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Lipid diversity among botulinum neurotoxin-producing clostridia

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Clostridium botulinum has been classified into four groupings (groups I to IV) based on physiological characteristics and 16S rRNA sequencing. We have examined the lipid compositions of 11 representative strains of *C. botulinum* and a strain of *Clostridium sporogenes* by 2D-TLC and by MS. All strains contained phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylethanolamine (PE) in both the all-acyl and the alk-1′-enyl (plasmalogen) forms. Five strains in proteolytic group I, which are related to *C. sporogenes*, contained varying amounts of an ethanolamine-phosphate derivative of *N*-acetylglucosaminyl-diradylglycerol, which is also present in *C. sporogenes*. Three strains in group II, which are related to *Clostridium butyricum*, *Clostridium beijerinckii and Clostridium acetobutylicum*, contained lipids characteristic of these saccharolytic species: a glycerol acetal and a PG acetal of the plasmalogen form of PE. Two group III strains, which are related to *Clostridium novyi*, contained amino-acyl derivatives of PG, which are also found in *C. novyi*. A strain in group IV had PE, PG and CL, but none of the distinguishing lipids. This work shows that the lipidome of *C. botulinum* is consistent with its classification by other methods.

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INTRODUCTION

Clostridium botulinum has been known as the cause of botulism, a potentially fatal foodborne toxinosis, since the late 19th century. Botulinum neurotoxins (BoNTs) are the most poisonous substances known and are classified into seven serologically distinct groups (serotypes A to G) (Hatheway, 1995). Most strains produce only one serotype of BoNT, but some produce more than one toxin serotype, including Ab, Ba, Af and Bf (Barash & Arnon, 2004; Gimenez & Gimenez, 1993; Santos-Buelga *et al.*, 1998), whereby the highest quantity of BoNT produced is designated by the upper-case letter. Many strains also contain unexpressed (silent) BoNT genes, where the silent gene is shown in parentheses, e.g. A(B) (Franciosa *et al.*, 1994). Human botulism is commonly caused by groups I and II *C. botulinum* (Sebaihia *et al.*, 2007). Other species of clostridia have been isolated that produce BoNTs, including *Clostridium argentinense*, and rare strains of *Clostridium baratii* and *Clostridium butyricum* (Hatheway & Johnson, 1998). Neurotoxin-producing clostridia are paraphyletic, and are designated solely by their ability to produce their characteristic neurotoxin.

Early biochemical and physiological studies of *C. botulinum* indicated that they were distinct physiologically and could be divided into four groups, which were designated groups I, II, III and IV (Hatheway, 1990). These physiological groups were based on proteolytic and saccharolytic characteristics, acid and alcohol fermentation end products, ability to grow at low temperatures, heat resistance of spores, and ability to grow in the presence of acids, alcohols and salts (Hatheway & Johnson, 1998).

The diversity of the neurotoxin-producing clostridia has been supported by molecular taxonomic studies (Collins & East, 1998; Collins *et al.*, 1994; Hill *et al.*, 2007). Johnson

†Deceased.

Abbreviations: BoNT, botulinum neurotoxin; CL, cardiolipin: EtnP-GlcNAcDRG, phosphoethanolamine GlcNAc-diradylglycerol; GAPlaE, glycerol acetal of plasmenylethanolamine; GlcNAcDRG, GlcNAc-diradylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGAPlaE, phosphatidylglycerol acetal of plasmenylethanolamine; PlaE, plasmenylethanolamine; PlaG, plasmenylglycerol.

Authors' note: In designations of the molecular species the number before the colon is the sum of the chain lengths and the number after the colon is the number of double bonds. The modifier diradyl is used to indicate molecular species that contain both diacyl and 1-*O*-alk-1′-enyl chains.

A supplementary figure is available with the online version of this paper.

and Francis divided the genus *Clostridium* into four major groups, I to IV, based on the G+C content of their DNA and rRNA gene homologies. *C. botulinum* strains were all in the low %G+C group I, and were separated into groups I-A, I-F and I-H based on rRNA homologies (Johnson & Francis, 1975). Further 16S rRNA analyses of the genus *Clostridium* confirmed the classification of *C. botulinum* into four groupings which corresponded to the four physiological groups, I to IV (Collins & East, 1998; Hutson *et al.*, 1993a, 1993b). An analysis of 174 *C. botulinum* strains by amplified fragment length polymorphism (AFLP) and by sequencing of 16S rRNA and BoNT genes confirmed the existence of at least four distinct genomic backgrounds, each of which has likely independently acquired BoNT genes through horizontal gene transfer (Hill *et al.*, 2007).

