Growth characteristics of pneumococcus vary with the chemical composition of the capsule and with environmental conditions

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14

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33

Abstract

Background: Pneumococcus, a bacterium that typically resides in the nasopharynx, is exposed to a variety of temperature and oxygen levels in the upper respiratory tract and as it invades the lung, tissues, and blood. The response to these variations likely varies by strain and could influence the fitness of a strain and its virulence. We sought to determine the effect of environmental variability on the growth characteristics of pneumococcus and to evaluate correlations between variability in growth characteristics between strains and biological and epidemiological characteristics.

Methods: We evaluated the effect of temperature and oxygen on the growth of 256 pneumococcal isolates representing 53 serotypes, recovered from healthy carriers and from disease patients. Strains were grown at a range of temperatures anaerobically or in ambient air with and without catalase and were monitored by reading the optical density. Regression models were used to evaluate bacterial and environmental factors associated with characteristics of the growth curves.

Results: Most isolates grew to the maximal density at the temperature of the nasopharynx (~33C) and under aerobic conditions (with catalase). Maximum density achieved was positively associated with the presence of N-acetylated sugars in the capsule and negatively associated with the presence of uronic acids. Reaching a greater density at an early time point was positively associated with the prevalence of serotypes among healthy carriers in the pre-vaccine period.

Discussion: Environmental variability affects the growth of pneumococcus, with notable differences between isolates and by serotype. Such variability could be influenced by characteristics of the capsule and might affect virulence and transmissibility.

Keywords: pneumococcal infection, growth curves, temperature, carriage, anaerobic growth

59 *Streptococcus pneumoniae* (pneumococcus) is an opportunistic pathogen that resides in the
60 human nasopharynx. The nasopharynx is considered to be the reservoir of transmission between
61 individuals [1]. Pneumococci are diverse, with >90 serotypes (defined by the capsule
62 polysaccharide) and has tremendous genetic variation, resulting from recombination. Serotypes
63 vary in their prevalence among healthy carriers and in the likelihood that they will cause severe
64 disease [2]. As conjugate vaccines against 7, 10, and 13 pneumococcal serotypes have been
65 introduced, the vaccine-targeted serotypes have declined in frequency among healthy carriers and
66 as causes of disease, while serotypes not targeted by the vaccine have increased in importance
67 (serotype replacement). Next-generation conjugate vaccines are under development that target
68 additional serotypes, and it is likely that these vaccines will lead to further serotype replacement.
69 Understanding the factors that influence the fitness of these non-vaccine strains could help to
70 anticipate future patterns of serotypes replacement and could aid in the design of more optimal
71 vaccines.
72
73 The success of pneumococcus in the nasopharynx and the likelihood that it causes disease is likely
74 driven, in part, by how it responds to variations in its local environment. In different anatomical
75 sites within the human host, pneumococci are exposed to variable temperature and oxygen levels.
76 In the nasopharynx, considered its main niche, the average temperature is around 33°C, with some
77 differences between children and adults [3-6]. The core body temperature, which would be
78 encountered during invasion into tissues, is 37°C. The temperature in the lungs is constantly
79 changing based on the temperature of inhaled air but is generally lower than 37°C [7]. During
80 infection by pneumococci or during viral co-infection (such as influenza or RSV), both external
81 and internal temperature increases [8-12]. Oxygen levels also vary within the host. In the

82 nasopharynx, bacteria on top of the mucus layer are exposed to almost ambient air (20% O_2).
83 Pneumococci in biofilms in the nasopharynx encounter lower levels of oxygen [13]. Entering the
84 lower respiratory tract or the middle ear, pneumococci are exposed to micro-aerophilic conditions
85 and to almost anaerobic conditions when present in blood and the cerebrospinal fluid (CSF) [14-
86 17]. Likewise, mucus production during infection (i.e., due to influenza or RSV) can block the air
87 passage and form micro-aerophilic (around 5% O_2) or even anaerobic microenvironments [15, 16].
88
89 While pneumococci exist in a complex environment, simple *in vitro* experiments can be used to
90 isolate the response to specific environmental conditions. Laboratory studies have evaluated
91 variations in growth characteristics by serotype and have identified a relationship between *in vitro*
92 growth characteristics and prevalence of serotypes in the nasopharynx of healthy children [18, 19].
93 However, the effect of variation in temperature and oxygen on the growth of different strains and
94 serotypes has not been systematically explored.
95
96 The aim of this study was to investigate how environmental variability in temperature and oxygen
97 influences the growth characteristics of pneumococci and how the responses to these variations
98 correlates with the biological characteristics of the strains and the observed epidemiology of the
99 serotypes. Using a diverse set of clinical and nasopharyngeal isolates, as well as capsule-switch
100 and capsule-knockout variants generated in the lab, we quantified how the growth characteristics
101 of pneumococci *in vitro* vary under a range of temperatures and in aerobic and anaerobic
102 conditions. Using statistical models, we estimated the variation in these growth characteristics
103 associated with serotype (after adjusting for isolate-specific effects that could reflect culture

104 history or other characteristics) and evaluated the relationship between the serotype growth
105 characteristics and relevant serotype-specific epidemiological and biological variables.
106
107 **Results**
108 **Descriptive results**
109 In total, we performed more than 4,900 growth curves on 256 different pneumococcal strains,
110 representing 53 different serotypes **(Figure 1)**. Because of the relatively poor growth achieved in
111 aerobic conditions without catalase, all further analyses presented here focus on the results from
112 clinical samples grown either with catalase or anaerobically (N=3151). There was a weak, positive
113 correlation (rho=0.27, 95%CI: 0.24, 0.30) between the maximum density achieved and maximum
114 growth rate and no notable correlation between the length of the lag phase and either growth rate
115 (-0.06; 95%CI: -0.09, -0.02) or maximum density (0.04; 95%CI: 0.00, 0.07). Growth curves for
116 all isolates and conditions can be explored interactively at
117 https://weinbergerlab.shinyapps.io/ShinyGrowth_v2/.
118 The maximum density of the strains was similar at 30-35°C, and lower densities were
119 achieved at 37-39°C (**Figure 2A**); maximum density was greater in aerobic conditions (with
120 catalase) than in anaerobic conditions. The growth rates were fastest at 35-37°C, with slower
121 growth at lower or higher temperatures (**Figure 2B**); growth was faster in aerobic conditions (with
122 catalase) than anaerobic conditions. The length of the lag phase increased with temperature
123 (**Figure 2C**).
124
125 **Variation in growth characteristics associated with serotype**

126 We quantified variation in maximum density achieved, growth rate, and length of lag phase
127 associated with serotype. A number of serotypes differed from the reference (serotype 14) in
128 maximum density achieved, length of the lag phase, and the density at an early time point (**Figure**
129 **3)**. We therefore evaluated characteristics between these serotype-specific averages and
130 characteristics of the capsules. The presence of uronic acid (GlcA/GalA) in the capsular
131 polysaccharide was associated with lower densities of growth and longer lag phases **(**P=0.001**,**
132 **Figure 3)**. The presence of N-acetylated sugar in the capsule was associated with higher density
133 (P=0.002), but not with growth rate or length of the lag phase (**Figure 4**). The strength of these
134 effects did not differ appreciably between aerobic and anaerobic conditions.

135 We next sought to determine whether this variability in growth characteristics was due to
136 serotype or due to other genetic variability. Some of the serotypes in our collection were
137 represented by multiple genetic lineages (MLST types). There did appear to be variation in growth
138 characteristics associated with serotype that was similar across multiple isolates and MLST
139 lineages (**Figures S1, S2**). However, there was not enough diversity in our sample to do a formal
140 analysis. We also evaluated the growth characteristics of capsule-knockout strains as well as
141 several capsule-switch variants. While the results were ambiguous, they suggested that there was
142 an effect of capsule production on maximum density, and this effect was more pronounced during
143 anaerobic growth compared with aerobic growth with catalase (**Figure S3-S6**).

