Lipid Composition in the Classification of the Butyric Acid-producing Clostridia

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An examination of 20 strains of butyric acid-producing *Clostridium* species for phospholipid class compositions, plasmalogen content, and acyl and alk-1-enyl chains showed that the deoxyribonucleic acid homology groups I (*Clostridium butyricum*) and II (*Clostridium beijerinckii*) could be distinguished by their lipid compositions. The phospholipids of *C. butyricum* strains had ethanolamine as the major nitrogenous lipid polar head-group moiety, more octadecenoate plus C₁₉-cyclopropane than hexadecenoate plus C₁₇-cyclopropane acyl chains, and the predominant alk-1-enyl chain was C₁₈-monounsaturated. *Clostridium beijerinckii* strains had *N*-methylethanolamine plus ethanolamine in phospholipid head-groups, more hexadecenoate plus C₁₇-cyclopropane than octadecenoate plus C₁₉-cyclopropane acyl chains, and the major alk-1-enyl chain was C₁₆-saturated. Three species falling outside the two homology groups *Clostridium fallax*, *Clostridium pseudofallax* and *Clostridium acetobutylicum* had ethanolamine as the major phospholipid base, but these species could be distinguished from *C. butyricum* by their acyl and alk-1-enyl chain compositions. The lipid composition of *Clostridium pasteurianum* is even more distinct.

INTRODUCTION

On the basis of cell wall sugar compositions and DNA homologies, Cummins & Johnson (1971) gathered many strains of butyric acid-producing clostridia into two DNA homology groups. Organisms in homology group I had cell walls containing glucose and grew in a mineral salts-glucose medium supplemented with biotin. Organisms in homology group II had both glucose and galactose in their cell walls and required supplementation with yeast extract in the presence of amino acids and ten vitamins for growth. These authors recommended that homology group I strains retain the name *Clostridium butyricum*, and that strains in homology group II be designated *Clostridium beijerinckii*. Some strains labelled *Clostridium fallax* and strains of *Clostridium acetobutylicum*, *Clostridium aurantibutyricum*, *Clostridium pasteurianum*, and *Clostridium tyrobutyricum* were not placed in these two homology groups.

In studies on the lipid compositions of butyric acid-producing clostridia designated *C. butyricum*, differences in lipid composition have been noted. For example, the predominant nitrogenous polar head-group moiety in *C. butyricum* ATCC 6015 was found to be *N*-methylethanolamine (Goldfine, 1962; Baumann *et al.*, 1965), whereas that of *C. butyricum* IFO 3852 was found to be ethanolamine (Matsumoto *et al.*, 1971). Both strains are rich in plasmalogens and contain an unusual glycerol acetal of the major plasmalogen (Matsumoto *et al.*, 1971), which differs in its polar head-group base in the two strains as noted above. The proportion of this lipid has been found to increase in cells grown on certain unsaturated fatty acids in the absence of biotin (Khuller & Goldfine, 1975; Goldfine *et al.*, 1977), and its thermotropic phase behaviour has revealed an unusually large hysteresis of the liquid-crystalline

Abbreviation: FAME, Fatty acid methyl ester.

to gel phase transition (Goldfine *et al.*, 1981). We have, therefore, studied the distribution of ethanolamine and *N*-methylethanolamine-containing phospholipids and the fatty acyl and alk-1-enyl group compositions of the total phospholipids in a number of strains of butyric acid-producing clostridia. Our studies show that homology groups I and II of Cummins & Johnson (1971) can be clearly distinguished on the basis of their lipid compositions in addition to their cell wall sugars and nutritional requirements.

METHODS

The sources and designations of the strains we have studied are given in Table 1. ATCC or VPI strains were first grown on chopped meat medium (Scot Labs, Fiskville, Rhode Island, U.S.A.) then transferred to peptone/yeast extract/glucose medium (Holdeman *et al.*, 1977). Inocula for large cultures were also grown on peptone/yeast extract/glucose medium. The large cultures of cells (2–4 l) for lipid analysis were grown on the medium described by Cummins & Johnson (1971). Anaerobic conditions were maintained in tubes by cooling sterilized medium under CO₂. The tubes were inoculated and traces of oxygen were removed by double plugging using alkaline pyrogallol (Hardman & Stadtman, 1960). When the medium was prepared in flasks, the glucose, cysteine and sodium bicarbonate were sterilized separately and all solutions cooled under CO₂ by blowing the gas into the neck of the flask through a sterile syringe needle; the solutions were added when cool, before inoculating with a 10% (v/v) inoculum. The flasks were sparged with CO₂ before being incubated at 37 °C. Cells usually grew overnight and were harvested in late-exponential or stationary phase. The cell paste was washed twice with 0.05 M-potassium phosphate pH 7.2 and either extracted immediately or frozen at –20 °C.

