1   **Lipidomic analysis of roseobacters of the pelagic RCA cluster and their response**

2   **to phosphorus limitation**

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17

18 **Abstract**

19 The marine roseobacter-clade Affiliated cluster (RCA) represents one of the most

20 abundant groups of bacterioplankton in the global oceans, particularly in temperate and

21 sub-polar regions. They play a key role in the biogeochemical cycling of various elements

22 and are important players in oceanic climate-active trace gas metabolism. In contrast to

23 copiotrophic roseobacter counterparts such as *Ruegeria pomeroyi* DSS-3 and

24 *Phaeobacter* sp. MED193, RCA bacteria are truly pelagic and have smaller genomes. We

25 have previously shown that RCA bacteria do not appear to encode the PlcP-mediated lipid

26 remodelling pathway, whereby marine heterotrophic bacteria remodel their membrane

27 lipid composition in response to phosphorus (P) stress by substituting membrane

28 glycerophospholipids with alternative glycolipids or betaine lipids. In this study, we report

29 lipidomic analysis of six RCA isolates. In addition to the commonly found

30 glycerophospholipids such as phosphatidylglycerol and phosphatidylethanolamine, RCA

31 bacteria synthesise a relatively uncommon phospholipid, acylphosphatidylglycerol, which

32 is not found in copiotrophic roseobacters. Instead, like the abundant SAR11 clade, RCA

33 bacteria upregulate ornithine lipid biosynthesis in response to P stress, suggesting a key

34 role of this aminolipid in the adaptation of marine heterotrophs to oceanic nutrient limitation.

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Introduction

The marine roseobacter group of *Alphaproteobacteria* comprises an ecologically important group of marine bacteria involved in the biogeochemical cycling of carbon, nitrogen and sulphur (Buchan et al., 2014; Luo & Moran, 2014). Roseobacters are metabolically diverse, being able to use an array of organic molecules, perform anoxygenic photosynthesis and produce secondary metabolites (Buchan et al., 2014; Brinkhoff et al., 2008). Arguably however, they are most well known for their role in marine trace gas formation, including the metabolism of methylated sulfur compounds like dimethylsulfide and dimethylsulfoniopropionate, and methylated amines, which has global significance (Lidbury et al., 2014; Mausz & Chen, 2019; Curson et al., 2011).

Using model marine roseobacters, we have previously studied membrane lipids in *Ruegeria pomeroyi* DSS-3 and *Phaeobacter* sp. MED193 (Sebastian et al., 2016; Smith et al., 2019). In addition to glycerophospholipids such as phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), these marine roseobacters have several lipids which are not widely reported, including amino-acid containing lipids like glutamine lipid (Smith et al., 2019). Many of these roseobacters are capable of remodelling their membrane lipid composition in response to environmental change, such as adaptation to P stress through a phospholipase (PlcP)-mediated lipid renovation pathway by substituting glycerophospholipids with non-phosphorus containing surrogate lipids (Sebastian et al., 2016). Indeed, some roseobacter clade bacteria, including *Phaeobacter* sp. MED193 can produce betaine-containing membrane lipids to replace glycerophospholipids in response to phosphorus limitation (Sebastian et al., 2016).

In contrast to the aforementioned strains, some marine roseobacters are truly pelagic with small genomes, which are known to be numerically abundant in marine surface waters, along with several other bacterial groups, notably the SAR11 clade (Luo & Moran 2014; Luo & Moran 2015; Sun et al., 2017; Giebel et al., 2011; Giovannoni 2017).

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62 One of such cosmopolitan pelagic roseobacter groups is the RCA (roseobacter clade

63 affiliated cluster) group which is particularly prevalent in the bacterioplankton in temperate

64 and polar regions of the oceans, where they can reach up to 35% of total bacterial counts

65 (Selje et al., 2004; Giebel et al., 2009; 2011; Billerbeck et al., 2016; Zhang et al., 2016).

