1 # Biodiversity and habitats of polar region polyhydroxyalkanoic acid-producing

2 ## bacteria: bioprospection by popular screening methods

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12 **Abstract**

13 Polyhydroxyalkanoates (PHA), the intracellular polymers produced by various

14 microorganisms as carbon and energy storage, are of great technological potential as

15 biodegradable versions of common plastics. PHA-producing microbes are therefore in great

16 demand and a plethora of different environments, especially extreme habitats, have been

17 probed for the presence of PHA-accumulators. However, polar region have been neglected in

18 this regard, probably due to the low accessibility of the sampling material and unusual

19 cultivation regime. Here, we present the results of a screening procedure involving 200

20 bacterial strains isolated for 25 habitats of both polar regions. Agar-based, microscopy and

21 genetic tests were conducted to elucidate the biodiversity and potential of polar-region PHA-

22 accumulators. Microscopic observation of Nile Red stained cells proved to be the most

23 reliable screening method as it allowed to confirm the characteristic bright orange glow of the

24 Nile Red – PHA complex as well as the typical morphology of the PHA inclusions.

25 Psychrophilic PHA-producers belonged mostly to the *Comamonadaceae* family

26 (Betaproteobacteria) although actinobacterial PHA synthesizers of the families

27   *Microbacteriaceae* and *Micrococcaceae* also featured prominently. Glacial and postglacial

28   habitats as well as developed polar region soils were evaluated as promising for PHA-

29   producer bioprospection. This study highlights the importance of psychrophiles as a

30   biodiverse and potent polyhydroxyalkanote sources for scientific and application-aimed

31   research.

32   **Keywords:** psychrophiles, Nile Red, *Comamonadaceae*, glacier, feast/famine regime

33   **Introduction**

34   Polyhydroxyalkanoates (PHA) are a group of intracellular polymers synthesized by a

35   variety of prokaryotic microorganisms. Their primary function is that of a carbon and energy

36   storage to be used in starvation periods. They are produced from the excess carbon present in

37   the cells environment and consist of polyesters of hydroxyalkanoic acids with chains of

38   varying lengths. Key enzymes conducting the polymerization process are the PHA synthases

39   encoded by the *phaC* gene. Currently, there are four classes of this enzyme discovered and

40   described. Amino acid sequence overlap between enzymes representing different classes is

41   moderate at best, albeit their products can be divided into scl-PHA – short side chain length

42   PHAs produced mainly by the actions of the I, III and IV class synthases and mcl-PHAs –

43   medium side chain length PHAs produced mainly by the class II synthases harbored *inter alia*

44   by the *Pseudomonas* genus (Reddy *et al*. 2003; Rehm and Steinbüchel 1999).

45   Polyhydroxyalkanoic acids are of great technological potential, because they are seen

46   as possible petroleum based plastic substitute: biodegradable, biocompatible and derived from

47   biowaste products (Chee et al. 2010). Therefore, PHA producing microbes are highly sought

48   after in terms of quality and quantity of the synthesized polymer (Kumar et al. 2020).

49   Screening procedures involve mainly the detection of the PHA granule themselves and/or the

50   detection of genes coding the PHA synthesis pathway enzymes (Kung et al. 2007). Staining of

51   the granules with lipophilic dyes is the most popular of the methods, with the fluorescent Nile

52 Red stain being frequently used to differentiate PHA positive bacterial colonies on a Petri dish

53 or to visualize granules within bacterial cells using an adequately equipped microscope. The

54 PHA positive strains display a bright orange glow when irradiated with UV or green (510-560

55 nm) light (Spiekermann et al. 1999; Kitamura and Doi 1994). Genetic screening involves

56 mainly the PCR based amplification of a fragment of the *phaC* gene using degenerate primers

57 (Romo et al. 2007).

58   PHA producing bacteria can be found in a variety of environments, mainly those that

59 experience periodic nutrient limitations, as the granule-stored carbon helps to cope with

60 starvation issues (Alistair et al. 1990). Studies conducted on the survival of PHA-containing

61 bacterial cells reveal the involvement of these polymers in cell recovery from a number of

62 other abiotic stressors (Obruca et al. 2020). Therefore, habitats where the intensity of physical

63 and chemical factors change frequently have the potency to enrich the microbial community

64 in most resilient cells, *inter alia* in PHA producers (Pernicowa et al. 2020). Polar region

65 habitats seem in this regard to be a very promising source of novel PHA synthesizing bacterial

66 strains (Ciesielski et al. 2014; Ayub et al. 2004). Polar and circumpolar regions experience a

67 severe case of seasonal nutrient inputs caused by the occurrence of the yearly polar day/night

68 phenomenon. Furthermore, several other factors (not only low temperatures) such as: water

69 activity shifts, freeze-thaw cycles, intense UV radiation, free radical formation and sudden pH

70 changes also contribute to the harshness of this environment (Varin et al. 2012). Therefore,

71 prospecting for polar region cold-loving bacterial PHA producers might prove very fruitful.

72 Such endeavors have to date been undertaken only in a limited respect, presumably due to the

73 low accessibility of the sampling material and unusual cultivation regime (Koller, 2017).

74   In this study we present a screening of a 200 bacterial isolates derived from 25

75 different habitats all located in polar regions of both Arctic and Antarctica done by popular

76 testing methods. The aim of these analyses was to evaluate the factual potential of those

microbes to produce PHAs and additionally to find the most accurate method to do so. Our hypothesis states, that polar region habitats harbor a phylogenetically and metabolically diverse cultivable bacteria communities capable of PHA production at low temperatures.

Materials and methods

Strain cultivation

Bacterial strains used in this study (See Tab. 1 for details) are part of the Central Collection of Strains of Institute of Biochemistry and Biophysics Polish Academy of Sciences. The strains are stored as glycerol stocks at -80°C. They were revived by streaking aliquots of the stocks on an appropriate medium. For the saltwater isolates R3A medium was used containing (g/L): Peptone -1, Tryptone – 1, Yeast extract – 1, Beef extract – 1, Glucose – 1, K_2HPO_4 – 1, NaH_2PO_4 – 0.5, $MgSO_4$ – 0.1, prepared with artificial seawater (g/L): NaCl – 27.5, $MgCl_2·6H_2O$ – 5.38, $MgSO_4·7H_2O$ – 6.78, KCl – 0.72, $NaHCO_3$ – 0.2, $CaCl_2·2H_2O$ – 1.4. For all other strains the R3A medium (agar and broth) was prepared with distilled water. The plates were incubated at 15°C in the dark (Liebher Thermostat Cabinet) for a period of 3 weeks. Strains were checked for purity by repeated transfer to fresh agar plates. Single colonies were picked and transferred to test tubes with 3 mL of cool, sterile R3A broth and incubated for 1 week to attain a saturated bacterial culture. The cultures were the basis for further research.