Extraction, separation and analysis of lipids. The cell paste was suspended in chloroform/methanol (2:1, v/v) and extracted for 1 h as described by Goldfine & Bloch (1961). The crude lipid extract was chromatographed on silicic acid columns (Bio-Sil A, Bio-Rad); non-polar lipids and fatty acids were eluted with chloroform followed by acetone, and the phospholipids with chloroform/methanol (1:2, v/v).

The intact phospholipids were analysed by TLC on silica gel G plates (Prekote, Applied Science, State College, Pa., U.S.A.) or on high-performance thin-layer (HPTLC) plates (Merck, no. 5631) using the following solvent systems: (a) chloroform/methanol/7 M-ammonium hydroxide (12:7:1, by vol.) (Skidmore & Entenman, 1962); (b) *n*-butanol/acetic acid/water (3:1:1, by vol.) (Kates, 1972); (c) chloroform/methanol/acetic acid (65:25:8, by vol.). Sequential hydrolysis of the phospholipids began with acid hydrolysis. Lipid (2–5 mg) was treated with 90% (v/v) acetic acid (1 ml) at 37 °C overnight. After removal of acetic acid by lyophilization, the aldehydes, produced by hydrolysis of plasmalogens and glycerol acetals, and phospholipids were separated on small (0.2–0.5 g) silicic acid columns. Chloroform (4–5 ml) plus 1% (v/v) methanol in chloroform (1 ml) eluted the aldehydes and 5–6 ml of chloroform/methanol (1:2, v/v) eluted the diacyl- and lyso-phospholipids. The phospholipids recovered from acid hydrolysis were further hydrolysed by alkaline methanolysis (Kates, 1972). Glycerophosphate esters were analysed by two-dimensional TLC by the method of Short *et al.* (1969). The aldehyde and fatty acid methyl ester (FAME) fractions were purified when necessary by TLC on pre-washed silica gel (Merck silica gel 60 HR, no. 7744) plates in the systems hexane/chloroform/methanol (27:12:1, by vol.) (Gilbertson *et al.*, 1967) for the aldehydes and petroleum ether/diethyl ether/acetic acid (90:10:1, by vol.) for the esters; the spots were scraped into glass-wool plugged Pasteur pipettes and eluted with chloroform (aldehydes) or diethyl ether (FAME). Reagents for detection of spots on chromatograms were: (a) the molybdate reagent of Dittmer & Lester (1964) for all phosphate-containing compounds; (b) ninhydrin (0.3% in ethanol) for ethanolamine compounds; (c) 0.4% dinitrophenylhydrazine in 2 M-hydrochloric acid for plasmalogens and glycerol acetals; (d) Rhodamine B (0.03% in ethanol) (Skipski & Barclay, 1969) for FAME; (e) plates were sprayed with water to detect aldehydes. The FAME and aldehyde fractions were analysed by GLC using a Perkin Elmer 990 instrument equipped with a hydrogen flame ionization detector. The column, 1.83 m × 3.2 mm (o.d.), of 10% (w/w) diethylene glycol succinate on 80/100 Supelcoport (Supelco, Bellefonte, Pa., U.S.A.) was operated at 170 °C with N₂ as carrier gas. The proportions of each component were determined by multiplying the peak height by the retention time (Kates, 1972). Phospholipid composition was determined as follows: after two-dimensional TLC in solvent systems (a) and (c), the spots were located by brief exposure to iodine vapour and scraped into acid-washed screw-capped glass tubes; lipid phosphorus was determined by the method of Bartlett (1959). The presence of the glycerol acetal of the plasmalogen form of phosphatidylethanolamine or the *N*-methylethanolamine analogue was demonstrated using detection method (c) by TLC in system (a) of parallel lipid samples before and after acid hydrolysis. Vinyl ether content was determined by the method of Gottfried & Rapport (1962), modified to give a final volume of 2.0 ml.