The proteolytic group I strains of *C. botulinum* expressing toxin types A, B and F are closely related to *Clostridium sporogenes*. The saccharolytic group II strains expressing toxin types B, E and F are related to *Clostridium butyricum*, *Clostridium beijerinckii* and *Clostridium acetobutylicum*. Group III strains expressing toxin types C and D cluster with *Clostridium novyi* and *Clostridium haemolyticum*, and group IV strains expressing toxin type G cluster with *Clostridium subterminale*, *Clostridium proteolyticus*, *C. argentinense* and *Clostridium schirmacherense* (Collins *et al.*, 1994; Hill *et al.*, 2007). The lipids of *C. butyricum*, *C. beijerinckii* and *C. acetobutylicum* have been studied intensively (Baumann *et al.*, 1965; Johnston & Goldfine, 1983, 1992; Oulevey *et al.*, 1986; Matsumoto *et al.*, 1971) and the polar lipids of *C. novyi* were recently elucidated (Guan *et al.*, 2011). We have also studied the polar lipids of *C. sporogenes*. Thus it became possible to compare the lipid compositions of a number of strains of *C. botulinum* with previously completed analyses of clostridial lipids. The results of a detailed study of the lipids of 11 strains of *C. botulinum* by 2D-TLC and by MS are here presented. These lipidomic studies demonstrate clear relationships of *C. botulinum* strains with those that have been found to be related by DNA sequence and physiological analyses.

METHODS

Strains. The *C. botulinum* strains examined in this study listed in Table 1 are from our (E. A. Johnson) culture collection. They were stored in 50 % glycerol/50 % TPGY (trypticase-peptone-glucose-yeast extract) medium at −80 °C.

Lipid isolation. Frozen stocks were thawed and grown anaerobically overnight in 10 ml TPGY medium at 30 °C (strains 17B, Alaska E, Beluga E and type G) or 37 °C (strains Loch Maree, Hall A Hyper, Okra B, ATCC 3502, 62A and *C. sporogenes*). These cultures were inoculated at 1 %, v/v, into 500 ml anaerobic TPGY, and grown statically for 18 h at 30 or 37 °C, and the cells were harvested by centrifugation at 4 °C. The wet cell pellets were extracted with chloroform/methanol/water by the method of Bligh & Dyer (1959), with modifications. The lipid extracts were dried under a stream of nitrogen, while being warmed in a heating block. They were dissolved in chloroform and stored at −20 °C.

Table 1. *C. botulinum* strains analysed

Strain	Serotype	Group	Source
Loch Maree	A-3	I	CDC
Hall A Hyper	A	I	Our laboratory
Okra B	B	I	Our laboratory
ATCC 3502	A	I	Our laboratory
62A	A	I	Our laboratory
Eklund 17B	B	II	Mel Eklund
Alaska E	E	II	Mel Eklund
Beluga E	E	II	Our laboratory
ATCC 1873	D	III	Our laboratory
Africa C	C/D	III	Our laboratory
G89	G	IV	CDC
C. sporogenes ATCC 3584	NA	NA	ATCC

TLC. 2D-TLC was performed on silica gel 60, 10 × 10 cm thin-layer plates. The solvents used were chloroform/methanol/concentrated ammonia/water, 65 : 30 : 2.5 : 2.5 (by vol.), in the first dimension and chloroform/methanol/acetic acid/water, 80 : 18 : 12 : 5 (by vol.), in the second dimension. Acid hydrolysis of lipids on TLC plates using HCl fumes for detection of plasmalogens has been described previously (Johnston *et al.*, 2010). Amine-containing lipids were detected using 0.3 % ninhydrin in ethanol, followed by heating at 120 °C for 5–10 min. Phosphorus-containing lipids were detected with 0.3 % (w/v) molybdenum blue (Sigma). Standards of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL) from Sigma-Aldrich, and of glycerol acetal of plasmenylethanolamine (GAPlaE) isolated from *C. butyricum*, were run alongside the lipid samples in both the first and second dimensions. The standards were spotted in two lanes at the left side of the plate for the first dimension run and at the right side of the top of the plate for the second dimension. HCl hydrolysis of plasmalogens results in the formation of the corresponding lyso lipid, i.e. with the remaining *sn*-2 acyl chain and long-chain aldehydes. HCl hydrolysis of the GAPlaE results in the formation of lyso-PE, and hydrolysis of phosphatidylglycerol acetal of plasmenylethanolamine (PGAPlaE) results in the formation of lyso-PE and PG in addition to long-chain aldehydes (Johnston & Goldfine, 1988; MacDonald & Goldfine, 1990).