144

145 **Effect of oxygen on growth varies by serotype**

146 We considered whether the effect of oxygen on growth patterns varied by serotype (**Figure 5**).
147 Overall, isolates producing certain capsules (e.g., 2, 4, 13, 35B) grew to a higher density under
148 aerobic conditions (with catalase), while isolates producing other capsules (e.g., 6B, 8, 9N, 12F)

149 did not show a difference between aerobic and anaerobic growth (**Figure 5**). This pattern was
150 similar for maximum growth rate. For lag phase, there was little difference among the serotypes
151 in how they responded to the presence of oxygen, with only the serotype 6C and 13 isolates
152 exhibiting a longer lag phase in aerobic conditions.
153

154 **Relationship of growth characteristics to serotype-specific characteristics**

155 Finally, we evaluated associations between the characteristics of the growth curves and serotype-
156 specific epidemiological characteristics. There was a correlation between the density of a serotype
157 at an early time point and the prevalence of the serotype among healthy carriers during the pre-
158 vaccination period in several settings (**Figure 6).** The association was marginally stronger when
159 looking at aerobic growth compared to anaerobic growth. There was no association between
160 prevalence in carriage and maximum density achieved, length of the lag phase, or maximum
161 growth rate. There was also no notable association between these growth characteristics and the
162 invasiveness of the serotypes or the case fatality ratio.
163

164 **Discussion**

165 We provide novel information about how the response to environmental conditions can differ
166 between pneumococcal isolates. Testing a diverse set of strains, pneumococci grew to the highest
167 density under conditions that mimic the normal environment of the nasopharynx in terms of
168 temperature and oxygen level. Important differences in these patterns were observed between
169 isolates. While, some of this variability could be due to the culturing history of the individual
170 isolates, patterns of variation were apparent by serotype. The presence of specific polysaccharide
171 components in the capsule was associated with higher or lower density. And the density of growth

172 at an early time point was correlated with higher prevalence of those serotypes among healthy
173 carriers.
174
175 Serotype is a major determinant of pneumococcal epidemiology and biology. We identified a novel
176 association between the capsule composition and the growth phenotype of the isolates, with
177 serotypes containing uronic acids in the capsule growing to a lower density and serotypes with N-
178 acetylated sugars growing to a higher density. Further work is needed to understand the mechanism
179 underlying these patterns, but the reliance on metabolic pathways shared between central
180 metabolism and capsule production could influence the growth phenotypes. This could result from
181 metabolic fluxes that restrict growth or through feedback loops that lead to increased metabolic
182 activity. When comparing growth in aerobic conditions (with catalase) with growth in anaerobic
183 conditions, the benefit of oxygen varied by serotype (**Figure 4, 5**). Serotypes 2, 4, 13, 23B, 35B
184 and 38 grew better with additional catalase than in anaerobic conditions, whereas serotypes 6B, 8,
185 9N, 12F, 22F and 42 grew similarly in both environments. These patterns did not correlate with
186 capsule characteristics. We tested a relatively small number of isolates per serotype, so it is
187 possible that non-capsular genetic variations or differences in the culture history of the isolates
188 could influence the observed responses to oxygen.
189
190 As the nasopharynx is the normal habitat of pneumococcus, we had hypothesized that
191 pneumococci would grow optimally at temperatures in the low-30C range, similar to the
192 temperature of the nasopharynx. Indeed, temperature played an important role in terms of the
193 maximal density achieved and how quickly the isolates started growing (lag phase). Isolates grew
194 to the highest density and had the shortest lag at temperatures resembling those of the nasopharynx;

195 these patterns were particularly pronounced for those isolates obtained from the nasopharynx.
196 These patterns among the nasopharyngeal isolates are unlikely to be a result of the *in vitro* culture
197 history of the strains alone—pneumococcal isolates are typically cultivated at 37°C, and thus if
198 the patterns were a result of adaptation, we would expect more efficient growth at that temperature
199 rather than at 33°C. These findings, along with recent work on the effect of lower temperatures on
200 the immune response to pathogens in the upper respiratory tract [20], suggest that the environment
201 of the nasopharynx is optimal for pneumococcal growth. IPD isolates responded differently to
202 environmental variations.
203
204 During the invasion process, when the pneumococcus transitions from the nasopharyngeal
205 environment to the internal body environment, it has to adapt to many changes, including nutrient,
206 temperature and oxygen levels. Temperatures vary from the low-30°C range in the upper
207 respiratory tract to 37°C in core body sites and even higher during fever. Likewise, oxygen levels
208 can vary considerably during infection. Increased mucus production (due to co-infection with
209 viruses or other pathogens in the upper respiratory tract) leads to lower oxygen levels and may
210 generate a local hypoxic environment [21-23]. The availability of oxygen is also decreased in
211 pneumonia, empyema, and otitis media. Oxygen levels in the uninflamed middle ear space, for
212 example, resemble that of venous blood, are less than a third that of the airway, and may be further
213 reduced by the presence of effusion [14, 15, 24]. Some isolates appeared to be strongly influenced
214 by these variations in oxygen and temperature, indicating that the strains have the capability of
215 adapting (either in the host or during *in vitro* growth) to thrive under different conditions.
216

217 Some of the differences observed in growth phenotypes between carriage and disease isolates
218 could reflect opaque/transparent phase variation [25]. Opaque variants are generally isolated from
219 IPD and have increased capsule production and decreased production of certain surface proteins.
220 Phenotypically, the presence of oxygen accentuates differences in capsule production between
221 opaque and transparent variants [24]. This effect could be mediated via the pathways involved in
222 converting pyruvate to acetyl-CoA, an important biochemical precursor for capsule production for
223 many serotypes [26]. Variations in the use of this pathway between serotypes or the efficiency of
224 this pathway between lineages could influence some of the patterns that were observed in the
225 growth curves.
226
227 This study had certain limitations. For the growth curves, we used BHI broth which is an artificial
228 growth medium that differs in nutrient composition from the host. We evaluated several minimal
229 media but found that growth was generally poor, making comparisons between strains difficult.
230 While we tested a large number of strains representing many serotypes, some serotypes were only
231 represented by a single isolate (i.e., 11B, 12F, 13). This could limit the generalizability of serotype-
232 specific findings in these instances, making it difficult to make inferences about whether variability
233 was due to serotype, site of isolation, or lineage effects. The strains used in this study were largely
234 a convenience sample from clinical studies. The genetic diversity of pneumococcus makes it
235 difficult to draw conclusions about the cause of differences between strains. The growth curves
236 with the capsule-knockout strains and capsule-switch variants suggests that the capsule itself could
237 influence these phenotypes. We did not perform any gene expression studies, which could be
238 highly influenced by environmental conditions [27]. Further work could explore the genetic basis
239 (both capsular and non-capsular factors) for the differences in growth phenotypes between strains.

240

241 In conclusion, we demonstrate that the growth characteristics of pneumococcus are influenced by
242 environmental variations, that the effect of these variations depend on strains, and that the optimal
243 growth conditions for carriage isolates resemble the conditions of the nasopharynx. Moreover, we
244 demonstrate that the growth patterns among serotypes are associated with carriage prevalence and
245 other epidemiological and biological characteristics. These results could help in understanding
246 which of the serotypes has the greatest capacity to emerge - in both carriage and disease - in the
247 future.

248

249 **Methods**

250 **Bacterial strains, culture media, and chemicals**

251 *Strains*

252 Invasive pneumococcal disease (IPD) isolates were obtained from the isolate bank at the Centers
253 for Disease Control/Active Bacterial Core surveillance; carriage isolates were provided by Ron
254 Dagan (Ben-Gurion University, Israel), Adrienn Tothpal and Eszter Kovacs (Semmelweis
255 University, Hungary [28, 29]) and Debby Bogaert and Anne Wyllie (UMC, Utrecht [30]) (**Table**
256 **1**). Capsule-switch variants generated on the TIGR4 genetic background and the serotype 6B
257 knockout strain were provided by Marc Lipsitch and generated as previously described [31].
258 Additional capsule-knockout strains were generated by replacement of the capsule biosynthesis
259 locus with the Sweet Janus cassette [32].