RESULTS AND DISCUSSION

Nineteen strains of the butyric acid group of *Clostridium* previously studied by Cummins & Johnson (1971) and *C. butyricum* IFO 3852 were examined for their phospholipid polar head-

Fig. 1. Two-dimensional thin-layer chromatograms of phospholipids from *Clostridium butyricum* ATCC 860 and *Clostridium beijerinckii* ATCC 11914. Chloroform/methanol/7 M-NH₄OH (12:7:1, by vol.) was used in the first dimension, and *n*-butanol/acetic acid/water (3:1:1, by vol.) in the second dimension. Abbreviations: CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PME, phosphatidyl-*N*-methylethanolamine; GA, glycerol acetal lipids; O, origin.

groups both by TLC of the intact lipids (Fig. 1) and by TLC of the glycerophosphate esters obtained by sequential mild acid hydrolysis and alkaline methanolysis of the polar lipids. Analyses of the aldehydes obtained by mild acid hydrolysis of the alk-1-enyl and glycerol acetal bonds and of the fatty acid methyl esters, both obtained from the polar lipids, were also done. The results of these analyses are presented in Tables 1 to 3.

All of the strains placed in homology group I according to their DNA homologies, cell wall sugars, and nutritional requirements (Cummins & Johnson, 1971) are characterized by the presence of ethanolamine as the major nitrogenous base in the phospholipids and a ratio of (16:1 + 17:cyc) to (18:1 + 19:cyc) acyl chains below 1.0. Most of these strains had 18:1 as the predominant alk-1-enyl chain except for *C. butyricum* (*multifermentans*) VPI 2399, in which the sum of 18:1 plus 19:cyc was slightly higher than the 16:0 alk-1-enyl chain (Table 3). All strains in homology group II had both *N*-methylethanolamine and ethanolamine as phospholipid nitrogenous moieties, a ratio of (16:1 + 17:cyc) to (18:1 + 19:cyc) acyl chains greater than 1.5, and 16:0 as the major alk-1-enyl chain.

When vinyl ether was measured by an iodometric procedure, the molar ratio of vinyl ether to lipid phosphorus was lower in homology group I than in homology group II strains (Table 1). This assay does not measure the glycerol acetal lipid which represented from 5.9 to 29.2% of the lipid phosphorus (Table 4). We observed that when a strain was grown on the Casamino acid medium of Broquist & Snell (1951), it contained a higher proportion of the glycerol acetal lipid than when it was grown on the richer medium used by Cummins & Johnson (1971) (data not shown). Thus, the differences in glycerol acetal content seen in Table 4 were probably more related to the media than to the strains studied. We have found the glycerol acetal class of lipid in all of the butyric acid-producing clostridia we have studied, with the exception of *C. pasteurianum* ATCC 6013, which has a lipid composition very different from those seen in other clostridia of this group (A. Kaufman and H. Goldfine, unpublished observations).

With the exception of 17:cyc acyl and alk-1-enyl chains, which were not found or were seen in only small amounts in homology group I strains, and some longer chain alkenyl and acyl groups seen in strains of both groups, the major components of the alkenyl and acyl chains were qualitatively alike (Tables 2 and 3). Both groups had high proportions of 16:0 acyl chains and in

Table 1. *Principal phospholipid nitrogenous base moieties, major alkenyl chains, unsaturated acyl chains, and vinyl ether contents of butyric acid-producing clostridia*