66 RCA group bacteria were first isolated from the North Sea represented by the type strain

67 *Planktomarina temperata* RCA23 (Giebel et al., 2013). Other RCA isolates include

68 *Roseobacter* sp. LE17, isolated from an algal culture off the coast of California (Mayali et

69 al., 2008), three strains (IMCC1909, IMCC1923, IMCC1933) isolated from the Yellow Sea

70 (Giebel et al., 2013) and, more recently, three RCA strains obtained using high-throughput

71 dilution-to-extinction culturing method using seawater from the East China Sea (strains

72 FZCC0023, FZCC0040 and FZCC0043) (Zhang et al., 2019). The genome sequence of

73 strains RCA23 and LE17 has been reported and, interestingly, neither genome contains

74 the genes involved in PlcP-mediated lipid remodelling, which are prevalent in other marine

75 roseobacters (Sebastian et al., 2016). Furthermore, three single-cell amplified genomes

76 (SAGs) belonging to the RCA were recently reported, none of which encodes the PlcP

77 enzyme (Sun et al., 2017). This prompted us to characterise the intact membrane lipids in

78 this ecologically important group of pelagic marine bacteria.

79

80 **Results and Discussion**

81 We brought together six RCA strains isolated from geographically distinct locations around

82 the world (**Figure 1**). Due to their unique growth requirements it was not feasible to use

83 the same growth medium to cultivate all six strains. Indeed, genome sequence and

84 comparative genomics studies have previously shown that each RCA strain has a unique

85 requirement for vitamins (Voget et al., 2015; Zhang et al., 2019). Thus, strains RCA23,

86 LE17 and IMCC1933 were cultivated in a diluted marine broth medium (40% w/v marine

87 broth in seawater), whereas the strains isolated from the East China Sea (strains

88   FZCC0023, FZCC0040 and FZCC0043) do not grow in marine broth or diluted marine

89   broth medium. Instead, they were grown in autoclaved natural seawater amended with

90   mixed vitamins, ammonium (1 mM), phosphate (100 µM), iron and a mixed carbon source

91   (Zhang et al., 2019). To determine the impact of P depletion on the lipid composition in

92   these latter three RCA strains, they were also grown on the same natural seawater

93   amended medium without adding additional phosphate. The final cell density of these six

94   RCA strains before harvest is shown in **Figure 1B**. Without phosphate, the final cell

95   density only reached ~10^7 cells/ml whereas with phosphate amended, they reached ~10^8

96   cells/ml or more (**Figure 1B**), suggesting P can be a limiting factor for their growth.

97   The intact membrane lipids were extracted from these six bacterial cultures using

98   a modified Folch method and their lipid composition analysed by liquid chromatography

99   coupled with mass spectrometry (LC-MS) in both positive (+ve) and negative (-ve)

100   ionisation mode (Smith et al., 2019). These lipids were separated using a hydrophilic

101   interaction column prior to MS fragmentation and subsequent identification and

102   quantification. Overall, four major phospholipids (phosphatidic acid (PA),

103   phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and acyl-PG (APG)) and one

104   aminolipid (ornithine-containing) were consistently identified in these RCA bacterial

105   isolates (**Table 1**). A representative LC-MS chromatograph of lipids from the RCA type

106   strain (RCA23) is presented in **Figure 2**. The major fatty acids in these

107   glycerophospholipids are C16:1, C18:1 and C19:1 (Table S1) although their composition

108   varies depending on the individual lipids (top panel). This is consistent with previous

109   estimations of fatty acid methyl esters by gas chromatography, showing that the two

110   monounsaturated C16:1 and C18:1 species accounted for more than 70% of the total fatty

111   acids in strain RCA23 (Giebel et al., 2013).