Quantification of *C. sporogenes* lipids. The relative amounts of the polar lipids of *C. sporogenes* were determined as previously described for the lipids of *Clostridium tetani* (Johnston *et al.*, 2010). Briefly, triplicate 10 ml cultures were prelabelled with 10 μCi (370 kBq) [1-^{14}C]acetate and grown overnight. The cells were harvested by centrifugation and the lipids extracted with chloroform/methanol. They were subjected to 2D-TLC as described above and quantified with a phosphorimager.

Liquid chromatography/MS (LC/MS). Normal-phase LC-electrospray ionization (ESI)/MS of lipids was performed using an Agilent 1200 Quaternary LC system, coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems). LC was performed on an Ascentis Si HPLC column (5 μm, 25 cm × 2.1 mm). The elution program has been described previously (Guan *et al.*, 2011). The post-column splitter diverted ~10 % of the LC flow to the ESI source of the QSTAR XL mass spectrometer, with MS settings as follows: IS=−4500 V, CUR=20 p.s.i., GS1=20 p.s.i., DP=−55 V and FP=−150 V. Nitrogen was used as the collision gas for MS/MS experiments. Data acquisition and analysis were performed using the Analyst QS software (Applied Biosystems). Polar lipids emerged from this column as follows: PG, 12–14 min; CL, 14–15 min; PE and phosphatidylmonomethylethanolamine (PMME), 16.5–17.5 min;

PGAPlaE, 17–18 min; GAPlaE, 18–20 min; lysyl-PG, 20–22 min; EtnP-GlcNAcDAG, 22–24 min. In some runs there was partial overlap between PG and CL.

RESULTS

The *C. botulinum* strains used in this study are a representative sample of the four physiological groupings. At least one strain from each major group is represented. Although the sample size is small, the results so far indicate that lipid composition mirrors taxonomy based on other physiological characteristics and DNA sequencing.

Group I

This group, which secretes BoNT types A, B or F and is proteolytic, is closely related to *C. sporogenes* and *Clostridium putrificum*, as revealed by 16S rRNA sequencing (Hill *et al.* 2007; Collins *et al.*, 1994; Collins & East, 1998). Analysis of the polar lipids of *C. sporogenes* revealed the presence of PE, PG and CL in both the all-acyl forms and as plasmalogens. The presence of the acyl forms of these lipids is predicted based on the presence of genes annotated as *pss*, *psd*, *pgpA* or *-B* and *cls*. All three phospholipids are rich in plasmalogens (1-*O*-alk-1′-enyl ether lipids), which have been found in many anaerobic, but not in aerobic or facultative bacteria (Goldfine & Johnston, 2005; Goldfine, 2010b). In addition, 5.4 % of the polar lipids of *C. sporogenes* were *N*-acetylglucosylaminyl-diradylglycerol (GlcNAcDRG) and 3.0 % were a recently discovered phosphoethanolamine-modified GlcNAcDRG (EtnP-GlcNAcDRG; Johnston *et al.*, 2010), both of which contained substantial amounts of the plasmalogen form (Fig. 1). EtnP-GlcNAcDRG was originally identified in *C. tetani* (Johnston *et al.*, 2010), which is moderately related to *C. sporogenes* (Collins *et al.*, 1994). We found EtnP-GlcNAcDRG in five group I *C. botulinum* strains: Hall A Hyper (Fig. 2a), 62A (Fig. 2b), Loch Maree, Okra B and ATCC 3502 (not shown) (Table 2). Upon MS/MS, EtnP-GlcNAcDRG produced a fragment (EtnP) ion at *m/z* 140.0, which was seen in the MS/MS spectra of these lipids from *C. sporogenes* and the group I *C. botulinum* strains.

Group II

Members of this group, which secrete BoNT types B, E or F, are typically saccharolytic and non-proteolytic. According to 16S rRNA sequencing, they cluster with *C. acetobutylicum*, *C. beijerinckii* and *C. butyricum* (Collins *et al.*, 1994; Hill