Strain and number	Principal phospholipid bases*	Major alk-1-enyl chain†	Ratio of acyl chains:	Molar ratio vinyl ether/P
			(16:1 + 17:cyc)/ (18:1 + 19:cyc)	
Homology group I				
<i>C. butyricum</i> IFO 3852‡	Etn	18:1	0.46	
<i>C. butyricum</i> ATCC 860§	Etn	18:1	0.34	
<i>C. butyricum</i> ATCC 19398§	Etn	18:1	0.26	
<i>C. butyricum</i> (multifermentans) VPI 2399	Etn	18:1 + 19:cyc	0.43	
<i>C. butyricum</i> (fallax) VPI 5982	Etn	18:1	0.48	
<i>C. butyricum</i> VPI 3005	Etn	18:1	0.59	
<i>C. butyricum</i> (multifermentans) VPI 2402	Etn	18:1	0.61	0.17
<i>C. butyricum</i> VPI 2419	Etn	18:1	0.67	0.16
Homology group II				
<i>C. butyricum</i> ATCC 6015	MeEtn, Etn	16:0	5.5	0.49
<i>C. beijerinckii</i> ATCC 11914§	MeEtn, Etn	16:0	3.5	
<i>C. beijerinckii</i> ATCC 14949§	MeEtn, Etn	16:0	3.1	
<i>C. beijerinckii</i> (butyricum) VPI 2983	MeEtn, Etn	16:0	2.8	
<i>C. beijerinckii</i> (amylolyticum) VPI 2697	MeEtn, Etn	16:0	5.1	
<i>C. beijerinckii</i> (multifermentans) VPI 2408	MeEtn, Etn	16:0	1.7	
<i>C. beijerinckii</i> ATCC 6014	MeEtn, Etn	16:0	3.1	0.34
<i>C. beijerinckii</i> (butyricum) VPI 2793	MeEtn, Etn	16:0	6.1	0.55
Other strains in the 'butyric acid group'				
<i>C. fallax</i> ATCC 19400	Etn	16:0	1.1	0.12
<i>C. pseudofallax</i> VPI 2410	Etn	16:0	0.89	0.16
<i>C. acetobutylicum</i> ATCC 824	Etn	19:cyc	0.37	0.67
<i>C. pasteurianum</i> ATCC 6013		17:cyc	∞	

* Etn, ethanolamine; MeEtn, *N*-methylethanolamine.

† The number before the colon is the chain length, the number after is the number of double bonds; cyc, cyclopropane.

‡ IFO 3852 is listed in homology group I based on its lipid composition. No studies on its DNA homology have been done.

§ These acyl chain compositions represent only the diacyl phospholipids. The plasmalogen and glycerol acetal lipid acyl chains were analysed separately. The latter are both qualitatively and quantitatively similar to the acyl chains of the diacyl lipids and, since they represent about one-fourth of the total acyl chains in *C. beijerinckii* strains and one-eighth in *C. butyricum* strains, the approximate weighted average composition calculated is nearly indistinguishable from those presented.

|| Earlier designations listed by Cummins & Johnson (1971). ATCC, American Type Culture Collection, Rockville, Md., U.S.A.; IFO, Institute of Fermentation, Osaka, Japan; VPI, Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va., U.S.A.

homology group II 16:0 was the major alk-1-enyl chain. The high (16:1 + 17:cyc) to (18:1 + 19:cyc) ratio seen in the acyl chains of homology group II strains, was also seen in the alk-1-enyl chains of all strains in this group, with the exception of VPI 2408. The longer 18:1 chains were predominant among the unsaturated alk-1-enyl chains of strains in homology group I. Three of the strains not in homology groups I and II had ethanolamine, but no *N*-methylethanolamine in their phospholipids. *Clostridium pasteurianum* ATCC 6013 had no or very small amounts of phosphatidylethanolamine. The acyl chains of *C. fallax* ATCC 19400 and *C. pseudofallax* ATCC 2410 are intermediate in composition between homology groups I and II (Table 2). Palmitic acid was the predominant species, but the ratios of (16:1 + 17:cyc) to (18:1 + 19:cyc) fell between those of groups I and II. The alkenyl chains were predominantly 16:0 in both strains (Table 3). *Clostridium acetobutylicum* was also characterized by a high proportion of 16:0 acyl chains, but 18:1 + 19:cyc were the predominant alk-1-enyl chains.