Agar based screening

The R3A agar plates for PHA screening were prepared according to Spiekermann et al. 1999. Stock solutions of Nile Blue A and Nile Red dyes were prepared in DMSO and added to the agar *post* autoclaving to give a final concentration of 0.5 μg dye per mL of medium. Strains were drop plated (5 μL) from the active culture onto the plates, with controls on R3A agar without the dyes. After 1 week of incubation at 15°C the plates were exposed to

101 ultraviolet light (312 nm) and the drop-spots were examined for the bright orange

102 fluorescence indicative for PHA presence. Those exhibiting the glow were scored as positive.

103 *DNA-based analysis*

104   For DNA extraction from bacterial cells 100µL of the liquid culture was centrifuged at

105 12000 rpm for 3min. in a 2 mL Eppendorf type tube and the pellet was suspended in 200µL of

106 sterile MiliQ water and a small amount (approx. 10% w/v) of Chelex100 resin and sharp

107 garnet sand were added. The suspension was further amended with 1.5µL of lysozyme

108 solution (10 mg/mL) and incubated at 37°C for 2.5h. Then 10µL of a 10% SDS solution was

109 added to the suspension and the tubes were placed in a Qiagen Retsch TissueLyser II for 5

110 min. at 39Hz – 1800 oscillation per min. The tubes were centrifuged briefly, amended with

111 1µL proteinase K solution and incubated at 55°C for 1h. After centrifugation at 12000 rpm for

112 3min. the DNA in the supernatant was purified using the Clean-up Concentrator kit (A&A

113 Biotechnology) according to manufacturer's protocol. Yield and purity of the extracted DNA

114 was checked in a NanoPhotometer® NP80 (Implen).

115   Amplification of 16S rRNA gene fragment was performed using universal primers

116 27F and 1492R (Lane 1991). PCR amplification reaction conditions were as follows: 1 min of

117 95 °C initial denaturation followed by 30 cycles of 95 °C for 15 s, 55 °C annealing for 15 s

118 and elongation 72 °C for 1min and 30 seconds, using DreamTaq polymerase (Thermo

119 Scientific-Fermentas). Obtained PCR products (~1500 bp for 16S rRNA gene fragment) were

120 checked on 0,8% agarose gel and purified using Clean-up Concentrator kit (A&A

121 Biotechnology). 16S rRNA gene amplicons were sequenced using the 27F 16S rRNA gene

122 primer with the use of BigDye Terminator v.3.1 chemistry and ABI3730xl genetic analyzer at

123 the DNA Sequencing Laboratory (Institute of Biochemistry and Biophysics PAS).

124   Amplification of *phaC* gene fragment was performed using primers G-D

125 (5'GTGCCGCCSYRSATCAACAAGT3')   and   G-1R

126 (5'GTTCCAGWACAGSAKRTCGAA3') by Romo et al. 2007 targeting both the type I and

127 type II synthase gene. PCR amplification reaction conditions were as follows: 1 cycle of 94ºC

128 for 10 min, 60ºC for 2 min and 72ºC for 2 min followed by 40 cycles at 94ºC for 20, 55.5ºC

129 for 45 sec and 72ºC for 1 min and a final cycle at 72ºC for 5 min using DreamTaq polymerase

130 (Thermo Scientific-Fermentas). Obtained PCR products (~550 bp for *phaC* gene fragment)

131 were checked on 0,8% agarose gel. Samples containing the desired length product were

132 scored as positive.

133 *Feast-famine regime implementation*

134   Three types of PHA-inducing media (PIM) were introduced to create a feast/famine

135 regime based on lowering the nitrogen to carbon ratio. The basis was a 7-fold diluted (0.5g/L)

136 R3A medium amended with several carbon sources. PIM1 contained carbon sources that were

137 unrelated to PHA structure: glucose (2.5g/L), sodium lactate (2.5g/L), glycerol (2.5g/L) and

138 sodium acetate (1g/L). PIM2 contained short-chain fatty acids: sodium butyrate and sodium

139 valerate (3.5g/L each). PIM3 contained a mix of medium-chain fatty acids derived from the

140 ultrasonic-initiated saponification of coconut oil (Mercantili et al. 2014). 10g of coconut oil

141 (generic brand) was amended with 30 mL of 1.5M NaOH (in excess) in a 50 mL Falcon-type

142 tube, heated in a water bath at 50°C until the oil completely melted. The ingredients were than

143 shaken for 5 min on a Tornado Vortexer until a homogenic emulsion was achieved. Then the

144 tube was placed in a VWR Ultrasonic Cleaner USC-TH set to 50°C and sonicated at 40kH for

145 45 min to facilitate the saponification reaction. The tube was placed in a thermostat at 37°C

146 for 6 weeks to allow the saponification reaction to proceed to completion. The saponified oil

147 was dissolved in ddH$_2$O, pH was adjusted to 5.0 with HCl upon which the free fatty acids

148 precipitated. The suspension was sedimented by centrifugation (10 min, 8000 rpms) and

149 washed three times with ddH$_2$O to remove the excess alkali and the glycerol byproduct. Free

150 fatty acids were air-died and suspended in water at a concentration of 7g/L with Tween80

151 (0.1g/L). The mixture was emulsified by heating to 45°C and vigorous shaking. All media

152 were adjusted to pH 7.2.

153   Bacterial cultures on R3A broth were harvested by centrifugation (0.5 mL) in separate

154 Eppendorf tubes, resuspended in 3ml of each of the PIMs and incubated at 14°C for 1 week.

155 Resulting suspension were subjected to microscopy based screening.

156 *Nile red staining and microscopy*

157   Bacterial suspensions were washed with a washing buffer (NaCl -9 g/L, methanol 10

158 g/L, Tween80 – 50 µg/L, tertrasodium pyrophosphate – 2.6 g/L) to remove media components

159 and slightly perforate cell walls for dye penetration. Bacterial cells were resuspended in 0.9%

160 saline and stained with Nile Red in DMSO (80µg/mL) to give a final concentration of 3.1

161 µg/mL for 30 min (Zuriani et al. 2013). Stained bacterial suspensions were trapped under a

162 microscope slide and observed under 1000x magnification with green (510-560 nm) and blue

163 light (450-490nm) excitation on a Nikon E-200 microscope with a 100 W Hg lamp and 100×

164 CFI 60 oil immersion objective, with a digital DS-Fi3 high-definition color microscope

165 camera equipped with a 5.9 megapixel CMOS image sensor and a filter block of wavelengths:

166 EX 330–380, DM 400, BA 420.

167 *Data analysis*

168   Calculations charts and graphs were made in Excel (MS Office for Windows). Simple

169 Matching Coefficient was calculated using the following formula: $SMC = \frac{M_{00}+M_{11}}{M_{00}+M_{01}+M_{10}+M_{11}}$

170 where: M_{00} - total number of attributes where both have a value of 0, M_{11} - total number of

171 attributes where both have a value of 1, M_{01} and M_{10} - total number of attributes where one

172 has a value of 1 and the other a value of 0. 16S rRNA gene fragments were identified using

173 the Blastn algorithm with the 'sequences from type material' option, identifying the strain as

174 the closest match to validly described species. Phylogenetic trees were made using the Mega-