260 The multi-locus sequence type was inferred for a subset of the isolates. These strains were
261 subjected to Illumina NovaSeq sequencing [33], and MLST was determined using SRST2 [34].

262

263

264 *Culture media*

265 Pneumococcal isolates were stored at -80 °C on Cryobeads (Cryobank, Copan Diagnostics,
266 Murrieta, CA). Strains were routinely grown at 37°C and 5% CO_2 overnight on tryptic soy agar
267 plates supplemented with 5% sheep blood (TSAII) (Thermo Fisher Scientific). Growth in broth
268 culture was performed in BHI (Becton, Dickinson, and Co., Sparks, MD) with and without catalase
269 (5000 units, Worthington Biochemical Corporation, Lakewood, NJ) for aerobic cultivation and
270 with Oxyrase® (Oxyrase, Inc., West Mansfield, OH) diluted 1:10 to create an anaerobic
271 environment.

272

273 **Growth experiment**

274 Strains were streaked onto TSAII plates and incubated at 37°C in a 5% CO2-enriched atmosphere
275 overnight, then harvested into PBS to OD600 0.2 and diluted (6 fold) in BHI with or without
276 catalase or Oxyrase. Growth was monitored in sterile flat-bottomed 96-well microtiter plates
277 (BRAND GMBH, Wertheim, Germany) for 24 hours in a microplate reader (BioTek ELx808) with
278 a built-in incubator, reading the optical density at 600 nm every 30 minutes, after 5s shaking (Gen5
279 program). Each strain was tested in all three oxygen conditions and across the full range of
280 temperatures (30-39°C). An anaerobic control strain (*Bacteroides thetaiotaomicron*) was used to
281 confirm the elimination of oxygen by Oxyrase.

282

283 **Data analysis**

Each growth curve was blanked by subtracting the OD600 reading at t=0 for that well. In instances where the t=30 minutes measurement was lower than the t=0 measurement due to measurement error at the first time point, the OD600 at t=30m was subtracted instead.

We extracted three characteristics from each of the growth curves: maximum density achieved, length of the lag phase, and density achieved at an early time point. The length of the lag phase was determined by fitting a smoothing spline to the log-transformed growth curves (smooth.spline function in R, with a smoothing parameter of 0.5) and calculating the second derivative of this curve. The maximum of the second derivative gives the point at which the growth rate increases the most, corresponding to the transition from stationary phase to log phase. We were also interested in evaluating the density at an early time point. However, what constitutes an 'early' time point likely varies by environmental conditions and source of the isolates. Therefore, we determined the mean time when log-phase growth began for each temperature/oxygen/site of isolate combination, and then determined the OD600 for the corresponding isolates 1 hour after this point.

To quantify variations in the growth characteristics by serotype, site of isolation, and environmental condition while adjusting for repeated measurements and strain-to-strain variations, we used linear mixed effects models (lme4 package in R) [35]. The outcome variable was maximum OD600, fixed effects variables included serotype, temperature (categorical), oxygen (aerobic+catalase, aerobic without catalase, anaerobic), site of isolation (categorical), and a random intercept for each isolate. Certain interactions among the fixed effects were also evaluated to test specific hypotheses (site of isolate*oxygen; oxygen*temperature; serotype*oxygen). The significance of these interactions at different levels was evaluated using the interactionMeans function in the phia package in R [36].

307

308 *Linkage to serotype-specific characteristics*

309 We sought to evaluate the link between serotype-specific growth curve characteristics and
310 previously published serotype-specific characteristics (capsule structure, disease severity,
311 invasiveness, pre-vaccine carriage prevalence) [37-40]. We first fit a linear mixed effects model
312 as described above that had dummy variables for serotype (reference: serotype 14) and controlled
313 for temperature (categorical), presence of oxygen, site of isolation, and interactions between site
314 of isolation and presence of oxygen and temperature and presence of oxygen. We then extracted
315 the serotype-specific regression coefficients, which represent variation in the growth
316 characteristics compared to serotype 14. These coefficients were used in second stage analyses to
317 assess correlations with serotype-specific biological and epidemiological variables.

318 Associations with the presence of uronic acids or N-acetylated sugars were assessed using
319 a Monte Carlo approach, where the mean maximum density was compared in the serotypes with
320 or without the sugar components. The sugar component labels were randomly scrambled 999
321 times, and the difference was calculated for each of these samples. P-values were calculated by
322 comparing the observed difference in means with the distribution obtained from the resampling.
323 The association with carriage prevalence was assessed with Poisson regression. In a series of linear
324 regressions, we evaluated the association case-fatality rate, complexity of the capsular
325 polysaccharide (carbons/polysaccharide repeat) and invasiveness (log transformed).

326

327 *Data availability*

The raw data and an R Markdown file are available in a github repository (https://github.com/weinbergerlab/GrowthVariation) and can be used to fully re-create the analyses presented here.

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442 **in Streptococcus pneumoniae isolated in Greece**. *Antimicrob Agents Chemother* 2001,
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444

445

446
447 **Figure legends**

448 **Figure 1. Variation in growth curves among all isolates used in this study.** Growth was
449 measured every 30 minutes over 24 hours at 30-39°C. (A) Ambient air, (B) ambient air with
450 catalase, and (C) anaerobic conditions. Each line corresponds to an individual growth curve.

451

452 **Figure 2. Effect of temperature and oxygen on growth phenotypes. (A) Maximum density**
453 **achieved, (B) Maximum growth rate and (C) length of lag phase.** Anaerobic (green squares)
454 and aerobic+catalase (orange circles). These estimates are based on 3151 growth curves.
455 Mean+/- 95% confidence intervals, calculated from a regression model adjusting for serotypes,
456 presence of oxygen, temperature, site of isolation, and an interaction between temperature and
457 presence of oxygen.

458

459 **Figure 3. Variation in growth characteristics associated with serotype.** Variation in (A)
460 maximum density (B) length of the lag phase (C) maximum growth rates and (D) density at an
461 early time point. The dots represent regression coefficients +/- 95% confidence intervals, as
462 calculated from a regression model that controls for presence of oxygen, temperature, site of
463 isolation, and interactions among these. The reference in the regressions is serotype 14.

464

465 **Figure 4. Variation in the maximum density achieved by serotypes based on**
466 **characteristics of the capsule.** The y-axis denotes the value of the serotype-specific
467 regression coefficient, estimated for a model that adjusts for environmental conditions. The
468 reference in the regression is serotype 14. Serotypes with GlcA/GalA in the capsule grew to

469 a lower density on average, while serotypes with N-acetylated sugars in the capsule grew to a
470 higher density. P-values are calculated with Monte Carlo resampling.
471
472
473 **Figure 5. Difference in maximum density by serotypes when grown in aerobic conditions**
474 **with catalase versus anaerobic conditions.** Positive values indicate that a higher density (as
475 measured by optical density) was achieved under aerobic conditions. A value of 0 indicates no
476 difference. Mean+/- 95% confidence interval, as calculated using a regression model with
477 covariates for serotype, presence of oxygen, temperature, an interaction between serotype and
478 presence of oxygen, and a random intercept for the isolate. The reference in the regression is
479 serotype 14.
480
481 **Figure 6. Correlation between carriage prevalence in the pre-vaccine period in different**
482 **locations and the density of growth at an early time point.** The serotype-specific estimates
483 of the density of growth at an early time point are calculated from a regression model that
484 controls for environmental variation and site of isolation. The Y-axis shows the number of
485 carriers of a serotype, and the X axis shows the serotype-specific regression coefficient, which
486 represents the variation in density for that serotype compared to serotype 14, after adjusting
487 for environmental variables. The reference in the regressions is serotype 14.
488
489 **Supplementary Figure 1.** Isolate-specific random effects, organized by serotype. The random
490 effect indicates how far above or below a strain is in maximum density achieved, lag length,
491 growth rate, or density at an early time point. Serotype is not adjusted for in this model. The

492 random effects are effectively an average residual for the isolate, after adjusting for
493 temperature, site of isolation, and presence of oxygen. Each dot represents an individual
494 isolate.
495
496 **Supplementary Figure 2.** Isolate-specific random effects, organized by serotypes and genetic
497 lineage. The random effect indicates how far above or below a strain is in maximum density
498 achieved, on average, after adjusting for temperature, site of isolation, and presence of oxygen.
499 Serotype is not adjusted for in this model. Each dot represents an individual isolate; within a
500 serotype dots of the same shape share an MLST type. Only isolates for which we have sequence
501 data are included in the plot.
502
503 **Supplementary Figures 3-6.** Growth curves for capsule-switch variants on the TIGR4
504 background and capsule knockout strains.
505

506

Table 1. Pneumococcal strains used in this study.

