *C. beijerinckii*. Chromatography group A is more heterogeneous, including all the *C. butyricum* strains, several *C. beijerinckii* strains, and the non-hybridizing strains.

In order to test the reproducibility of this chromatographic method, 10 independent experiments after growth in SIGA and TYGA were done with *C. butyricum* VPI 3266 and *C.*

Fig. 3. Diagrammatic representation of the values of the butyric acid/acetic acid ratio (calculated from molar concentrations) measured after 3 d growth in (a) SIGA broth and (b) TYGA broth.

beijerinckii VPI 5481, which respectively fit in chromatography groups A and B, and with strain 1106, belonging to DNA hybridization group III and to chromatography group A. The results are shown in Fig. 4. The butyric acid/acetic acid ratio of *C. butyricum* VPI 3266 varied from 0.12 to 0.14 in SIGA and from 0.21 to 0.24 in TYGA (Fig. 4a); variations for *C. beijerinckii* VPI 5481 were 0.35 to 0.43 in SIGA, and 0.49 to 0.60 in TYGA (Fig. 4b); strain 1106 showed variations ranging from 0.09 to 0.11 in SIGA, and from 0.11 to 0.13 in TYGA (Fig. 4c). These data represent a maximum variation of 6 to 10% from the mean value for each strain in each medium. On the other hand, several other strains were tested twice or three times (including all strains of CF group A3, see later). Similar results were obtained (data not shown). The most important feature is that no strain ever fitted into a chromatography group other than that reported for each strain in Table 1 and Fig. 3.

Fermentation patterns

The ability to ferment glycerol, inositol and ribose was determined for each strain following the strictly defined conditions described in Methods. The 65 strains showed the three different fermentation patterns listed in Table 3. These results, combined with those of GLC analysis, allow the definition of six 'chromatography-fermentation groups' (CF groups) obtained by the combination of the chromatography groups A and B with the fermentation patterns 1 to 3. The CF group of each strain is given in Table 1. The distribution of the 65 strains in the CF groups is shown in Table 4.

DISCUSSION

The DNA hybridization experiments allowed the preliminary identification of the strains included in this study. The results are in complete agreement with those of Cummins & Johnson

Fig. 4. Reproducibility of the determination of the butyric acid/acetic acid molar ratio in SIGA broth (□) and TYGA broth (■).

Table 3. *Fermentation patterns*

Fermentation group	Fermentation of:		
	Inositol	Ribose	Glycerol
1	—	+	+
2	+	—	—
3	—	—	—

+, Production of acid under the conditions described in Methods; —, no production of acid.

Table 4. *Distribution of the 65 strains in the CF groups*

See text for definition of CF groups.

CF group	No. of strains of:		
	<i>C. butyricum</i>	<i>C. beijerinckii</i>	Non-hybridizing strains
A1	39	0	0
A2	0	5	0
A3	1	2	5
B1	0	0	0
B2	0	7	0
B3	0	6	0
Total no. studied	40	20	5

Table 5. *Properties of strains belonging to CF group A3*

Strain	Butyric acid/acetic acid ratio* in:		Gel type
	SIGA	TYGA	
<i>C. butyricum</i> Meb 125	0.17	0.15	α
<i>C. beijerinckii</i> CNRZ 653	0.24	0.26	β
<i>C. beijerinckii</i> CNRZ 530	0.24	0.24	β
Non-hybridizing strains: 1104	0.13	0.12	γ
1106	0.10	0.13	γ
103282	0.11	0.16	γ
105082	0.14	0.10	γ
105282	0.13	0.17	γ

* Calculated from molar concentrations.

(1971), who showed that clostridia of the butyricum group belong to two main species, *C. butyricum* and *C. beijerinckii*. Five strains in this study are not related to the reference strain of either of these two species, although they are phenotypically indistinguishable. Further studies are needed to determine whether they constitute a homogeneous hybridization group.

A new method of differential identification of *C. butyricum* and *C. beijerinckii*, based on quantitative GLC and the determination of fermentation patterns of glycerol, inositol and ribose, is presented in this paper. Two chromatography groups are defined by the determination of the butyric acid/acetic acid ratio after 3 d incubation in SIGA or TYGA. Of the 20 *C. beijerinckii* strains studied, 13 belong to chromatography group B, which does not include any other strains (Fig. 3). Strains of the butyricum group showing a high butyric acid/acetic acid ratio can thus be identified with this test alone. Chromatography group A is more heterogeneous, since it includes representatives of the three DNA homology groups (Fig. 3, Table 4), and the examination of sugar fermentation by strains of this group is necessary for their identification.

Clostridia of the butyricum group are highly saccharolytic, fermenting a great variety of carbohydrates. Fermentation of glycerol and mannitol has been used by several authors to distinguish between species (Breed *et al.*, 1957; Beerens *et al.*, 1962), but many strains cannot be identified by this technique (Cummins & Johnson, 1971). Nevertheless, the fermentation patterns for glycerol, inositol and ribose are a useful test for the identification of strains of this group, when the test is done under strictly defined conditions. Three different fermentation patterns were obtained with the 65 strains studied (Table 3). The combination of chromatography groups with the fermentation patterns allowed the definition of six CF groups, A1, A2, A3, B1, B2 and B3. The CF grouping permits the classification of many strains of chromatography group A (Table 4), since strains of CF group A1 belong to *C. butyricum*, and strains of CF group A2 are all *C. beijerinckii*. However, CF group A3 includes phenotypically indistinguishable strains of all three hybridization groups, for which it is necessary to perform a DNA hybridization experiment or, more conveniently, to determine their protein PAGE pattern (Fig. 2) in order to make a precise identification. The eight strains of CF group A3 share all the phenotypic properties of clostridia of the butyricum group as defined in Methods. The 'non-hybridizing' strains have a very low butyric acid/acetic acid ratio, particularly in SIGA broth where they show the five lowest values of all strains tested (Fig. 3, Table 5). However, there are too few strains in CF group A3 to show whether this result is statistically significant.

Until now, no method of differential identification of *C. butyricum* and *C. beijerinckii* has been available for the routine diagnostic laboratory. A PAGE analysis of native cell proteins was recently described by Cato *et al.* (1982), but this method was poorly reproducible in our hands and results were very dependent on the growth medium employed. We also tried to perform rapid PAGE techniques of SDS-denatured proteins, as described, for example, by Moore *et al.* (1980), but a gel densitometer appears to be necessary for clear results. The main advantages of the more tedious techniques described in this paper are that differences between the four gel types are visible to the naked eye, and that the results are not dependent on the growth medium.

Using the 'CF groups' method, 57 (88%) of the 65 strains of the butyricum group included in this study can be easily and reliably identified with techniques that are currently used in the anaerobe diagnostic laboratory. Quantitative GLC analysis of fermentation products allows the identification of *C. beijerinckii* when the butyric acid/acetic acid ratio is high; if this ratio is low, determination of the fermentation patterns allows the identification of the other strains, except those belonging to CF group A3.

This work also showed that all our β -lactamase-producing strains of the butyricum group are 'true' *C. butyricum*, and that *C. butyricum* is mainly isolated from samples of human origin (Table 1). This raises the question of the potential pathogenicity of this organism (Popoff & Sebald, 1981). The 20 strains of *C. beijerinckii* included in this study were obtained mainly from contaminated dairy products (Table 1), and we have never found any strain of this species from a human source.

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