112   In addition to PG, PE and a small amount of PA, we also observed phospholipids

113   that eluted at 3.9 mins with a m/z ranging from 925.8-967.8 (**Table 1, Figure 2**). To

5

114   elucidate the identity of these lipids, we isolated the major ion species with a m/z of 925.8

115   for further fragmentation analysis in the negative (-ve) ionisation model (**Figure 3**). The

116   MS^n fragmentation pattern suggests that this lipid is a modified C34:2 PG (C16:1/C18:1)

117   by acylation (APG) with a third acyl fatty acid R3' of C12:1. Interestingly, 12-carbon fatty

118   acids were previously found in RCA23 using gas chromatography analysis of fatty acid

119   methyl esters (Giebel et al., 2013). APG lipids are rarely reported as a major lipid in

120   bacteria (Yague et al., 1997; Hines & Xu 2019; Luo et al., 2018). Indeed, APG lipids were

121   not detected in our previous studies of the lipidomes of marine roseobacters, including

122   *Ruegeria pomeroyi* DSS-3 and *Phaeobacter* sp. MED193 (Sebastian et al., 2016; Smith

123   et al., 2019) nor in pelagic SAR11 clade bacteria (Carini et al., 2015). APG is produced as

124   a minor lipid in *E. coli* through direct acylation of the headgroup of PG using an acyl donor,

125   with the outer membrane lipase PldB involved in generating this donor (Nishijima et al.,

126   1975; Hines & Liu, 2019). Indeed, the genome sequences of RCA isolates RCA23 and

127   LE17 also contain a PldB homologue showing 30% sequence identity (e^{-20}) to that of *E.*

128   *coli*, suggesting that APG in RCA strains is synthesized in a similar manner. However, it

129   should be noted that the role of PldB is not limited to APG biosynthesis. Indeed, PldB of

130   *Sinorhizobium meliloti* (SMc04041) also has thioesterase activity which is able to

131   hydrolyse palmitoyl-CoA (Sahonero-Canavesi et al., 2015). Clearly the pathway for APG

132   biosynthesis in RCA bacteria warrants further investigation.

133   In addition to these phospholipids, these RCA isolates also produced aminolipids.

134   Thus, ornithine lipid (OL) eluted at 13 min, a feature consistent with the production of OLs

135   in *R. pomeroyi* DSS-3 and *Phaeobacter* sp. MED193 (Smith et al., 2019). OLs in these

136   RCA isolates comprise two major species of m/z 703.5 and 717.5 respectively, and their

137   fragmentation pattern is consistent with a fatty acid composition of 3-hydroxyl20:1/C18:1

138   and 3-hydroxyl20:1/C19:1, respectively. Like *R. pomeroyi* DSS-3, the genomes of RCA

139   isolates RCA23 and LE17, and SAGs AB-661-I11 and AB-661-L17, all contain a two gene

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140 cluster *olsA* and *olsB* encoding the *O*-acetyltransferase and *N*-acetyltransferase,

141 respectively, which is likely responsible for the synthesis of OL. Glutamine lipid, which was

142 observed in both *R. pomeroyi* DSS-3 and *Phaeobacter* sp. MED193, was not found in

143 these RCA bacteria despite the fact that the *glsB* gene, which was previously shown to be

144 essential for glutamine lipid biosynthesis in *R. pomeroyi* DSS-3, is present in their

145 genomes (Smith et al., 2019).

146   To gain an insight into the role of P availability in modifying the lipidome of RCA

147 bacteria, we used the three strains isolated from the East China Sea (strains FZCC0023,

148 FZCC0040 and FZCC0043) and grew them in autoclaved natural seawater amended with

149 100 µM phosphate or without phosphate, as described previously (see Zhang et al., 2019).

150 Analysis of the lipidome revealed no statistical difference (student *t*-test, p=0.31) in the

151 relative abundance of the APG lipids. However, OLs were significantly more abundant

152 (student *t*-test, p=0.01) in the P-stressed cultures, suggesting a role of OLs in response to

153 P stress (**Figure 4**). It should be noted that, due to the lack of commercial standards of

154 OL, only the relative abundance of OL/PG is analysed in this study, which does not take

155 into account the potential differences in ionisation efficiency amongst different lipid classes.