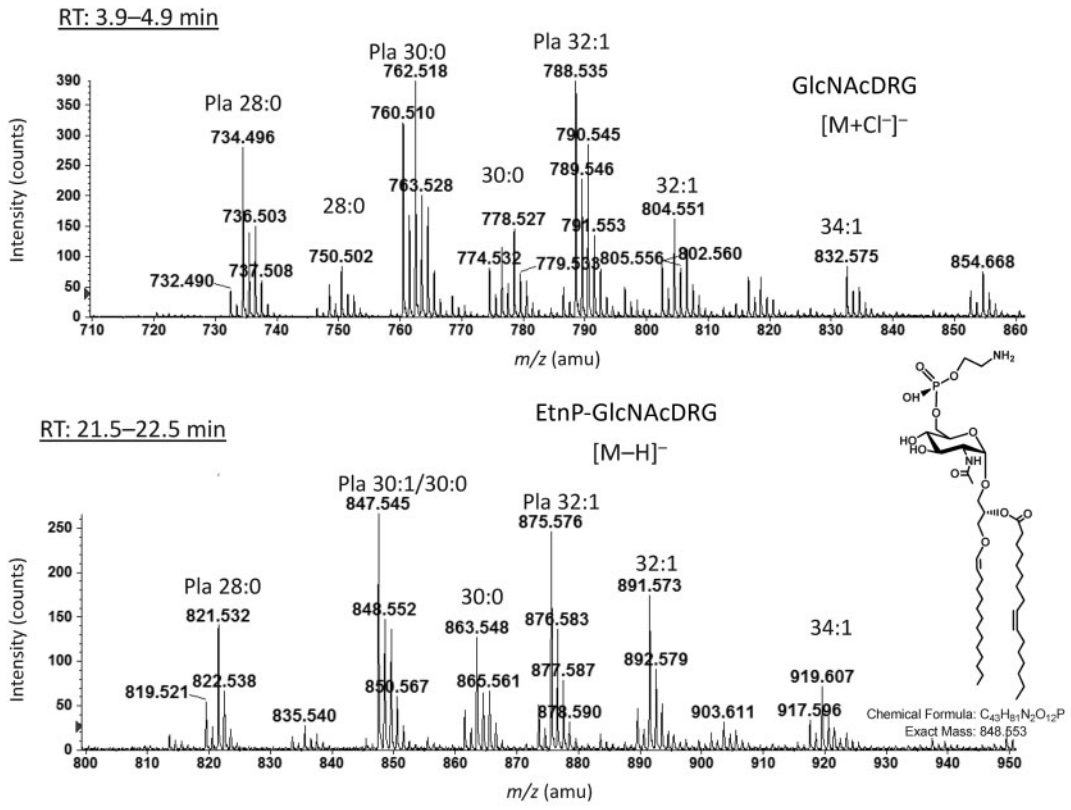

Fig. 1. Negative ion ESI/MS spectra of GlcNAcDRG and EtnP-GlcNAcDRG species from *C. sporogenes* strain ATCC 3584. Pla, plasmalogen species. The structure of the plasmalogen form of EtnP-GlcNAcDAG (30 : 1) is shown. The number before the colon is the sum of the chain lengths and the number after the colon is the number of double bonds.

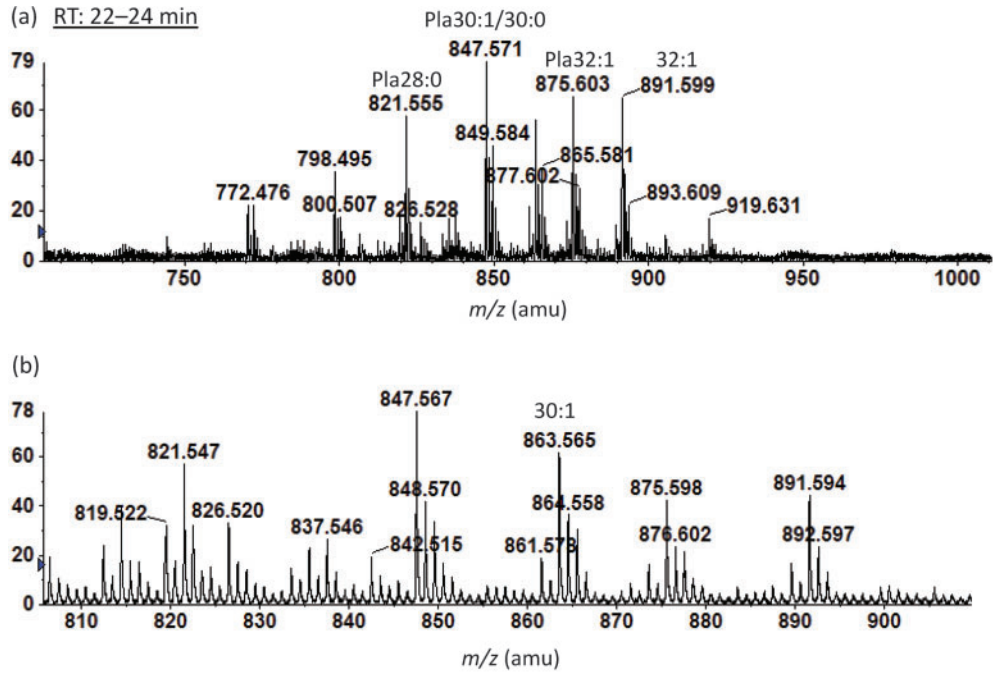

Fig. 2. Negative ion ESI/MS spectra of EtnP-GlcNAcDRG species from (a) *C. botulinum* strain Hall A Hyper and (b) strain 62A. Pla, plasmalogen species. The number before the colon is the sum of the chain lengths and the number after the colon is the number of double bonds.