Cummins & Johnson (1971) showed that these latter species had low degrees of homology with representative *C. butyricum* and *C. beijerinckii* strains and that *C. pasteurianum* strains had very low DNA homologies. The lipid composition of *C. pasteurianum* ATCC 6013 was indeed

Table 2. *Acyl chain compositions of butyric acid-producing clostridia*

Strain and number	Acyl chain composition (% w/w)*							
	14:0	14:1	16:0	16:1	17:cyc	18:0	18:1	19:cyc
Homology group I								
<i>C. butyricum</i> IFO 3852	14.8	—	43.2	13.0	—	2.8	24.4	3.6
<i>C. butyricum</i> ATCC 860	7.2	—	42.4	12.1	—	2.5	23.2	12.5
<i>C. butyricum</i> ATCC 19398	5.1	—	47.5	8.6	—	4.8	18.9	14.2
<i>C. butyricum</i> VPI 2399	10.0	tr	48.8	11.3	tr	3.2	11.9	14.3
<i>C. butyricum</i> VPI 5982	10.7	tr	46.7	13.2	—	1.9	17.5	9.8
<i>C. butyricum</i> VPI 3005	13.1	tr	35.2	18.1	tr	1.1	21.7	9.2
<i>C. butyricum</i> VPI 2402	11.2	tr	40.2	18.1	—	1.5	28.4	1.1
<i>C. butyricum</i> VPI 2419	16.5	0.5	33.0	19.6	—	0.6	27.2	2.2
Homology group II								
<i>C. butyricum</i> ATCC 6015	3.9	0.5	58.1	25.7	5.6	tr	4.6	1.1
<i>C. beijerinckii</i> ATCC 11914†	4.1	tr	52.05	20.8	12.2	1.4	3.6	5.9
<i>C. beijerinckii</i> ATCC 14949†	2.73	0.5	50.2	25.3	7.9	1.5	7.3	3.4
<i>C. beijerinckii</i> VPI 2983	4.14	tr	60.9	12.1	11.9	2.2	1.9	6.6
<i>C. beijerinckii</i> VPI 2697	5.5	tr	54.0	15.9	17.7	1.0	1.64	4.9
<i>C. beijerinckii</i> VPI 2408‡	7.6	6.4	35.5	9.6	10.1	8.3	2.3	9.58
<i>C. beijerinckii</i> ATCC 6014	3.9	0.8	48.3	28.0	6.4	1.25	8.2	3.03
<i>C. beijerinckii</i> VPI 2793	4.5	0.7	52.6	27.5	8.5	—	4.4	1.5
Other strains in the 'butyric acid group'								
<i>C. fallax</i> ATCC 19400	7.5	1.55	45.5	20.1	2.12	1.5	17.1	3.0
<i>C. pseudofallax</i> VPI 2410	6.4	0.8	51.4	17.8	0.7	1.8	19.8	0.9
<i>C. acetobutylicum</i> ATCC 824	8.1	tr	64.5	5.6	1.0	2.9	8.8	8.8
<i>C. pasteurianum</i> ATCC 6013	—	—	92.7	3.3	4.0	—	—	—

* Each value represents the mean of at least two analyses of a single sample of lipid from each strain; —, not detectable; tr, trace.

† See footnote §, Table 1.

‡ This strain also had 9.8% 20:0.