156 Nevertheless, a similar response of up-regulation of OLs in response to P stress was also

157 observed in the marine heterotroph SAR11 strain HTCC7211 (Carini et al., 2015;

158 Sebastian et al., 2016). In SAR11 clade bacteria, lipid renovation in response to P stress

159 also involves the formation of several glycolipids, which are synthesized to replace

160 glycerophospholipids. A bifunctional glycosyltransferase (Agt) is responsible for the

161 formation of monoglycosyldiacylglycerol (MGDG) and glucuronic acid diacylglycerol

162 (GADG). However, no homologues of Agt were present in the genomes of RCA clade

163 bacteria and these glycolipids were also absent in their lipidomes in our analysis.

164   Together, the data presented in this study reports the lipidome of six strains of RCA

165 bacteria isolated from widely different geographical locations. These bacteria produce a

166 relatively uncommon phospholipid, APG, although this lipid does not appear to play a role

167 in the response to P stress. On the other hand, all RCA bacteria produce an ornithine-

168 containing aminolipid which was significantly up-regulated under P stress. How these OLs

169 facilitate membrane homeostasis in response to abiotic and biotic stresses certainly

170 warrants further investigation.

171

172

173 **Materials and Methods**

174 **Bacterial strains and cultivation**

175 Strains RCA23 (North Sea), IMCC1933 (Yellow Sea) and LE17 (Pacific, West coast) were

176 grown in 50 ml 40% (w/v) marine broth medium at 15°C in light-dark cycles for 20 days as

177 described by Giebel et al. (2013). End point cell density was obtained by using a BD Accuri

178 C6 flow cytometer (BD Biosciences, San Jose, CA, USA) after the protocol of Giebel et al.

179 (2019). Cell pellets were harvested from a 50 ml culture by centrifugation at 4°C. Cells

180 were stored at -80°C prior to lipid extraction.

181 Strains FZCC0023, FZCC0040 and FZCC0043 were isolated from the East China

182 Sea and do not grow in marine broth or diluted marine broth medium. The seawater-based

183 medium used for culturing these strains was prepared as follows. Coastal seawater was

184 filtered using a 0.2-μm pore-size filter and autoclaved for 90 min. After autoclaving,

185 seawater was sparged with 0.1 μm-filtered CO_2 for 6 h followed by aeration overnight.

186 After autoclaving and sparging, the seawater was amended with 1 mM NH_4Cl, 1 μM $FeCl_3$,

187 mixed carbon sources (Cho and Giovannoni, 2014) and vitamins (Carini et al., 2013).

188 Cultures were grown in one of the following conditions: (i) phosphate-addition (100 μM

189 K_2HPO_4 added); or (ii) without additional phosphate (no phosphate added) (Zhang et al.,

190 2019). Cultures were grown in the dark at 23℃ without shaking. The cells were not washed

191 after pre-culture; instead, the bacteria were inoculated into the same medium (0.2% v/v)

192 and the cell density was monitored using a Guava EasyCyte flow cytometer before

193 harvesting (Merck Millipore, Billerica, MA, USA). Cell pellets were harvested from 10 ml

194 culture by centrifugation which were stored at -80°C prior to lipid extraction.

195

Lipid extraction and HPLC-MS

197 Lipid extraction from 10 ml bacterial culture was carried out using a modified Folch

198 extraction method (Folch et al., 1957) using HPLC-grade chloroform (1 ml), Milli-Q water

199 (0.3 ml) and LC-MS grade methanol (0.5 ml) in a 2 ml glass Chromacol vial (Smith et al.,

200 2019). After phase separation by centrifugation, the lower chloroform phase containing

201 the lipids was dried under nitrogen before resuspending in 0.5-1 mL of solvent (0.05 ml of

202 10 mM ammonium acetate in water, pH 9.2 and 0.95 ml acetonitrile). The lipid d17:1/12:0

203 sphingosylphosphoethanolamine (Sigma-Aldrich, 50 nM) was added to the samples and

204 used as internal standard. Five µl of the lipid extract was injected onto the LC-MS and

205 separated by a Dionex 3400RS HPLC using a hydrophilic interaction column (XBridge