Table 2. Polar lipids of *C. botulinum* strains

The '+' symbols indicate the relative amounts of these lipids as judged by molybdate staining and/or charring. Abbreviation: Pla, plasmalogen form.

Strain	Group	PE/PlaE	PG/PlaG	CL/PlaCL	GAPlaE	PGAPlaE	Ala- or Lys-PG	EtnP-GlcNAc-DRG	GlcNAcDRG
HAH*	I	++/+++	++/+++	+/+++†				+ (Mostly Pla)	
LM A-3‡	I	++/+++	+/++	++/+++				Trace	
Okra B	I	+/+++	+/+++	+/+++				Trace	
62A	I	+++/++	++/+++	++/+++				Trace	
ATCC 3502 (type A)	I	++/++	+/+++	++/+++†				+ (Mostly Pla)	
C. sporogenes		++/+++	++/+++	++/+++				+	
Eklund 17B	II	++/+++	+/++	++/++ +†	++	+			+ (Diacyl)
BE§	II	+/+++	+/++	++/+++	++	++‖			+ (Diacyl)
Alaska E	II	+/++	+++/+	++/++	++	++	Lys-PG		
D1873	III	+++/+++	++/++	++/++			Lys-PG/Pla Ala-PG/Pla		
Africa C	III	+++/+	+++/+	+++/+			Lys-PG		
G89	IV	+/+++	++/+++	++/+++					

*Hall A Hyper.
†Di-alkenyl species found.
‡Loch Maree A3.
§Beluga E.
‖Both PG and PlaG acetals of PlaE were found.

et al., 2007; Collins & East, 1998). In terms of lipid composition this is the best-studied group of the genus. Like most clostridia and indeed many bacteria, these species contain PE, PG and CL. *C. acetobutylicum* differs from the other two in having large amounts of glycosyl-diradylglycerols in addition to phospholipids (Johnston & Goldfine, 1992; Oulevey *et al.*, 1986). A distinguishing feature of these species is the presence of significant levels of glycerol-acetals of plasmenylethanolamine (PlaE) (Matsumoto *et al.*, 1971; Khuller & Goldfine, 1974) and smaller amounts of PG-acetals of PlaE (Johnston & Goldfine, 1988; MacDonald & Goldfine, 1990). Glycerol and PG acetals of PlaE were seen by 2D-TLC (Fig. 3) and MS (Fig. 4) in strain Beluga E, and in Eklund 17B and Alaska E (data not shown) (Table 2). Interestingly, strain Beluga E has both PG and plasmenylglycerol (PlaG) acetals of PlaE (Fig. 4a). This represents the first identification of PlaG acetals of PlaE.

Group III

C. botulinum strains in this group produce type C and D toxins and are genetically related to *C. novyi* (Collins *et al.*, 1994; Collins & East, 1998; Hill *et al.*, 2007). Members of this group are non-proteolytic and have higher optimal growth temperatures than members of groups I and II (Collins & East, 1998). A distinguishing feature of the lipidome of *C. novyi* is the presence of the aminoacyl-PGs lysyl- and alanyl-PG (Guan *et al.*, 2011). Like *C. novyi*, strain D1873 contains both lysyl-PG and alanyl-PG (Fig. 5), and strain C. Africa CP contains only lysyl-PG. Lysyl-PG was also observed in *C. botulinum* Alaska E (Fig. S1).

Group IV

There are relatively few *C. botulinum* strains in group IV, which is characterized by secretion of the G type toxin. These strains do not ferment sugars and differ from the other groups by lack of lipase production. By 16S rRNA sequence analysis they cluster most closely with *C. subterminale* and *Clostridium estertheticum* (Collins & East, 1998) and also with *C. proteolyticus*, *C. argentinense* and *C. schirmacherense* (Hill *et al.*, 2007). The strain G that we examined contained PE, PG and CL with abundant plasmalogen forms (Table 2).