175 X software. Sequences were deposited under the accession numbers: MT585825-MT586024.

Results

Polar region strains screened with the Nile Red-amended agar technique displayed varying shades of the expected bright orange fluorescence. Some strains displayed a fluorescence with a pinkish hue (like *Polaromonas* sp. 1701), some with a more yellowish hue (like *Chrysoebacterium* sp. 966). In others, like the *Rhodanobacter* sp. 2793 only a faint trace of the orange fluorescence could be observed at the rim of the drop-growth (Fig. 1A). Several strains displayed growth retardation on the Nile Red amended agar in comparison to the Nile Red free control. Agar with the Nile Blue A dye did not yield any specific fluorescence. Amplification of the *phaC* gene resulted in the single, specific 550bp long fragment like in the case of *Polaromonas* sp. 1701 or in multiple fragments, including the specific one (Fig. 1B). Microscopic observations of the Nile Red – stained cells observed under green light excitation in most cases confirmed the orange-fluorescing intracellular granules with a clearly defined morphology thereof. The outline of cells without PHA granules was also visible. Most strains belonging to the Bacteroidetes phylum, like *Flavobacterium* sp. 1052 and also marine bacteria (*inter alia Psychromonas* sp. 1212) displayed an orange fluorescence of the whole cell, without any granule-characteristic morphology.

Examined isolated belonged to four phyla: Proteobacteria (fig. 2), Actinobacteria, Firmicutes and Bacteroidetes (fig. 3). Proteobacterial isolates belonged to three classes: Alphaproteobacteria (n=8) consisting mostly of *Sphingomonas* sp. strains. Betaproteobacteria were more numerous consisting of 45 isolates clustered within roughly three families, the most important being the *Comamonadaceae* (n=27), with *Polaromonas* as the most frequent genus. Gammaproteobacterial isolates were the most numerous (n=53) and most diverse (>7 families). The family with the most members were the *Pseudomonadaceae* (n=27) consisting exclusively of the *Pseudomonas* genus (fig. 2). Actinobacteria (n=43), Firmicutes (n=6) and

201　Bacteroidetes (n=45) were less frequently isolated than Proteobacteria. Actinobacterial strains

202　belonged mostly to two families: *Microbacteriaceae* (n=22) and *Micrococcaceae* (n=17).

203　*Microbacteriaceae* consisted mostly of three genera: *Salinibacterium*, *Cryobacterium* and

204　*Glaciihabitans*. The *Micrococcaceae* consisted exclusively of two genera: *Arthrobacter* and

205　*Paeniglutamicibacter*. The Bacteroidetes isolates were mostly members of the

206　*Flavobacteriaceae* family (N=36).

207　　　To examine the reproducibility of the results obtained by the different methods a

208　Simple Matching Coefficient was calculated between each of the binary data sets, taking both

209　negative and positive responses into account (Fig. 2). As the microscopy-based method was

210　evaluated as the least bias-prone, strains were included into the PHA-positive group (POS)

211　when granules were present after cultivation on either of the liquid media used (R3A,

212　PIM1,2,3). None of the methods used showed a total result overlap. For the 'All strains' group

213　the highest overlap was achieved for the *phaC* gene detection and microscopy method for

214　R3A medium cultured strains (CON - constitutive PHA producers) – 0.67. Low overlap

215　values were achieved for the agar plate screening method (0.45-0.59). For Gram-negative

216　bacteria this was also apparent with high overlap of the *phaC* detection with strains scored as

217　positive (0.66). Gram-positive bacteria at the other hand had low overlap (0.34) of the *phaC*

218　detection scores with positive strains but high with microscopy based method for constitutive

219　producers. The plate method displayed in the case of Gram-positive bacteria greater overlap

220　with other methods (0.65 – 0.67). Proteobacteria displayed low to moderate overlap values,

221　especially low for the agar plate method (0.35 - 0.50). Highest overlap value was achieved for

222　the *phaC* detection and PHA positives. Actinobacteria displayed relatively high overlap

223　between *phaC* detection and microscopy method for constitutive producers (0.78) and low

224　when the overlap was calculated for PHA positive strains. Bacteroidetes displayed moderate

225 to high overlap of tested methods with highest between *phaC* detection and microscopy

226 granule detection after cultivation on R3A broth (Fig. 2.).

227   Percentage of PHA positive strains were based on microscopy observations (Fig. 5).

228 For all examined strains a value of 63% of PHA-granule positive were achieved. Within the

229 phylum-rank group high values were scored for Proteobacteria (75%) and Actinobacteria.

230 Bacteroidetes scored only 27% PHA-positive strains. Betaproteobacteria and

231 Gammaproteobacteria scored 91% and 60% positive strains respectively. Within the family

232 level groups (where n>10) the *Comamonadaceae* displayed highest values (96%) whereas the

233 *Flavobacteriaceae* the lowest (28%). The *Alcaligenaceae*, *Pseudomonadaceae*,

234 *Microbacteriaceae* and *Micrococcaceae* remained within a 70-80% threshold (Fig. 5). Half of

235 the PHA-producers (within all examined strains) were constitutive granule producers, whereas

236 the other half had to be induced with a high C/N ratio. Highest constitutive producer

237 percentage were found belonging to the *Alcaligenaceae* and *Comamonadaceae* families

238 within the Betaproteobacteria class (86 and 73% respectively), whereas the

239 *Microbacteriaceae* (Actinobacteria) and *Pseudomonadaceae* (Gammaproteobacteria)

240 displayed high percentage of strains that produced PHA only upon induction. The most

241 successful induction medium was the PIM1, inducing PHA-formation on 71% of all of the

242 non-constitutive PHA producers. It was the highest scoring induction medium for all groups

243 except for the Betaproteobacteria and *Alcaligenaceae*, where PIM2 was more successful in

244 inducing PHA formation. PIM3 medium containing medium-length fatty acid salts was the

245 most successful in PHA induction of the *Pseudomonadaceae* family members.

246   The inducible PHA-granule accumulating strains usually produced the storage

247 material on more than one type of induction media (Fig. 6). PIM1 and PIM2 shared the

248 greatest number of induced strains (23) whereas PIM2 and PIM3 the lowest (13). Twelve

249 strains managed to produce PHAs on all three induction media.

250   Strains were grouped according to the type of environment they were isolated from

251   (Fig. 7). Glacial environments harbored the most PHA-positive strains (91%), 52% of which

252   were constitutive granule accumulators. High contribution values of PHA positive strains

253   were also apparent for soil-associated (pedogenic) habitats (78%) but also post-glacial

254   deposits (67%). Marine and animal-influenced habitats contained mostly non-PHA producing

255   strains, albeit the PHA producing minority had to be induced to accumulate PHA (>70%).

256   Almost half of the freshwater habitat isolated strains had PHA synthesis abilities, 67% of

257   which did it without the high carbon to nitrogen ratio induction.

258   **Discussion**

259   *Evaluating screening methods*

260   Popular screening methods (both physiology- and gene based) displayed a varying

261   degree of accuracy when dealing with polar region-derived bacterial strains. The plate method

262   yielded results that were hard to evaluate. Most difficult to interpret were pigmented strains,

263   which was noted also by other researchers (Higuchi-Takeuch et al. 2016). In the case of

264   *Rhodanobacter* sp. isolates the presence of the pigment seemed to extinguish the Nile Red-

265   stained granule fluorescence. *Rhodanobacter* isolates often produce xanthomonadin-like

266   pigments, which absorb wavelengths in the blue-green range, those that that excite the

267   granule-bound Nile Red dye (He et al. 2020; Rajagopal et al. 1997; Zuriani et al. 2013).