Source of isolate	Number of isolates	Country	Serotypes
Invasive pneumococcal disease (sepsis, meningitis)	40	US (CDC)	1, 2, 3, 4, 6A, 6B, 6C, 6D, 7C, 7F, 8, 9N, 9V, 10A, 10F, 11A, 11B, 12F, 13, 14, 15A, 15B, 15C, 15F, 17F, 18A, 18B, 18C, 19A, 19B, 20, 22F, 23F, 29, 31, 33F, 34, 35A, 35B, 37
	2	Hungary	3, 6B/D
Pneumonia	8	Hungary	3, 8, 10A, 15A, 19F, 35B, 43/45/38, NT
Conjunctivitis	16		3, 21, 31, 34, 42, 11A, 15A, 15B, 16F, 19A, 19F, 23A, NT
Carriage	52	Israel	6B, 14, 15B/C, 19A, 19F, 23F, NT
	87	Hungary	1, 3, 8, 21, 31, 34, 38, 6A, 9V, 10A, 11A, 15A, 15B/C, 16F, 18C, 19A, 19F, 22A, 22F, 23A, 23B, 23F, 24F, 28F, 35F
	51	Netherlands	3, 27, 10A, 11A, 15B/C, 16F, 19A, 19F, 22A, 22F, 23B, 33F, 35B, 35F, NT
Laboratory-generated genetic variants	4	Various	TIGR4 (cps-, 5, 14, 19F), 603 (6B, cps-), CDC-10A (10A, cps-), CDC-15B (15B, cps-)

507

Figure 1

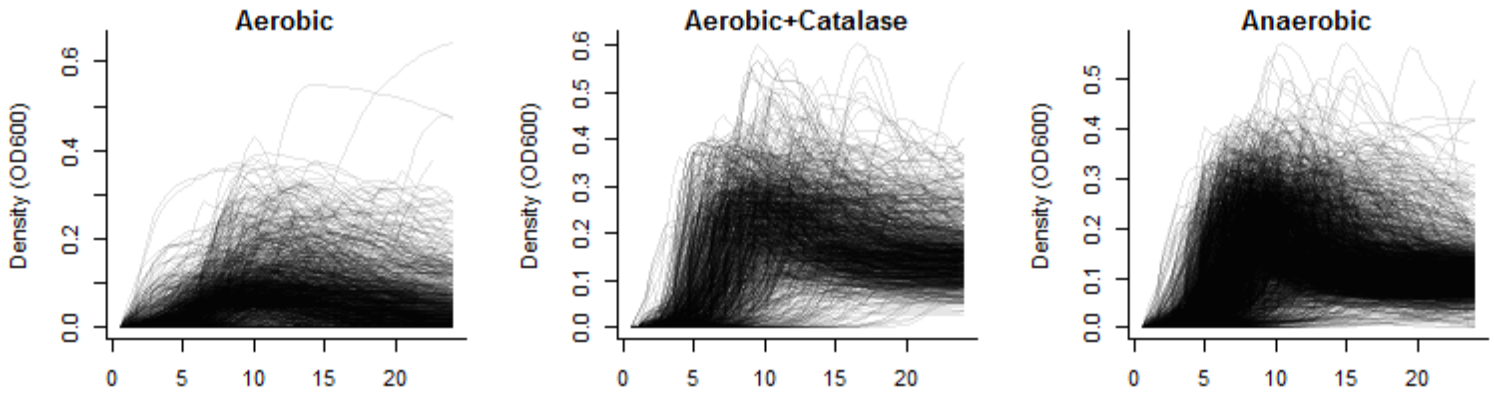

Aerobic
Aerobic+Catalase
Anaerobic
Density (OD600)
0.0
0.2
0.4
0.6
0.1
0.3
0.5
0
5
10
15
20

Figure 2

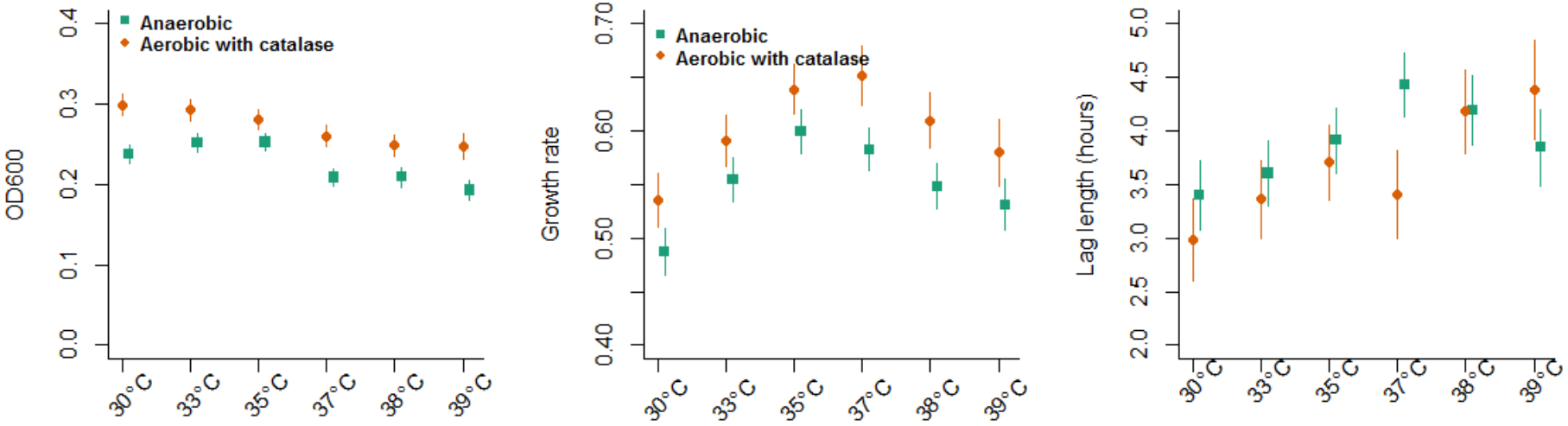

Anaerobic
Aerobic with catalase
OD600
0.0
0.1
0.2
0.3
0.4
30°C
33°C
35°C
37°C
38°C
39°C
Growth rate
0.40
0.50
0.60
0.70
Lag length (hours)
2.0
2.5
3.0
3.5
4.0
4.5
5.0