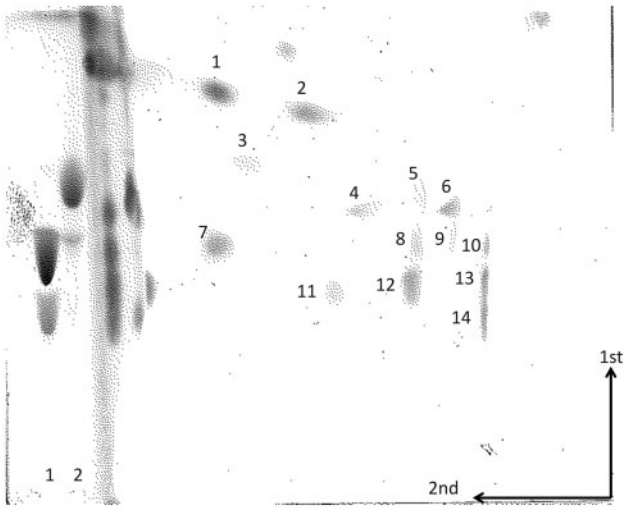

Fig. 3. Analysis by 2D-TLC of ^{14}C-labelled lipids from *C. botulinum* strain Beluga E. The lipids were subjected to hydrolysis with HCl fumes between the first and second chromatographies. Based on their positions and staining, the major lipids were tentatively identified as: 1, monoglycosyldiacylglycerol; 2, unknown phospholipid; 3, unknown phospholipid; 4, CL; 5, PG; 6. lyso-CL derived from PlaCL; 7, unknown (phosphorus-negative, ninhydrin-negative); 8, PE; 9, unknown; 10, lyso-PE derived from PlaE; 11, unknown (phosphorus-negative, ninhydrin-negative); 12, PG derived from PGAPlaE; 13, lyso-PE derived from PGAPlaE and lyso-PG derived from PlaGAPlaE; 14, lyso-PE derived from GAPlaE. Spots at the solvent front of the second dimension are aldehydes and their condensation products derived from the plasmalogens. A slightly slower-moving product is seen in the lane containing PGAPlaE and PlaGAPlaE. Standards on the left side were: 1, PE and GA; 2, PG. The arrows indicate the 1st and 2nd dimensions.

DISCUSSION

Polar lipid compositions clearly distinguish groups I, II and III strains of *C. botulinum*. Group I strains are closely related to *C. sporogenes* and more distantly related to *C. tetani* (Collins & East, 1998; Hill *et al.*, 2007; Sebaihia *et al.*, 2007). In addition to PG, CL and PE, phospholipids that are found in all strains of *C. botulinum* that we have examined, group I strains contain low amounts of EtnP-GlcNAcDRG, which was detected by MS of the total lipids. This phosphoethanolamine-modified diradylglycerol glycolipid was initially characterized in *C. tetani* (Johnston *et al.*, 2010). It is also found in *C. sporogenes* (Fig. 1). Since there is no rapid method to date for detecting this lipid, it would not serve as a useful diagnostic tool for this group at this time.

The saccharolytic group II strains cluster with *C. acetobutylicum*, *C. beijerinckii* and *C. butyricum* (Collins *et al.*, 1994; Hill *et al.*, 2007; Collins & East, 1998). The lipids that are unique to this group of organisms are glycerol acetals and PG acetals of PlaE (Goldfine & Johnston, 2005), and these lipids were found in the three group II strains of *C. botulinum* that we examined. These lipids are readily detectable on 2D-TLC of the total lipids, as they have distinct mobilities and produce specific products of HCl hydrolysis when plates are exposed to HCl fumes prior to chromatography in the second dimension (Fig. 3). *C. acetobutylicum* and *C. beijerinckii* are noted for their ability to produce acetone and butanol, and have been used for industrial production of these compounds (Jones & Woods, 1986; Keis *et al.*, 2001). In these solventogenic bacteria the conversion of PlaE to GAPlaE, possibly by way of the intermediate PGAPlaE, serves to stabilize the bilayer arrangement of the cell membrane in response to the