268   Drop-growth of most Bacteroidetes members (mostly *Flavobacterium* spp. and

269   *Chryseobacterium* spp. isolates) and several Proteobacteria of marine origin (e.g.

270   *Psychromonas* sp.) displayed strong orange fluorescence on the Nile Red amended agar. This

271   was checked by microscopy analysis, where whole cells had an orange glow in green light

272   excitation. Flavobacteria of polar origin produce substantial amounts of branched and

273   unsaturated fatty acids to maintain membrane fluidity (Králová 2017), whereas marine

274   psychrophiles produce polyunsaturated fatty acid for the same purpose (Russel and Nichols,

275   1999). Alonso and Mayzard (1999) state, that those fatty acids belong to the polar lipid group

276   and their fluorescence after Nile Red staining is in the range of 610 nm (orange). A feature of

277   the plate based-screening, besides color evaluation difficulty, was also visible growth

278   retardation on the Nile Red amended agar in comparison to the Nile Red-free control.

279   Presumably, strains that originate from polar regions, where there is low anthropogenic

280   impact and low nutrient concentrations, are more sensitive to xenobiotics, like the diluent

281   used (DMSO) or the dye itself (Znój et al. 2017; Speakermann et al. 1999). Surprisingly, agar

282   amended with the Nile Blue A dye did not yield any specific fluorescence. Presumably the

283   low temperature or the pH of the medium hampered its oxidation to the active derivative (Nile

284   Red) (Greenspan et al. 1985). Genetic screening methods have an advantage over the

285   metabolism based ones as they do not rely on granule presence, scoring however the strains

286   that are being examined only as potential producers. The primer set designed by Romo et al.

287   (2011) targets type I and II synthase genes. Amplification of the *phaC* fragment using those

288   primers frequently yielded multiple bands with or without the 550 bp specific band during the

289   screening of polar region bacteria. This could be explained either by non-specific binding of

290   the degenerate primers or the existence of other copies of the gene in close proximity to each

291   other, simultaneous carrying of two types of synthases or other unusual PHA synthase related

292   genes. Recent studies (Tan et al. 2020) have shown, that *Pseudomonas* spp. isolated from

293   Antarctica carried two classes of synthases (I and II) and some *Janthinobacterium* sp. isolates

294   contained genes of an usual PHA synthase (provisionally named class V) besides the class I

295   synthase. Sequencing of the genomes of several of strains used in this study (data not shown)

296   indicates presence of a variety of genes identified as polyhydroxyalkanoic acid synthases

297   seeded throughout the genome. Furthermore, genome sequencing revealed the presence of

298   type III and IV synthase genes in some of the examined strains. Those genes were not

299   detected by the primers used and currently there is no wide-range primer sets to detect them

300 (Montenegro et al. 2017). Microscopy observation of the Nile Red stained cells also required

301 cautious examination. However, microscopy-based approach has proven the most accurate

302 and the most reliable down the line, as it detected active PHA accumulators and granule

303 presence was not only confirmed by the characteristic fluorescence but also by their

304 morphology (Wältermann and Steinbüchel 2005). As already mentioned marine strains and

305 those belonging to the Bacteroidetes phylum displayed a whole-cell red fluorescence in green

306 light excitation. Those were scored as negative for PHA accumulation as the granules in

307 native producers tend not to exceed 40% of the cells volume (Mravec et al. 2016). Further

308 advantage of the microscopy-based approach was that the cells were stained *post* growth and

309 PHA production so there were no issues with proliferation-hampering effect of the dye or the

310 carrier diluent (DMSO). Furthermore, the feast-famine regime could easily be incorporated

311 into the method to further increase the number of PHA-positive strains.

312 Comparison of the data with the Simple Matching Coefficient clearly showed that

313 each method produced different results for the same sample group. Assuming that the

314 microscopic method is the most accurate one and considering the results of the feast/famine

315 regime, several observations can be made. The plate-based method displayed low overlap

316 with the microscopy method in Proteobacteria, suggesting that in this group the agar plate

317 approach potentially introduces a heavy bias. In Actinobacteria *phaC* detection method

318 showed high overlap with the microscopy-based technique only for constitutive producers and

319 low when also inducible producers were taken into account. This points towards failed

320 amplification of most of the actinobacterial *phaC* gene fragment. The reason being, that the

321 primers were designed mostly on the basis of proteobacterial sequences or that polar region

322 actinobacterial genes represent a novel quality among PHA synthases.

323 *Biodiversity of psychrophilic PHA producers*

324   The most frequently isolated polar region PHA producing strains belonged mostly to

325   the *Comamonadaceae* family. Members of this family have been frequently recognized as a

326   vital part of PHB – producing consortia enriched from a mixed microbial culture under

327   different feeding conditions (Dai et al. 2015; Cavaillé et al. 2016). Furthermore, members of

328   the *Comamonadaceae* like *Rhodoferax* spp. and *Polaromonas* spp. have been found to

329   produce PHA in sea ice and cold waters of the northern Baltic Sea (Pärnänen et al. 2015).

330   *Polaromonas* spp. are a frequent resident of glacial Arctic and Antarctic habitats and are

331   suspected to be involved in symbiosis with eukaryotic algae (Gawor et al. 2016). This is in

332   line with the conclusions drawn by Kadouri about the pivotal role of PHAs in bacterial

333   symbionts of eukaryotic organisms. Especially surprising is the constitutive feature of the

334   betaproteobacterial PHA synthesis. The need for constant granule presence might be dictated

335   by the sudden changes in nutrient availability or intensity of metabolism-challenging factors

336   as the PHAs are thought to protect extremophiles from environmental stressors (Koller 2017).

337   Gammaproteobacteria, especially the *Pseudomonadaceae* family members produced PHA

338   mostly under nitrogen limiting conditions. Those bacteria are a known group of

339   opportunitrophs, displaying a large variety of adaptive features like fast growth and vide

340   range of substrate utilization and lytic enzyme production (Prieto et al. 2016), so PHA

341   production may be an asset only in extremely harsh conditions (Ayub et al. 2009).

342   Actinobacteria were second in terms of isolate numbers of active PHA producers revealed in

343   this study. A vast majority of actinobacterial strains produced PHA only upon induction with

344   high C/N ratio, most notably the members of the *Microbacteriaceae* family (*Cryobacterium*

345   for eg.). *Micrococcaceae* were more evenly divided among constitutive and inducible PHA

346   producers (eg. *Arthrobacter* spp.). Information on members of those families as PHA

347   producers is limited, however polar region Actinobacteria were described as occupying

348   diverse niches (Zdanowski et al. 2013), so the scenario presented earlier for

349   *Pseudomonadaceae* might also be applicable. As reported by Matias et al. 2009

350   Actinobacteria tend to produce PHAs of unusual chemistry, therefore actinobacterial isolates

351   derived from polar region materials might represent an untapped source of novel,

352   biotechnologically relevant polyhydroxyalkanoates.