Figure 3

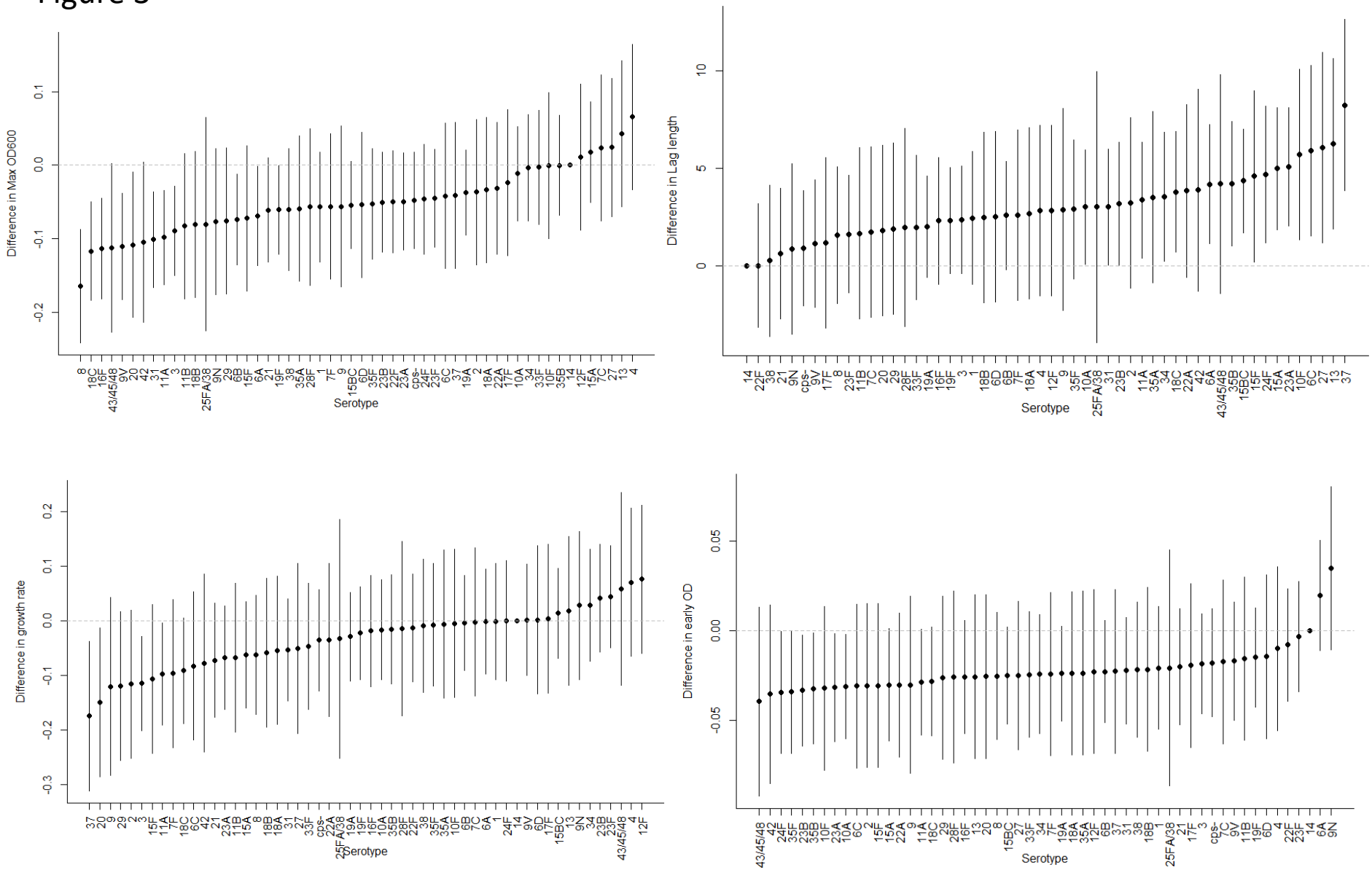

Difference in Max OD600
Serotype
Difference in Lag length
Serotype
Difference in growth rate
Serotype
Difference in early OD
Serotype

Figure 4

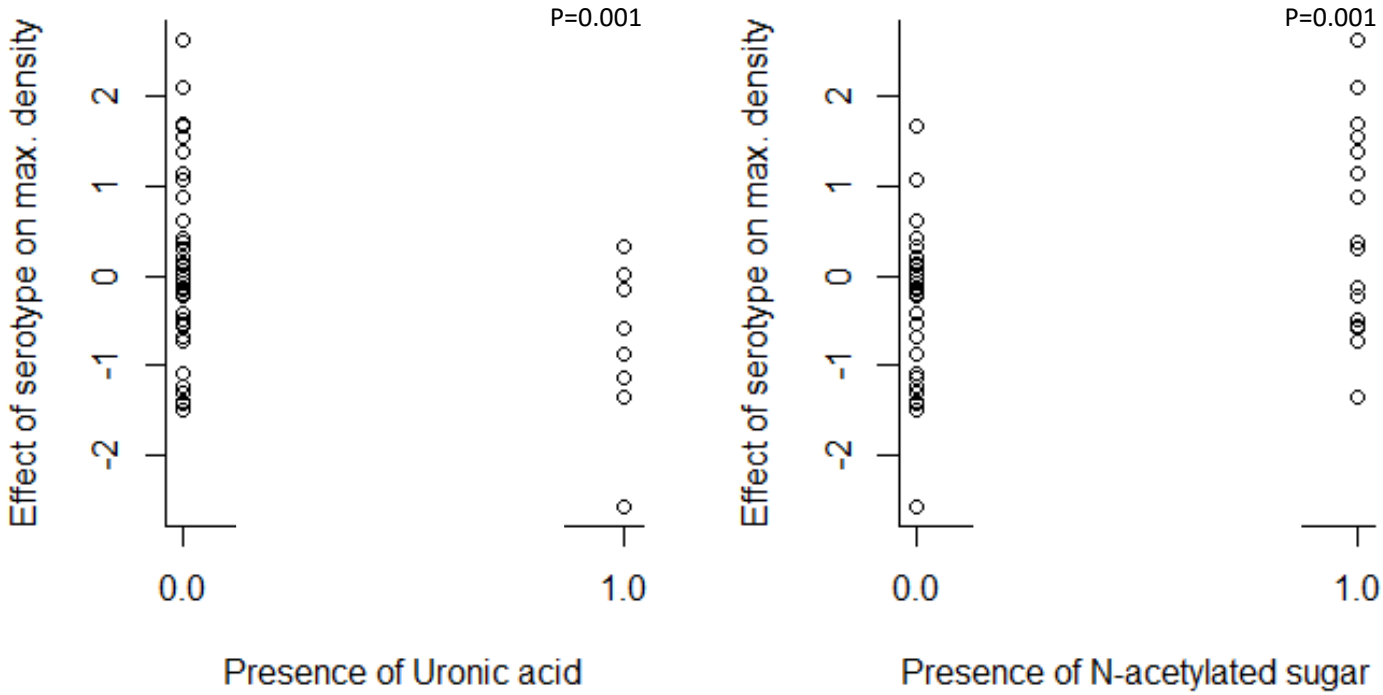

P=0.001
Effect of serotype on max. density
-2
-1
0
1
2
0.0
1.0
Presence of Uronic acid
P=0.001
Effect of serotype on max. density
-2
-1
0
1
2
0.0
1.0
Presence of N-acetylated sugar