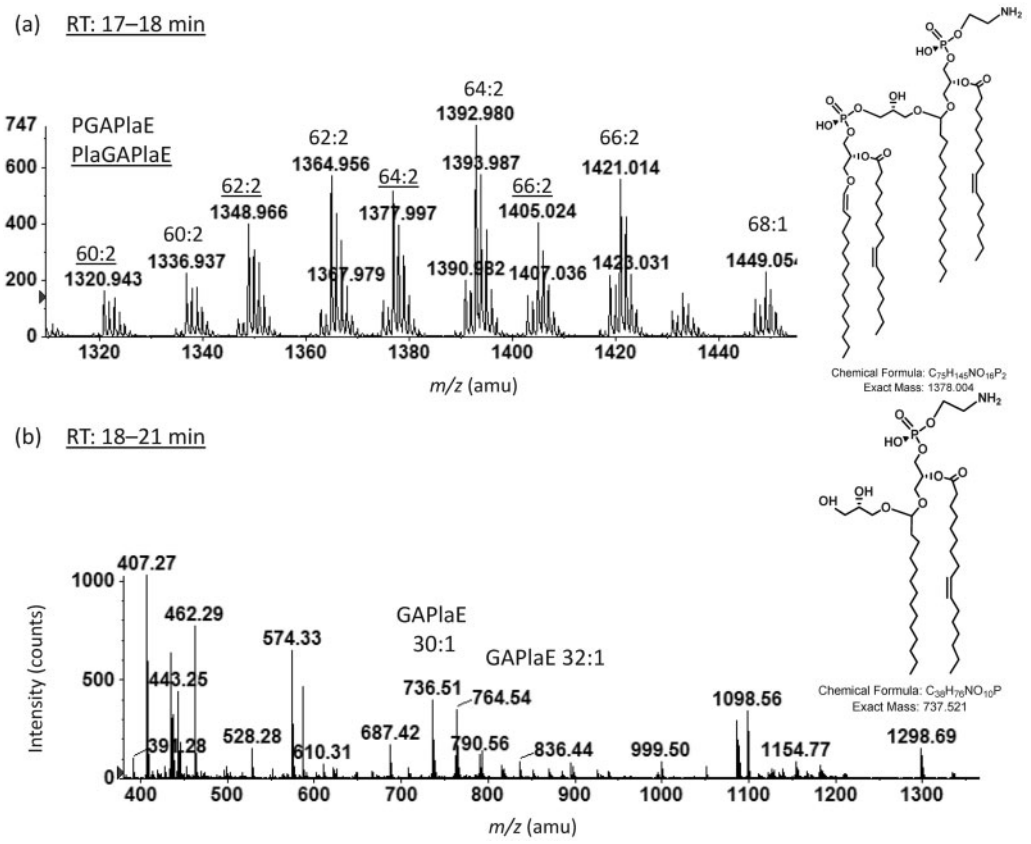

Fig. 4. (a) Negative ion ESI/MS spectra of PG and PlaG acetals of PlaE. PlaGAPlaE species are underlined. The structure of PlaGAPlaE (64 : 2) is shown. (b) Glycerol acetals of PlaE (GAPlaE) from *C. botulinum* strain Beluga E. The structure of GAPlaE (30 : 1) is shown. The number before the colon is the sum of the chain lengths and the number after the colon is the number of double bonds.

formation of solvents (MacDonald & Goldfine, 1990, 1991; Goldfine, 2010a; Goldfine *et al.*, 1987). The retention of these lipids in *C. botulinum* group II strains suggests that they meet similar challenges during and after growth in sugar-containing substrates.

The two group III strains, D1873 and Africa C, like *C. novyi* to which they are related, contain aminoacyl-PGs. The gene products responsible for the biosynthesis of aminoacyl-PG have been designated MprF (multiple peptide resistance factors), and are considered to be virulence factors that control cellular permeability to cationic antibiotics by transferring an amino acid from tRNA to the free distal hydroxyl group of the glycerol moiety of PG. Roy and Ibba found 117 sequences of MprF-related proteins in 22 genera of Gram-positive bacteria (Roy & Ibba, 2008). MprF is also widely distributed in Gram-negative species. In Gram-positive bacteria MprF is mostly found in Firmicutes (bacilli and clostridia), but not in Mollicutes. It is also found in Actinobacteria (Roy & Ibba, 2008).

MprF of *C. novyi* NT is 93 % identical to that of *C. botulinum* type C, 80 % identical to that of strain BKT015925, and 78 % identical to that of D strain 1873. We should also note that lysyl-PG is also present in the group II strain Alaska E.

There are only a few known strains of *C. botulinum* in group IV which produce BoNT/G (Suen *et al.*, 1988). They are related to *C. subterminale*, *C. proteolyticus*, *C. argentinense* and *C. schirmacherense* (Hill *et al.*, 2007). We have analysed strain G89, and found that it contains PE, PG and CL, some minor lipids, but none of the distinguishing polar lipids. None of these genomes has been sequenced, making it difficult to predict their phospholipid-synthesizing capabilities.