206 BEH amide XP column 2.5 µm 3.0x150 mm, Waters) according to their polar headgroup.

207 Samples were run on a 15 min gradient from 95% (v/v) acetonitrile/5% (w/v) ammonium

208 acetate (in water, 10 mM, pH 9.2) to 70% (v/v) acetonitrile/30% (w/v) ammonium acetate

209 (in water, 10 mM, pH 9.2), followed by 5 minutes of isocratic run 70% acetonitrile/30%

210 ammonium acetate. Ten minutes equilibration at the initial run condition were performed

211 between samples. The flow rate was maintained at 150 µL min^{-1} and the column

212 temperature at 30 °C. The injection volume was 5 µL for each run; the ionisation was done

213 in both positive and negative mode. Drying conditions were the same for both modes (8 L

214 min^{-1} drying gas at 300 °C; nebulising gas pressure of 15 psi), while the end cap voltage

215 was 4,500 V in positive mode and 3,500 V in negative mode, both with 500 V offset.

216 Identification of lipid classes was made through MS^{n} fragmentation using the amaZon SL

217 ion trap mass spectrometer. Data analyses were carried out using the Bruker Compass

218 Software with DataAnalysis for peak identification and lipid MS^n fragmentation and

219 QuantAnalysis for lipid quantification against internal standard

220 sphingosylphosphoethanolamine (SPE). The abundance of each lipid was normalized

221 against sphingosylphosphoethanolamine and expressed as relative abundance against

222 phosphatidylglycerol (PG).

223

224 **Bioinformatics and statistics**

225 The genome sequences of strains RCA23 and LE17 and the single-cell amplified

226 genomes (SAGs) of AB-661-I11, AB-661-L17 and AB-661-M21 were analysed using the

227 JGI IMG portal (https://img.jgi.doe.gov/). Genomes were searched for the presence of the

228 following genes involved in lipid renovation: *plcP* (MED193_17359), *olsA* (SPO1979), *glsB*

229 (SPO2489) and *olsB* (SPO1980). Student-*t* tests were performed using Excel version

230 2017.

231

232 **Acknowledgements**

237

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320

Figure legends

Figure 1 RCA isolates used in this study **A)** Geographical location of the six RCA strains; **B)** Culture medium used for each strain and the final cell density before harvesting.

Figure 2 Major lipids detected in the RCA bacterium, type strain RCA23. An overview of the LC-MS chromatogram showing the major lipids extracted from strain RCA23. Lipids were analysed in both negative (- ve) and positive (+ ve) ionisation mode. The pie chart above each lipid class shows the major intact lipid species within the class. The colours represent the sn-1/sn-2 fatty acid composition in the phospholipids. Two major ornithine lipids were found in negative ionisation mode (see Table 1) with a mass-to-charge ratio of 703.7 and 717.7, respectively. A list of all lipids detected in other RCA strains are shown in supplementary Table S1. PA, phosphatidic acid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; OL, ornithine lipids; APG, acyl-PG.

Figure 3 Fragmentation pattern of APG lipids eluted at 3.6 min and the proposed structure of the major APG lipids in these RCA bacterial isolates. Three APG lipids (Table 1) are found in all RCA strains with a mass-to-charge ratio of 925.8, 953.8 and 967.8, respectively. The mass spectra shown represents the APG lipids from the type strain RCA23. The dominant APG lipid species with a m/z ratio of 925.8 was selected for further fragmentation (MS^2) which produces a lyso-PA species (m/z 417). This lyso-PA was further fragmented (MS^3) to produce a C18:1 fatty acid and a characteristic ion of m/z 153. The mass difference between APG and the corresponding PG was 180.3, suggesting that the R3' chain is C12:1 fatty acid.
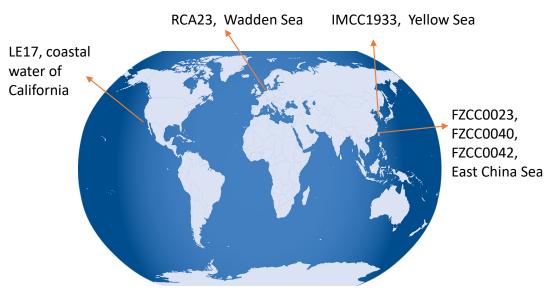
346   **Figure 4** Box-whisker plot of the relative abundance of (A) APG and (B) OL lipids (relative