Figure 5

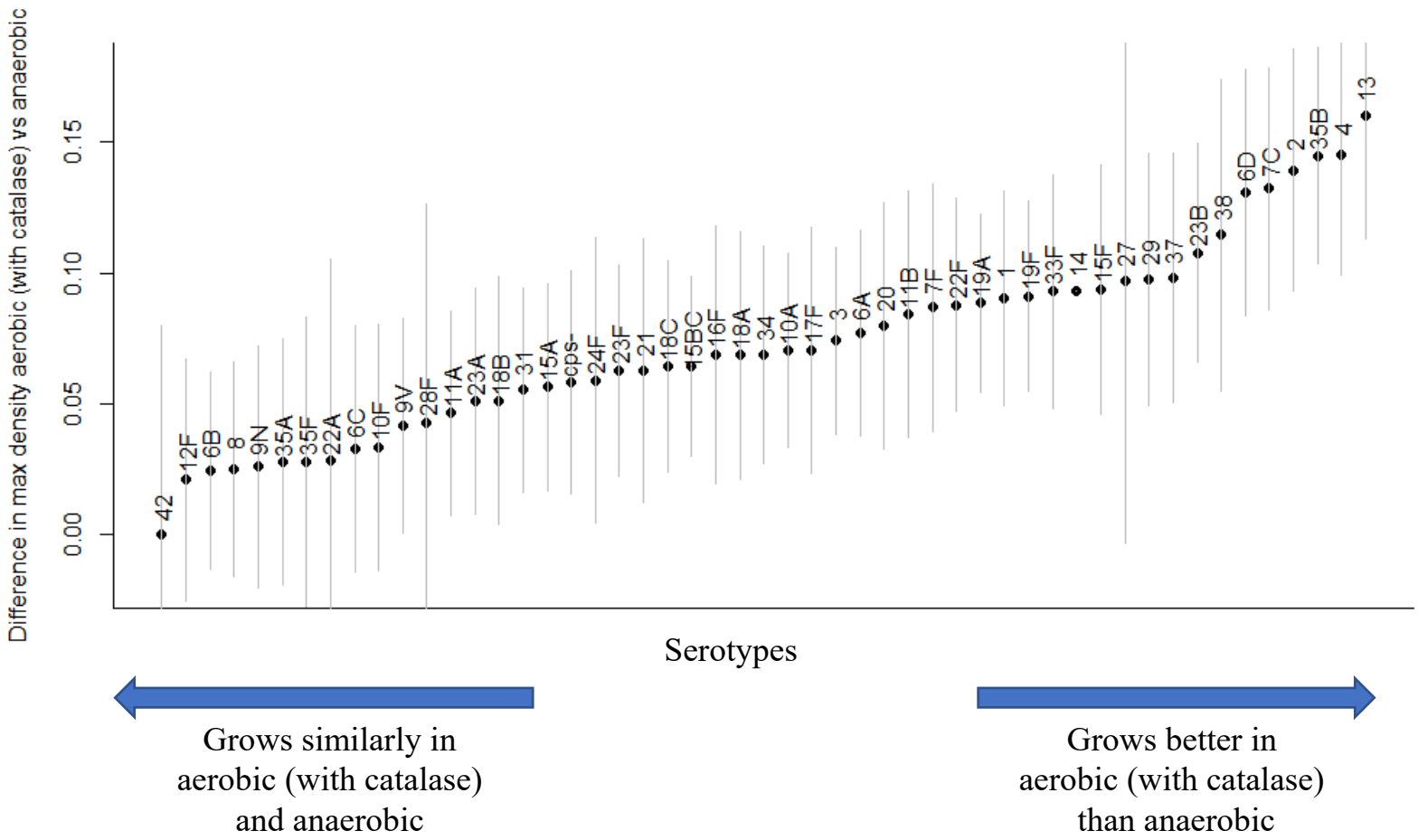

Difference in max density aerobic (with catalase) vs anaerobic
0.00
0.05
0.10
0.15
42
12F
6B
8
9N
35A
35F
22A
6C
10F
9V
28F
11A
23A
18B
31
15A
cps-
24F
23F
21
18C
15BC
16F
18A
34
10A
17F
3
6A
20
11B
7F
22F
19A
1
19F
33F
14
15F
27
29
37
23B
38
6D
7C
2
35B
4
13
Serotypes
Grows similarly in aerobic (with catalase) and anaerobic
Grows better in aerobic (with catalase) than anaerobic

Figure 6

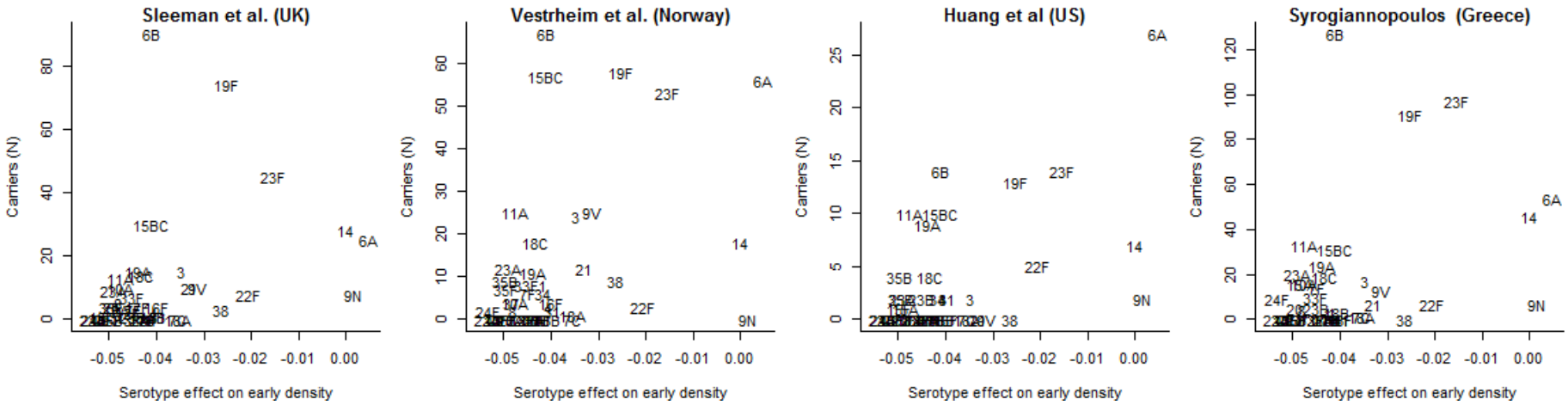

Sleeman et al. (UK)
Vestrheim et al. (Norway)
Huang et al (US)
Syrogiannopoulos (Greece)
Carriers (N)
Serotype effect on early density
-0.05 -0.04 -0.03 -0.02 -0.01 0.00
6B
19F
23F
15BC
14
6A
3
9V
22F
9N
38
11A
18C
21
19A
35B
24F
33F

Figure S1

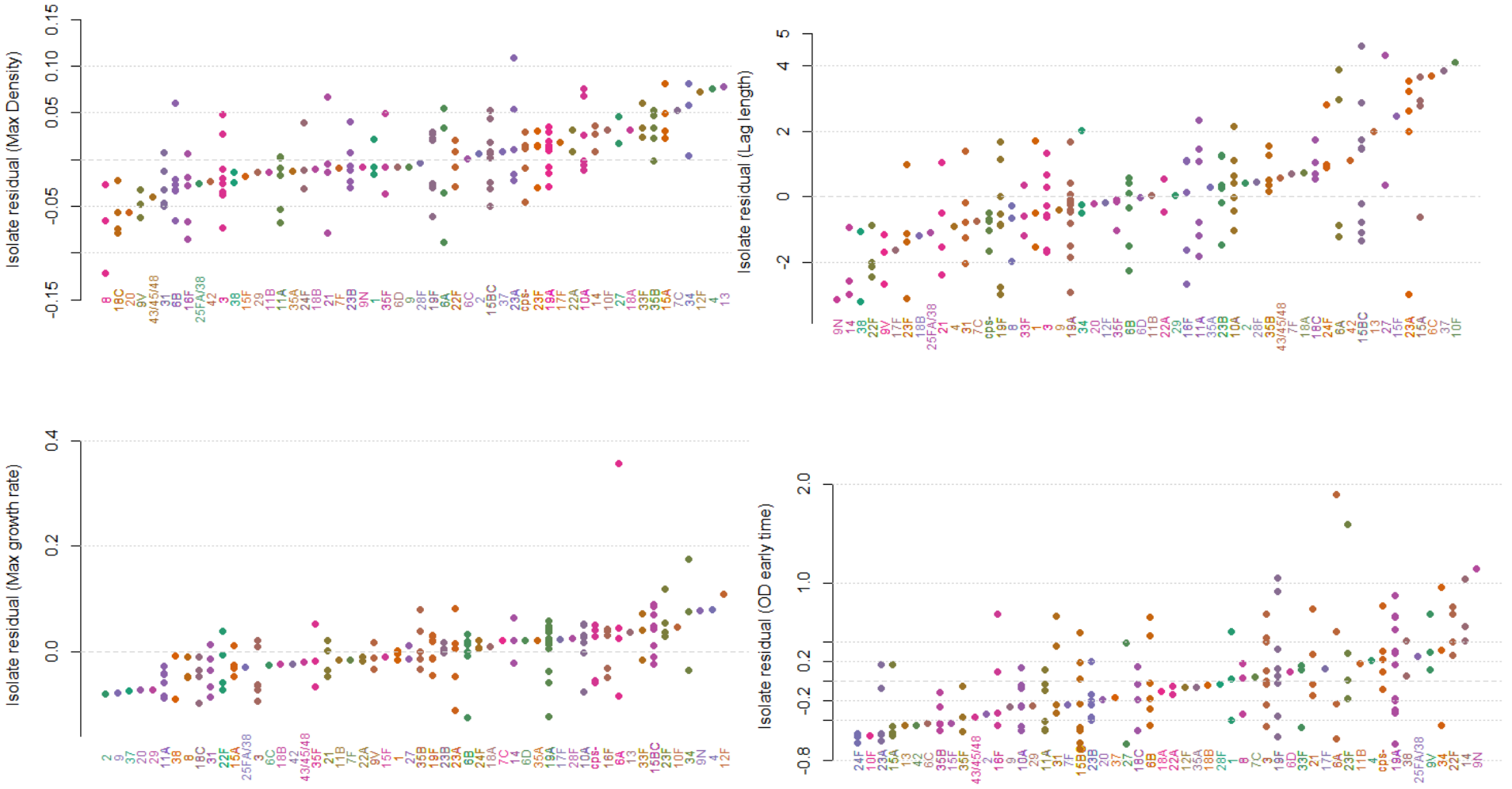

Isolate residual (Max Density)
Isolate residual (Lag length)
Isolate residual (Max growth rate)
Isolate residual (OD early time)