Notably, all strains of *C. botulinum* that we have examined have varying amounts of plasmalogen, as demonstrated both by 2D-TLC with HCl treatment and by MS. The only earlier report concerning the polar lipids of *C. botulinum* was on strain NCIB 4270 grown at 30 °C. This non-proteolytic strain, which was isolated by the Torry Research Station from sea-bottom deposits off the Scandinavian coast prior to 1967 (Hodgkiss *et al.*, 1967), contained PE, PG, CL, phosphatidylserine and a phosphorus-containing glycolipid of unknown structure (Evans *et al.*, 1998). None of these lipid classes had plasmalogen equivalents. A similar situation has been observed in *C. tetani*, in which one strain was found

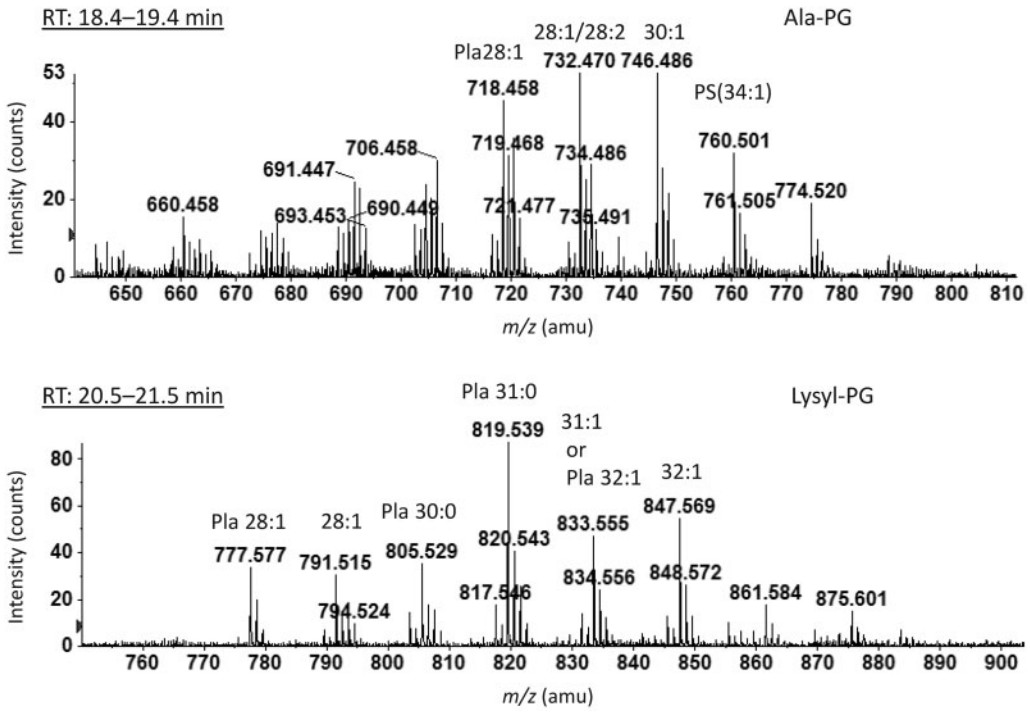

Fig. 5. Negative ion ESI/MS spectra of Ala-PG and Lys-PG from *C. botulinum* strain D1873. Pla, plasmalogen species. The number before the colon is the sum of the chain lengths and the number after the colon is the number of double bonds.

to be negative for plasmalogens, but four other strains were positive. The loss of plasmalogen-synthesizing capabilities has been noted in laboratory-passaged strains (Johnston *et al.*, 2010). Another possibility is that the cool marine environment permitted the loss of plasmalogens. It would be interesting to examine other clostridia isolated from this environment. Clearly, other *C. botulinum* strains with various histories have retained this capacity.

It has been acknowledged for several decades that 16S rRNA sequencing and more recently total genome sequencing provide the best evidence for taxonomic relationships among bacteria and Archaea. However, lipid analysis can provide significant additional information about these relationships (Goldfine, 1982). Indeed, before the distinct evolutionary position of Archaea was shown by 16S rRNA sequencing (Woese & Fox, 1977), it was known that halophiles, later classified among Archaea, contain unique 2,3-diphytanyl-*sn*-glycerol ether lipids, characteristic of this group (Kates, 1972). The distinct nature of the lipids was one of the first clues that this group of organisms was very different from bacteria, which are largely characterized by lipids containing fatty acids, and in the case of anaerobes, by vinyl-ethers (Goldfine, 1982). Since most bacteria contain PG, CL and PE, taxonomic relationships are better shown with lipids that are found in relatively small clusters of organisms. In this paper we have shown that for *C. botulinum*, EtnP-GlcNacDRG, glycerol- and PG-acetals of PlaE, and aminoacyl-PGs represent polar lipids that are of diagnostic value in bacterial taxonomy.

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