Table 3. *Alk-1-enyl chain compositions of butyric acid-producing clostridia*

Strain and number	Alk-1-enyl chain composition (% w/w)*								
	14:0	14:1	16:0	16:1	17:cyc	18:0	18:1	19:cyc	Unknown
Homology group I									
<i>C. butyricum</i> IFO 3852	0.3	—	20.3	24.7	—	4.6	44.6	5.3	
<i>C. butyricum</i> ATCC 860	tr	—	23.1	22.4	1.88	3.85	40.3	8.6	
<i>C. butyricum</i> ATCC 19398	<1	—	22.9	21.1	tr	4.6	43.6	7.1	
<i>C. butyricum</i> VPI 2399	1.8	—	32.3	14.7	—	7.5	24.4	9.6	5.25, 4.25
<i>C. butyricum</i> VPI 5982	—	—	13.0	14.35	—	5.4	49.3	5.9	11.9
<i>C. butyricum</i> VPI 3005	0.6	tr	8.8	15.25	0.45	5.5	44.4	3.2	17.4, 3.4
<i>C. butyricum</i> VPI 2402	0.9	—	12.7	19.4	—	4.8	58.4	3.6	
<i>C. butyricum</i> VPI 2419	tr	tr	10.0	35.9	—	2.2	49.4	3.7	
Homology group II									
<i>C. butyricum</i> ATCC 6015	0.55	tr	42.6	30.6	12.5	2.5	7.8	3.3	
<i>C. beijerinckii</i> ATCC 11914	tr	—	51.4	14.0	21.1	2.4	2.05	8.9	
<i>C. beijerinckii</i> ATCC 14949	0.5	—	43.9	20.5	21.05	—	5.9	8.0	
<i>C. beijerinckii</i> VPI 2983	0.5	—	55.6	6.6	18.5	6.0	2.3	10.3	
<i>C. beijerinckii</i> VPI 2697	0.4	—	68.8	4.9	15.7	4.2	tr	5.6	
<i>C. beijerinckii</i> VPI 2408	0.4	—	57.4	1.76	9.4	14.6	1.18	15.1	
<i>C. beijerinckii</i> ATCC 6014	0.4	—	45.2	27.7	9.2	3.5	9.2	4.7	
<i>C. beijerinckii</i> VPI 2793	0.5	—	52.9	22.6	15.3	tr	4.6	3.9	
Other strains in the 'butyric acid group'									
<i>C. fallax</i> ATCC 19400	0.75	tr	96.5	2.7	—	tr	tr	—	
<i>C. pseudofallax</i> VPI 2410	2.8	—	68.8	15.4	—	5.2	7.6	—	
<i>C. acetobutylicum</i> ATCC 824	2.3	tr	17.5	7.7	5.0	5.7	15.3	46.2	
<i>C. pasteurianum</i> ATCC 6013	—	—	23.5	3.0	73.6	—	—	—	

* Each value represents the mean of at least two analyses of a single sample of lipid from each strain; —, not detectable; tr, trace.

Table 4. *Phospholipid compositions of selected strains*

Strain and number	PE*	PME*	Glycerol acetal lipid	PG*	CL*†
Homology group I					
<i>C. butyricum</i> ATCC 19398‡	56.7	0	9.1	16.3	17.6
<i>C. butyricum</i> IFO 3852§	39	0	22.6	24.8	8.9
Homology group II					
<i>C. beijerinckii</i> ATCC 11914‡	47.3		5.9	21.5	21.7
<i>C. butyricum</i> ATCC 6015§	12.5	33.8	29.2	22.1	

* PE, phosphatidylethanolamine; PME, phosphatidyl-*N*-methylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin. All of these lipid classes contain plasmalogens (Baumann *et al.*, 1965; Matsumoto *et al.*, 1971).

† Includes a polyglycerol phosphatide tentatively identified as acyl phosphatidylglycerol (Y. Koga & H. Goldfine, unpublished observations).

‡ Grown on medium described by Cummins & Johnson (1971).

§ Grown on Casamino acid medium of Broquist & Snell (1951). Data for *C. butyricum* ATCC 6015 from Baumann *et al.* (1965) and Goldfine *et al.* (1977).

|| Mainly PME (see Fig. 1).

very different from that of other organisms in the 'butyric acid group'. It had 92.7% 16:0 acyl chains and 73.6% 17:cyc alk-1-enyl chains, but the structures of the polar lipids were not identified. On the basis of 23S rRNA homologies, Johnson & Francis (1975) placed *C. butyricum* and *C. pasteurianum* in separate subgroups of a very large rRNA homology group.

Clostridium butyricum IFO 3852, originally studied for lipid content by Matsumoto *et al.* (1971) represents an enigma. It is stated to be derived from *C. butyricum* ATCC 6014. However, ATCC 6014 is clearly a member of homology group II (Cummins & Johnson, 1971) and IFO 3852 closely resembles homology group I strains in its lipid composition. Based on its lipid composition and other biochemical tests, strain IFO 3852 can no longer be considered to be a subculture of ATCC 6014 and has been discarded from the collection (Dr K. Imai, Curator, Institute for Fermentation, Osaka, personal communication).

The DNA homology and cell wall sugar data of Cummins & Johnson (1971), together with the lipid composition presented here, suggest that the designation of *C. butyricum* ATCC 6015 should now be changed to *C. beijerinckii*, as was done earlier for other strains. Based on a review of its biochemical properties, the American Type Culture Collection will redesignate strain ATCC 6015 as *C. beijerinckii* (P. A. Pienta, Curator, ATCC, personal communication).

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