Figure S2

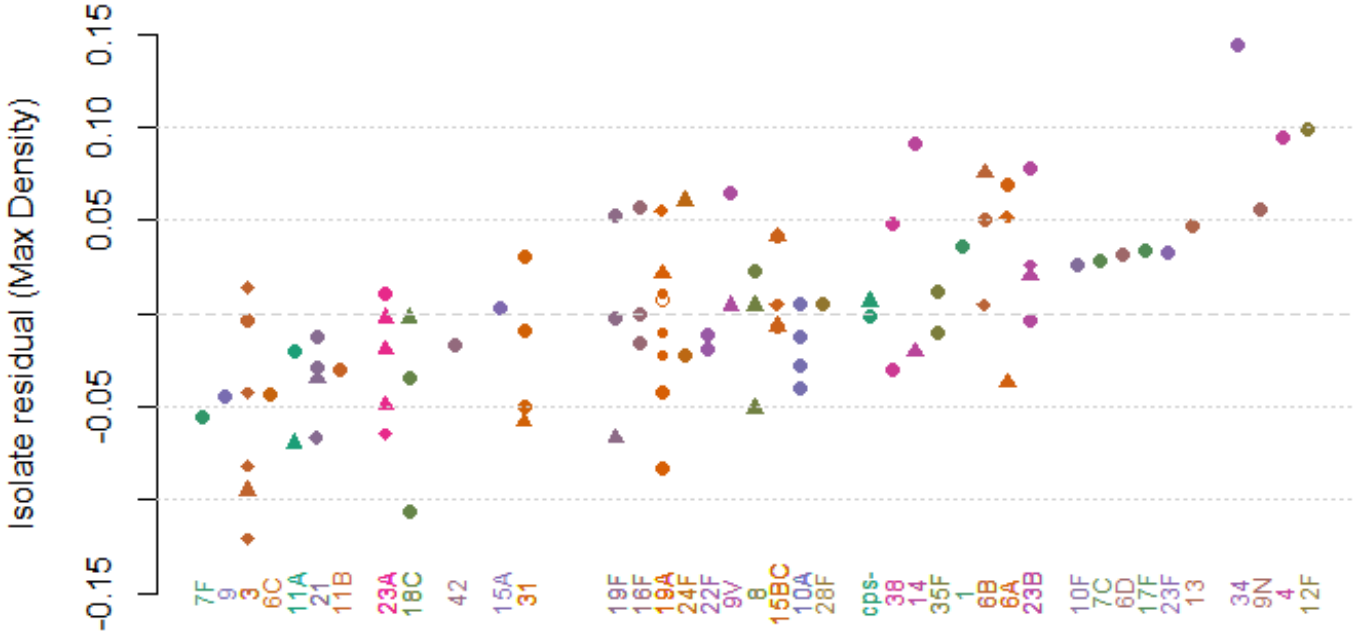

Isolate residual (Max Density)
-0.15
-0.05
0.05
0.10
0.15
7F
9
3
6C
11A
21
11B
23A
18C
42
15A
31
19F
16F
19A
24F
22F
9V
8
15BC
10A
28F
cps-
38
14
35F
1
6B
6A
23B
10F
7C
6D
17F
23F
13
34
9N
4
12F

Figure S3

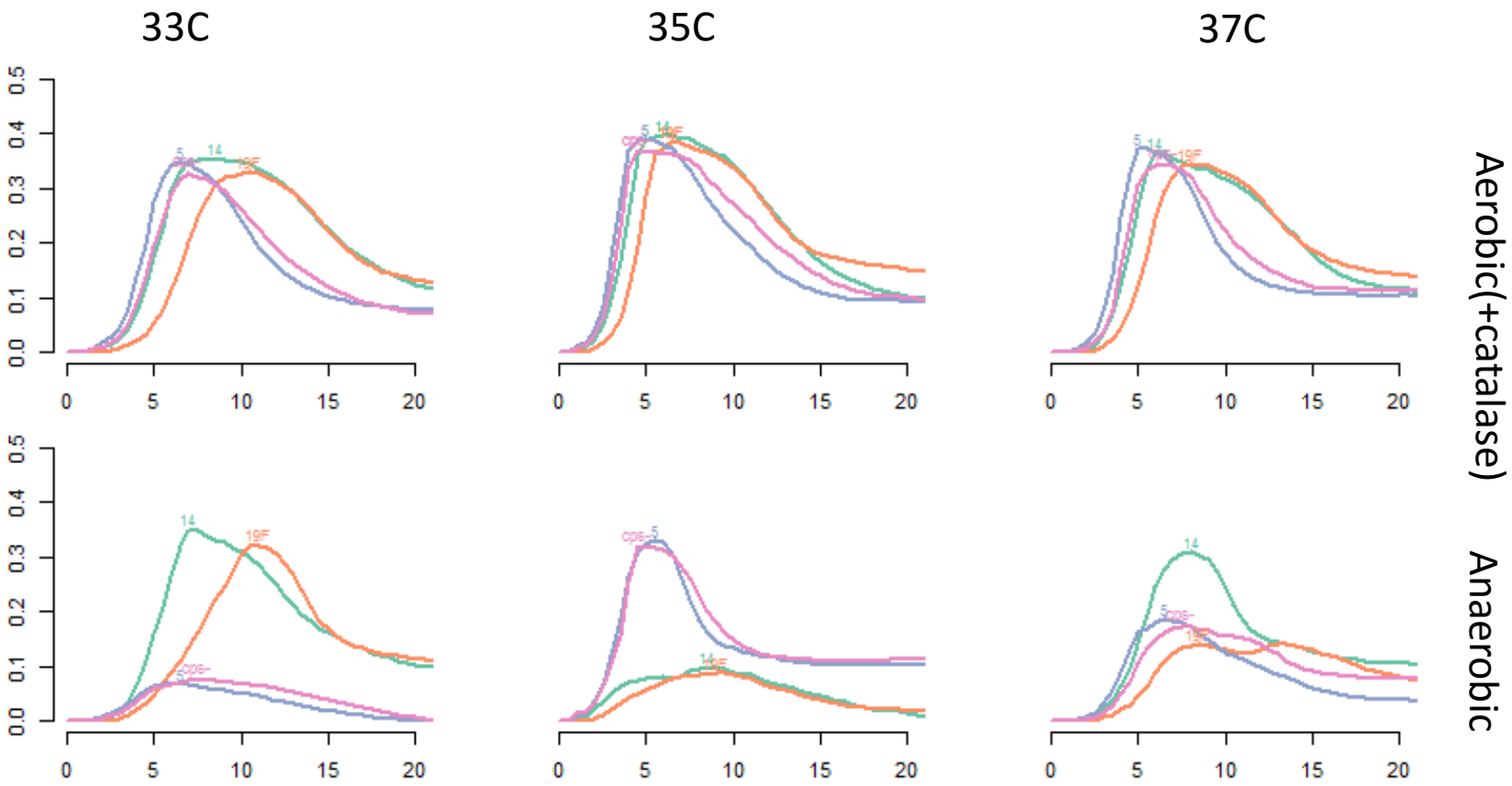

33C
35C
37C
Aerobic(+catalase)
Anaerobic
0.0
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20
5
14
19F
cps-