353   *PHA producers and their habitat*

354   Polar region habitat types differed quite substantially in cultivable bacterial PHA

355   accumulators' content. The zoogenic habitat type which was represented by sea birds'

356   (penguins and little auks) nesting sites and marine habitats represented mainly by decaying

357   seaweed heaps displayed the lowest contribution of PHA producers. Seemingly, polar

358   nutrient-rich substratum (Grzesiak et al. 2020, Zdanowski et al. 2005) does not promote

359   species that diverge energy and resources towards carbon-storage material synthesis (Pratt et

360   al. 2012; Wang and Yu, 2000). Furthermore, even among isolates displaying this ability, its

361   expression had to be triggered by nitrogen starvation with simultaneous carbon surplus.

362   Therefore, constant high nutrient availability negatively influences the contribution of PHA

363   producers to the community also in polar regions. Contrasting in this respect were the highly

364   oligotrophic glacial habitats. Vital nutrients, especially nitrogen and phosphorus are scarce in

365   those sites (Grzesiak et al. 2015a; Grzesiak et al. 2015b). Over 90% contribution of PHA

366   positive isolates among glacier-derived bacteria was therefore not surprising. Presumably,

367   labile carbon surplus is present only during the short summer season (Telling et al. 2014),

368   therefore PHA production is needed in regards of carbon deficit survival. Among glacier-

369   derived PHA+ strains there was an even distribution of constitutive and inducible PHA

370   producers, indicating different mechanisms for coping with extreme glacial conditions. As

371   mentioned before, some bacterial groups might use the granules as an abiotic stress-resistance

372   feature, whereas others might rely on different mechanisms, not related to PHA metabolism

373   like pigment synthesis (Dieser et al. 2010). Soils of varying complexity (developed and

postglacial) shared similar characteristics to each other in terms of percentage of PHA-producing strains and their ability to constitutive or inducible granule accumulation. Heterogeneity of such habitat enables an establishment of a multitude of different niches, even among closely related microorganisms (Gittel et al. 2014), therefore the fairly even distribution between non-producers, constitutive producers and inducible producers. Polar region freshwater habitats are strongly affected by seasonal changes in nutrient quantity and quality, creating a natural feast/famine regime (Crump et al. 2003). Consequently, high altitude Himalayan lakes were recently proclaimed as "bioplastics reservoir" by Kumar and associates (2018).

Conclusions

Polar region bacteria present a novel and potent source of PHA-producing microorganisms. Microscopic observation of Nile Red stained cells amended with a feast/famine regime implementation using different carbon sources was proven as the most reliable screening method. Caution is advised when evaluating members of the Bacteroidetes phylum and polar region marine bacteria as they often give false positive results in the Nile Red dye involving approach. Members of the *Comamonadaceae* family were the most numerous PHA producers by percentage among the 200 examined strains. Most abundant in cultivable PHA accumulators were glacier associated habitats of both polar regions, followed by well-developed soils and postglacial deposits. Nonetheless, to further expand the topic of polar region PHA producers additional investigations are in order: a culture independent approach of the native source material and an in depth laboratory analysis of bacterial strains.

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Strain numbers	Material of isolation	Place of origin	Environment type	Maintenance medium
2043, 2045, 2047, 2049, 2099, 2102, 2132, 2139, 2157	Little auk (Alle alle) guano	Hornsund Fiord, Spitsbergen Island, Arctic	Zoogenic	R3A agar
966, 967, 968, 969, 970, 972, 973, 974, 975	Adélie penguin guano	Point Thomas Rookery, King George Island, Antarctica	Zoogenic	R3A agar
1198, 1200, 1204, 1206, 1208, 1210, 1212, 1334, 1335, 1336, 1337, 1338, 1339, 1340, 1341, 1343, 2848	Decaying seaweeds	Hornsund Fiord, Spitsbergen Island, Arctic	Marine	R3A agar with artificial sea water
1639, 1640, 1641, 1642, 1643, 1662, 1663, 1664, 1666, 2861	Decaying seaweeds	Admiralty Bay shore, King George Island, Antarctica	Marine	R3A agar with artificial sea water
2529, 2539, 2543, 2556, 2561, 2574, 2579, 2595, 2600	Cryoconite	Hans Glacier, Spitsbergen, Arctic	Glacial	R3A agar
2720, 2722, 2724, 2728	Cryoconite	Werenskiold Glacier, Spitsbergen, Arctic	Glacial	R3A agar
301, 303, 304, 305, 330, 331, 332, 333, 334	Glacial surface ice	Ecology Glacier, King George Island, Antarctica	Glacial	R3A agar
473, 474, 475, 476, 477, 478, 479, 481, 482	Glacial surface ice	Baranowski Glacier, King George Island, Antarctica	Glacial	R3A agar
803, 805, 806, 807, 808, 809, 810, 811, 812	Plant-free postglacial soil	Ecology Glacier foreland, King George Island, Antarctica	Glacial	R3A agar
989, 990, 991, 992, 993, 994, 995, 996, 997, 998	Postglacial soil with plant debris	Ecology Glacier foreland, King George Island, Antarctica	Glacial	R3A agar
844, 847, 848, 851	Plant-free postglacial soil	Baranowski Glacier foreland, King George Island, Antarctica	Glacial	R3A agar
117, 123, 130, 139, 1258, 1259, 1261	Plant-free postglacial soil	Windy Glacier foreland, King George Island, Antarctica	Glacial	R3A agar
2176, 2180, 2181, 2183, 2186, 2191, 2196, 2199, 2200, 2201, 2203	Arctic tundra soil with moss/lichen debris	Hornsund Fiord, Spitsbergen Island, Arctic	Pedogenic	R3A agar
1044, 1051, 1052, 1053, 1058, 1062, 1065, 1079, 1081	Kettle lake water	Werenskiold Glacier forefield, Hornsund fiord, Spitsbergen, Arctic	Freshwater	R3A agar
1699, 1700, 1701, 1702, 1703, 1704, 1705, 1706, 1707, 1708, 2031, 2032, 2033, 2034, 2035, 2036, 2042, 2083, 2084, 2954, 2955	Freshwater microbial mat	Jasnorzewski Gardens, King George Island, Antarctica	Freshwater	R3A agar
867, 868, 869, 870, 871, 872,	Air	Point Thomas	Zoogenic	R3A agar

873, 874, 875, 876		Rookery, King George Island, Antarctica		
1160, 1162, 1165, 1166, 1167, 1169, 1170, 1191, 1192	Subglacial water	Subglacial stream, Hans Glacier, Spitsbergen, Arctic	Glacial	R3A agar
P12, 2778, 2779, 2785	*Deschampsia antarctica* rhizosphere soil	Arctowski Station vicinity, King George Island, Antarctica	Pedogenic	R3A agar
2792, 2793, 2794, 2795, 2797, 2799, 2800, 2860	Ornithogenic soil	Arctowski Station vicinity, King George Island, Antarctica	Pedogenic	R3A agar
2810, 2812, 2813, 2814, 2815, 2816, 2817, 2818, 2819	Moss rhizosphere	Arctowski Station vicinity, King George Island, Antarctica	Pedogenic	R3A agar
2921, 2923, 2924, 2926, 2927, 2928	Supraglacial water	Supraglacial stream, Ecology Glacier, King George Island, Antarctica	Glacial	R3A agar
2743, 2744, 2746, 2747, 2748, 2749, 2750	River water	Ariedalen stream, Spitsbergen, Arctic	Freshwater	R3A agar

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Table 1. Information on origin and cultivation of strains used in analysis.