347   to PG) in medium with and without phosphorus supplementation. The line in the middle of

348   the box represents the mean whereas the symbol x represents the median. Data were

349   from the three isolates (n=3) obtained from the East China Sea (strains FZCC0023,

350   FZCC0040 and FZCC0043). Student-*t* tests were performed in Excel. PG,

351   phosphatidylglycerol; OL, ornithine lipids; APG, acyl-PG.

352

Table 1 Major lipids identified from RCA bacteria

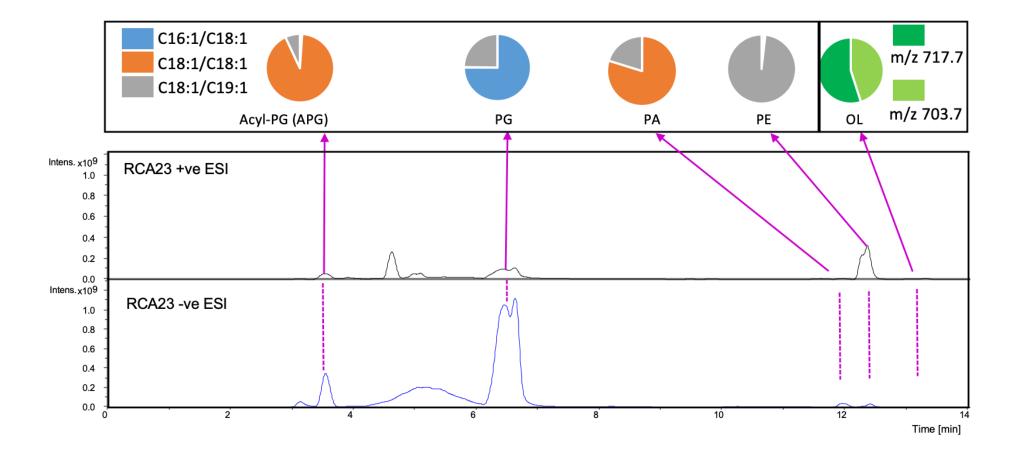| Retention time	Lipid class	m/z	Identity	Fatty acid species
3.6 min	Acyl-PG (APG)	925.8	[M-H]$^-$	C18:1/C16:1/C12:1
		953.8	[M-H]$^-$	C18:1/C18:1/C12:1
		967.8	[M-H]$^-$	C18:1/C19:1/C12:1
6.5 min	PG	745.7	[M-H]$^-$	C16:1/C18:1
		773.7	[M-H]$^-$	C18:1/C18:1
		787.7	[M-H]$^-$	C19:1/C18:1
11.3 min	PA	729.7	[M+Acetate]$^-$	C16:1/C18:1
		757.7	[M+Acetate]$^-$	C18:1/C18:1
		771.7	[M+Acetate]$^-$	C19:1/C18:1
11.8 min	PE	714.7	[M-H]$^-$	C16:1/C18:1
		742.7	[M-H]$^-$	C18:1/C18:1
		756.7	[M-H]$^-$	C19:1/C18:1
13.0 min	OL	703.7	[M-H]$^-$	OL3-OH20:1/C18:1
		717.7	[M-H]$^-$	OL3-OH20:1/C19:1