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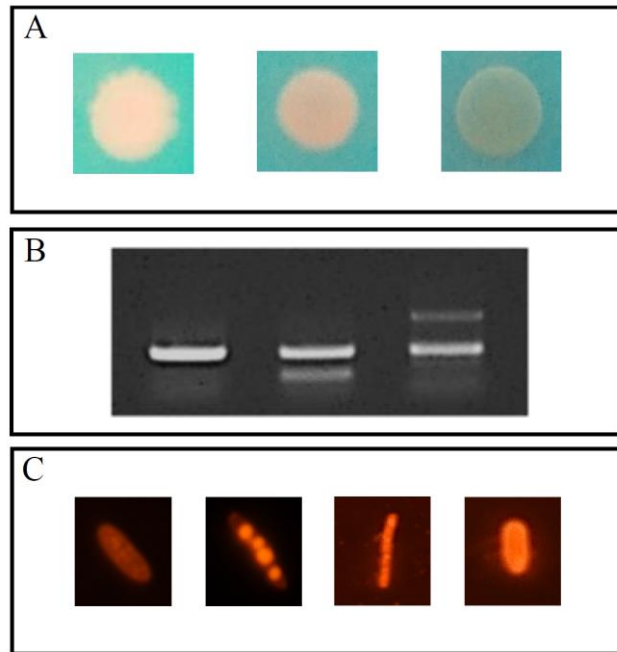
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Fig 1. (A) Examples of drop growth fluorescence of bacterial strains in UV light on a R3A agar supplemented with Nile Red (from left to right): *Janthinobacterium* sp. 1167, *Chryseobacterium* sp. 966, *Rhodanobacter* sp. 2793. (B) Examples of PCR-amplification results (bands) of a *phaC* gene fragment using the G-D and G-1R primers by Romo et al. 2007 (left to right): *Polaromonas* sp. 1701 (550bp band), *Acidovorax* sp. 1169 (300 bp and 550 bp band), *Janthinobacterium* sp. 1167 (550 bp and 1000 bp band), (C) Bacterial cell fluorescence in green light excitation (510-560 nm) after Nile Red staining (left to right): *Janthinobacterium* sp. 1167 – "empty" cell, *Janthinobacterium* sp. 1167 – granule-filled cell, *Flavobacterium* sp. 1052 cell, *Psychromonas* sp. 1212 cell.
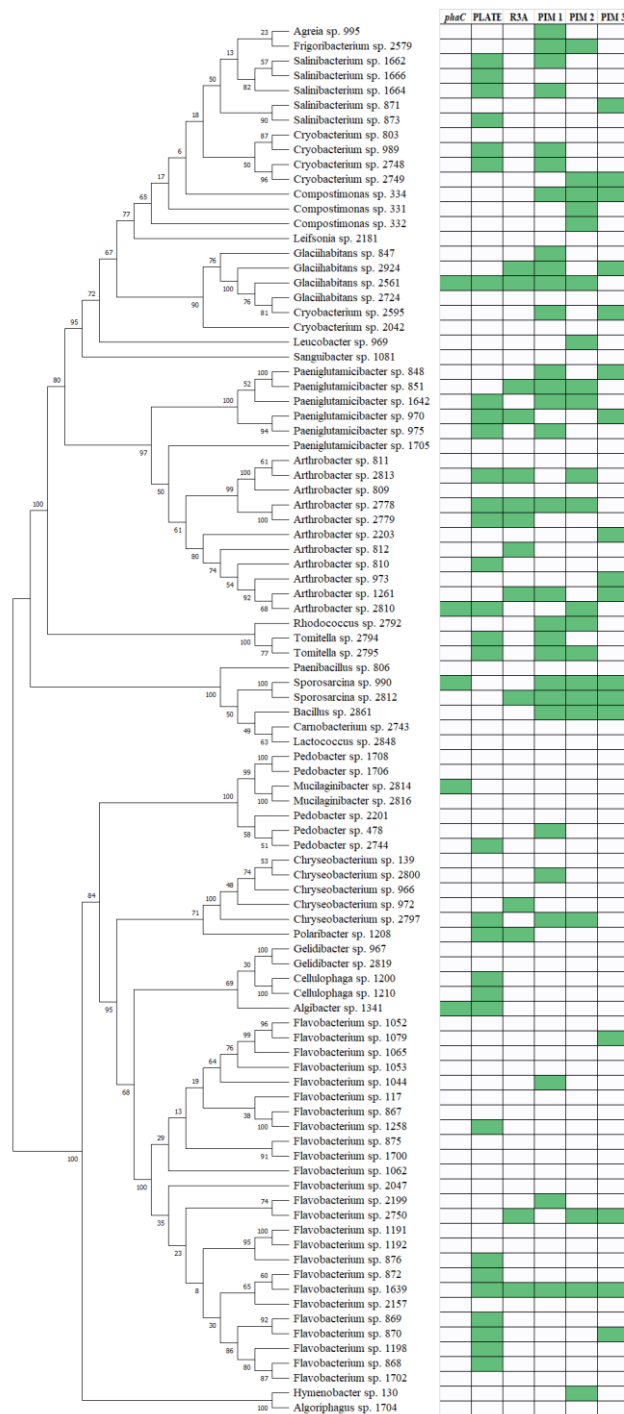
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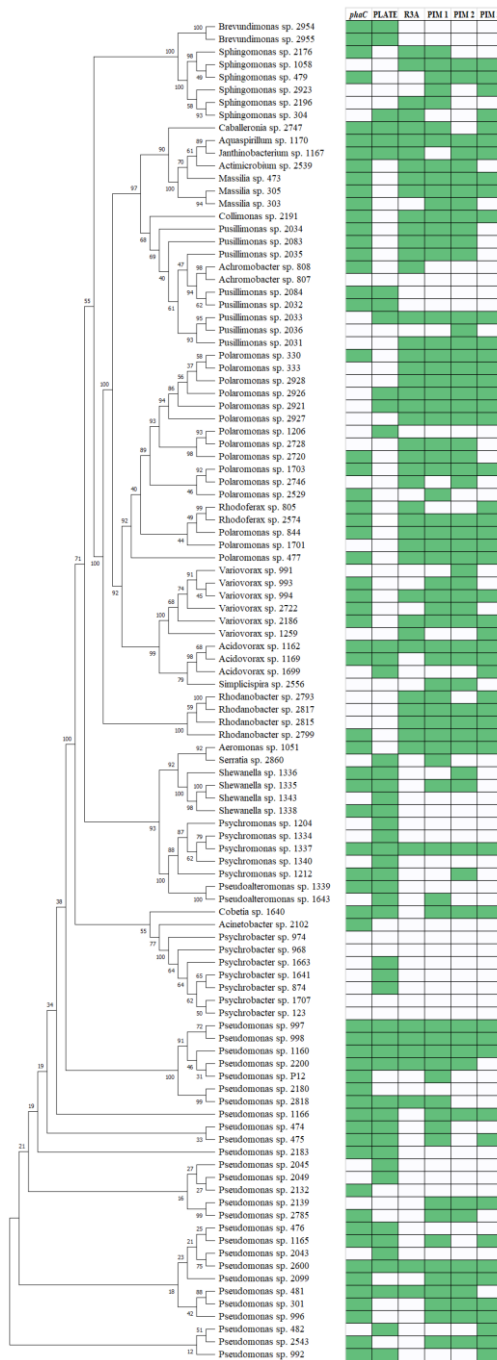
Fig 2. Phylogenetic tree based on partial 16S rRNA gene sequences belonging to the
Proteobacteria phylum lined up with the results from the PHA screening with various
methods. The tree was built using the neighbor-joining method. Bootstrap values are indicated
at the nodes. Green boxes indicate a positive score. *phaC* – presence of the 550 bp DNA
fragment of the *phaC* gene; PLATE – presence of characteristic fluorescence on R3A agar
plates with Nile Red after UV exposure; R3A – presence of red-fluorescing granules in Nile
Red stained cells cultured on R3A broth; PIM1 - presence of red-fluorescing granules in Nile
Red stained cells incubated in PIM1 medium; PIM2 - presence of red-fluorescing granules in
Nile Red stained cells incubated in PIM2 medium; PIM3 - presence of red-fluorescing
granules in Nile Red stained cells incubated in PIM3 medium.

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Fig 3. Phylogenetic tree based on on partial 16S rRNA gene sequences belonging to the
Actinobacteria, Firmicutes and Bacteroidetes phylum lined up with the results from the PHA
screening with various methods. The tree was built using the neighbor-joining method.
Bootstrap values are indicated at the nodes. Green boxes indicate a positive score. *phaC* –
presence of the 550 bp DNA fragment of the *phaC* gene; PLATE – presence of characteristic
fluorescence on R3A agar plates with Nile Red after UV exposure; R3A – presence of red-
fluorescing granules in Nile Red stained cells cultured on R3A broth; PIM1 - presence of red-
fluorescing granules in Nile Red stained cells incubated in PIM1 medium; PIM2 - presence
of red-fluorescing granules in Nile Red stained cells incubated in PIM2 medium; PIM3 -
presence of red-fluorescing granules in Nile Red stained cells incubated in PIM3 medium.

All strains

	PLATE	CON^M	POS^M
phaC	0.59	0.67	0.58
PLATE		0.52	0.45

Proteobacteria

	PLATE	CON^M	POS^M
phaC	0.50	0.54	0.65
PLATE		0.38	0.35

Gram-negative bacteria

	PLATE	CON^M	POS^M
phaC	0.55	0.64	0.66
PLATE		0.47	0.43

Actinobacteria

	PLATE	CON^M	POS^M
phaC	0.65	0.79	0.30
PLATE		0.63	0.51

Gram-positive bacteria

	PLATE	CON^M	POS^M
phaC	0.67	0.78	0.34
PLATE		0.65	0.51

Bacteroidetes

	PLATE	CON^M	POS^M
phaC	0.69	0.87	0.69
PLATE		0.69	0.60

Fig. 4. Simple Matching Coefficient calculated from the binary (positive/negative) data obtained from each screening method for the following subsets: All strains (n=200), Gram-negative bacteria (n=151), Gram-positive bacteria (n=49), Proteobacteria (n=106), Actinobacteria (n=43), Bacteroidetes (n=45). *phaC* – binary dataset of the presence/absence of the 550 bp DNA fragment of the *phaC* gene; PLATE – binary dataset of the presence/absence of the characteristic fluorescence on R3A agar plates with Nile Red after UV exposure; CON^M – microscopy-obtained (M) binary dataset of the presence/absence of granules after incubation in R3A broth (constitutive producers); POS^M - microscopy-obtained (M) binary dataset of the presence/absence of granules after incubation on ≥ 1 of the liquid media used (R3A, PIM1,2,3).