PG: phosphatidylglycerol; APG, acyl-phosphatidylglycerol; PA: phosphatidic acid; PE: phosphatidylethanolamine; OL, ornithine lipid.
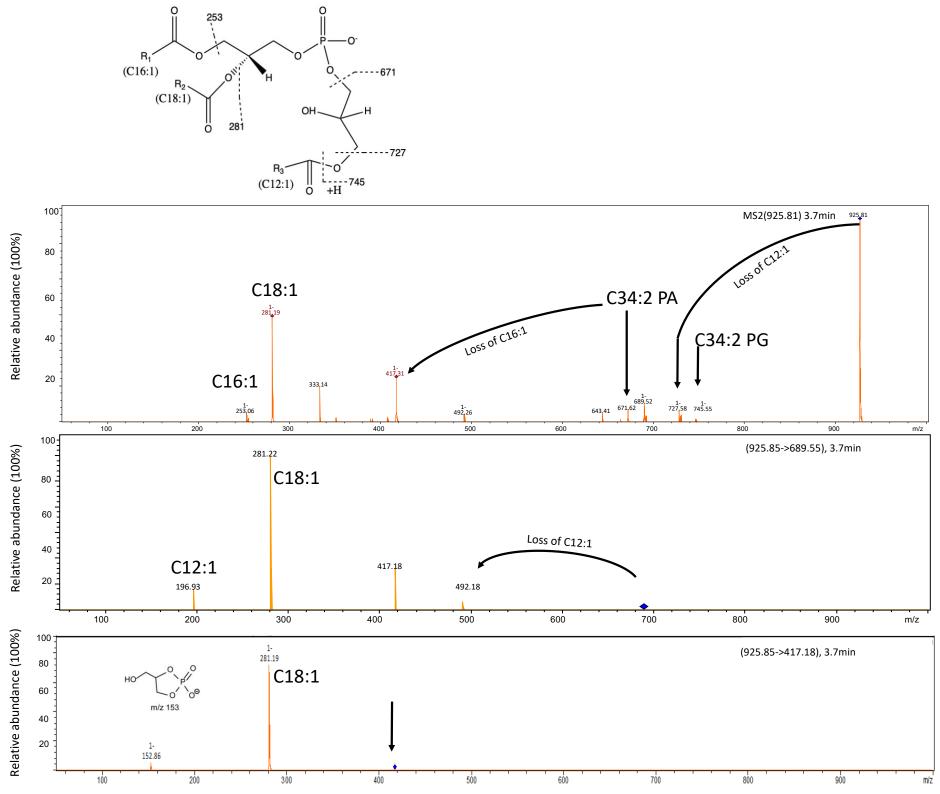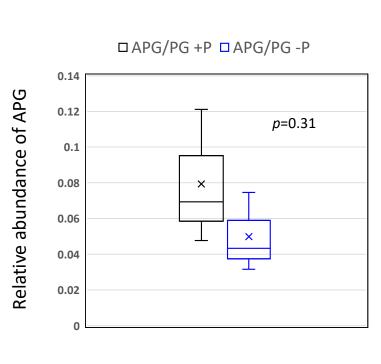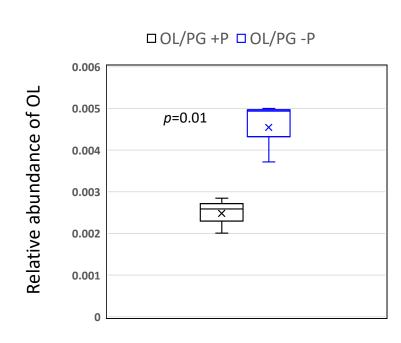
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A)

RCA23, Wadden Sea   IMCC1933, Yellow Sea

LE17, coastal water of California

FZCC0023, FZCC0040, FZCC0042, East China Sea

B)

Strains	Medium	Final cell density (ml^{-1})
RCA23	40% marine broth	6 X 10^9
IMCC1933	40% marine broth	4 X 10^9
LE17	40% marine broth	5 X 10^8
FZCC0023	Natural seawater	3 X 10^7
	Natural seawater + phosphate	1 X 10^8
FZCC0040	Natural seawater	2 X 10^7
	Natural seawater + phosphate	7 X 10^8
FZCC0042	Natural seawater	4 X 10^7
	Natural seawater + phosphate	7 X 10^8

A)

□ APG/PG +P □ APG/PG -P

Relative abundance of APG

p=0.31

B)

□ OL/PG +P □ OL/PG -P

Relative abundance of OL

p=0.01