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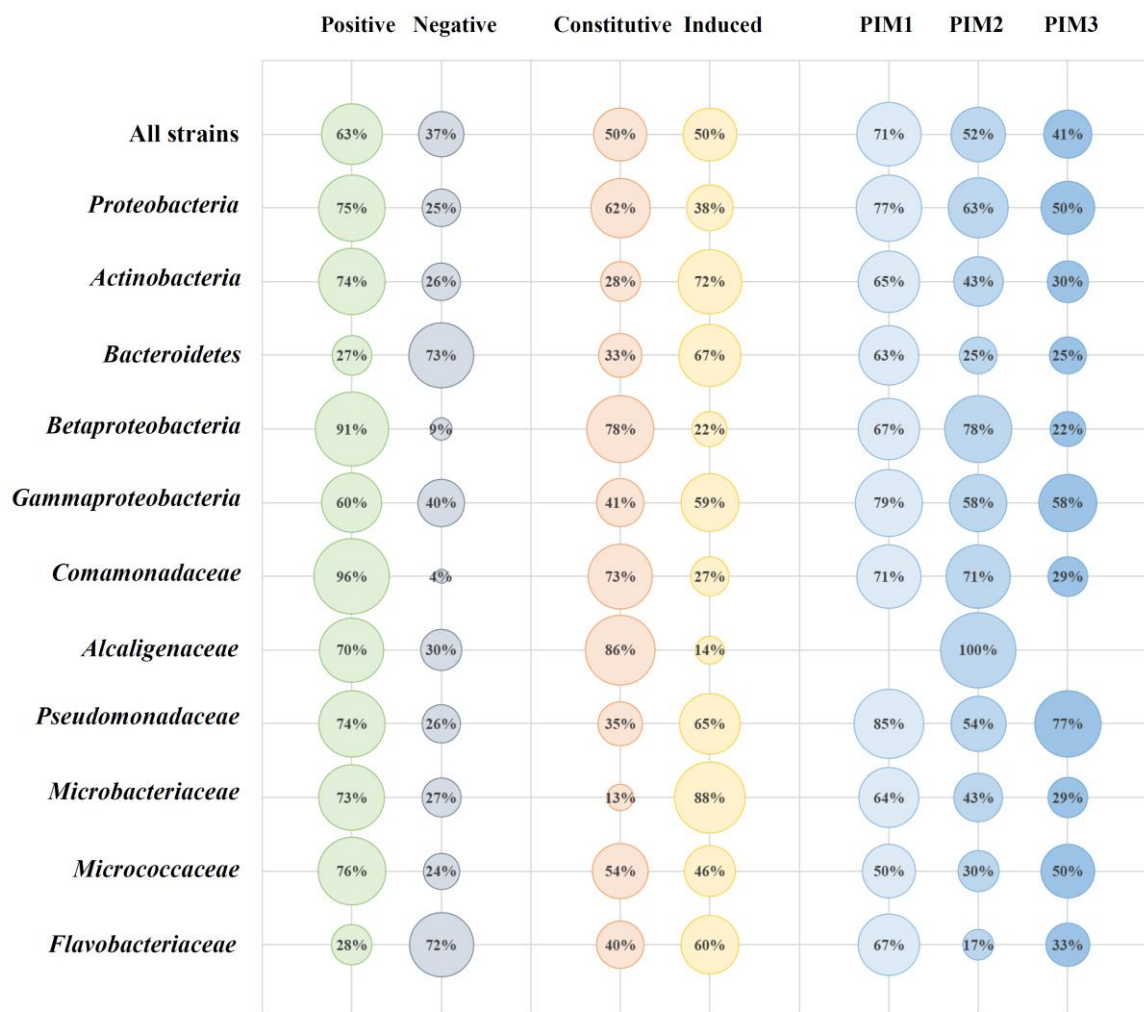
Fig 5. Percentage of strains (total and within selected taxonomic ranks) expressing the following traits: Positive - presence of red-fluorescing granules (on R3A broth and on PIMs); Negative – absence of red-fluorescing granules in any conditions; Constitutive – presence of red-fluorescing granules in R3A broth; Induced - presence of red-fluorescing granules only after incubation in PIMs; PIM1 - presence of red-fluorescing granules after incubation in PIM1 within the 'Induced' group; PIM2 - presence of red-fluorescing granules after incubation in PIM2 within the 'Induced' group; PIM3 - presence of red-fluorescing granules after incubation in PIM3 within the 'Induced' group.
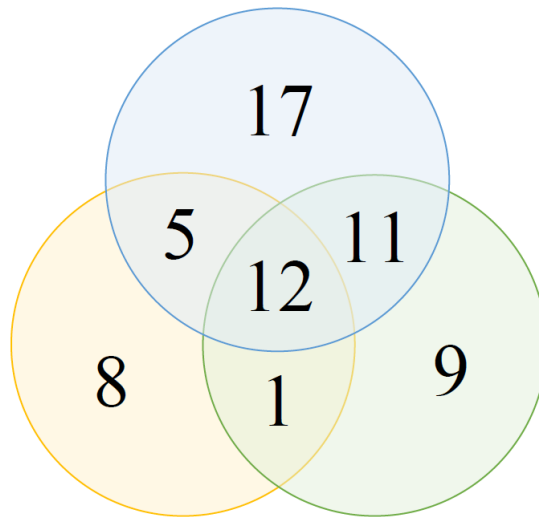
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Fig 6. Venn diagram displaying numbers of strains within the 'Induced' group that were PHA positive on different version of PIM. Blue circle – PIM1, green circle – PIM2, orange circle – PIM3.
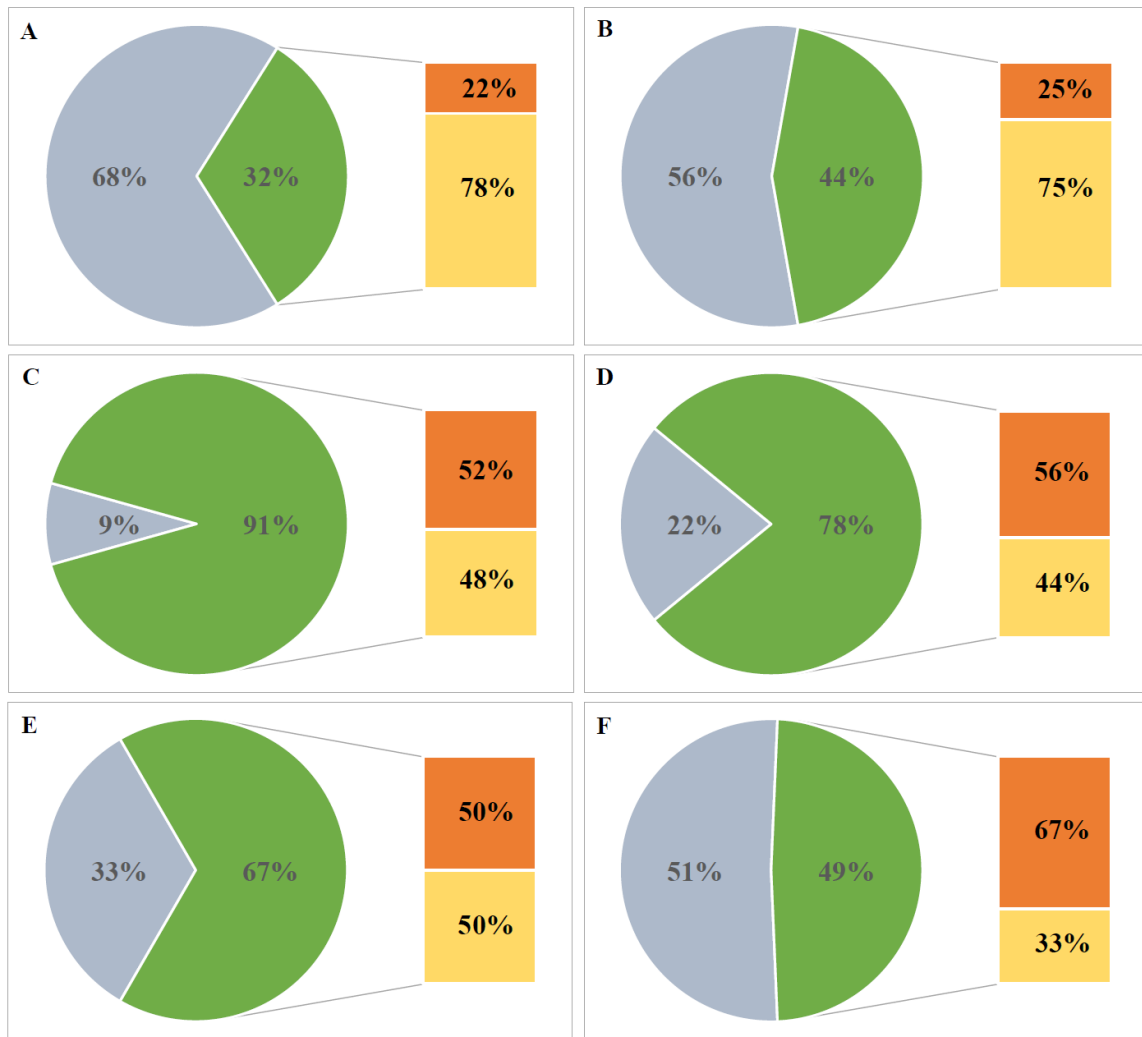
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679 Fig 7. Percentage of strains divided according to habitat-type of origin: A – zoogenic habitat,
680 B – marine habitat, C – glacial habitats, D – pedogenic habitats, E – postglacial habitats, F –
681 freshwater habitats. Percentage of strains positive for granule presence – green part of
682 diagram, negative – gray, putative PHA producers within the positives – orange, PIM-
683 inducible PHA producers within the positives – yellow.