Figure S4

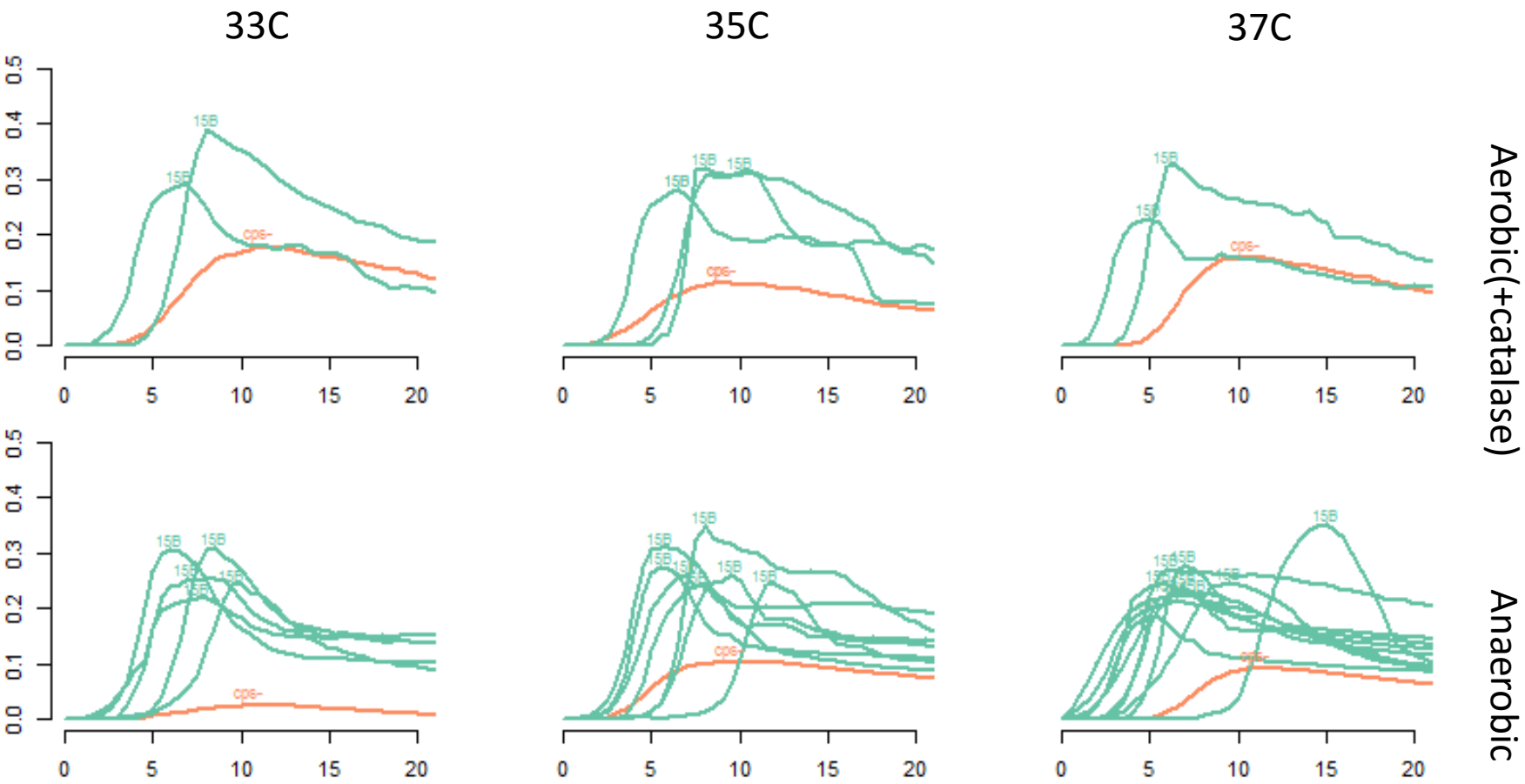

33C
35C
37C
Aerobic(+catalase)
Anaerobic
15B
cps-
0.0
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20

Figure S5

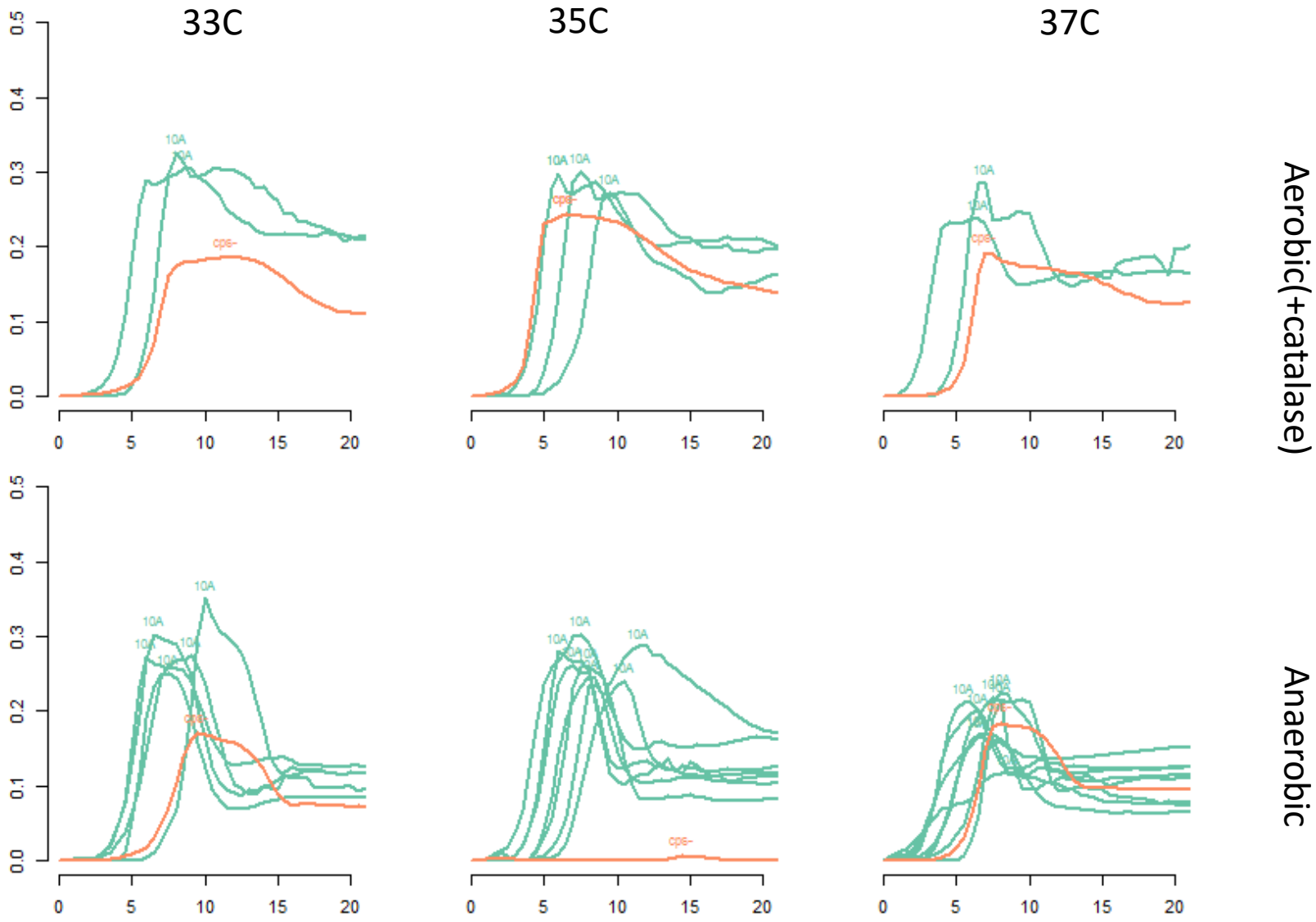
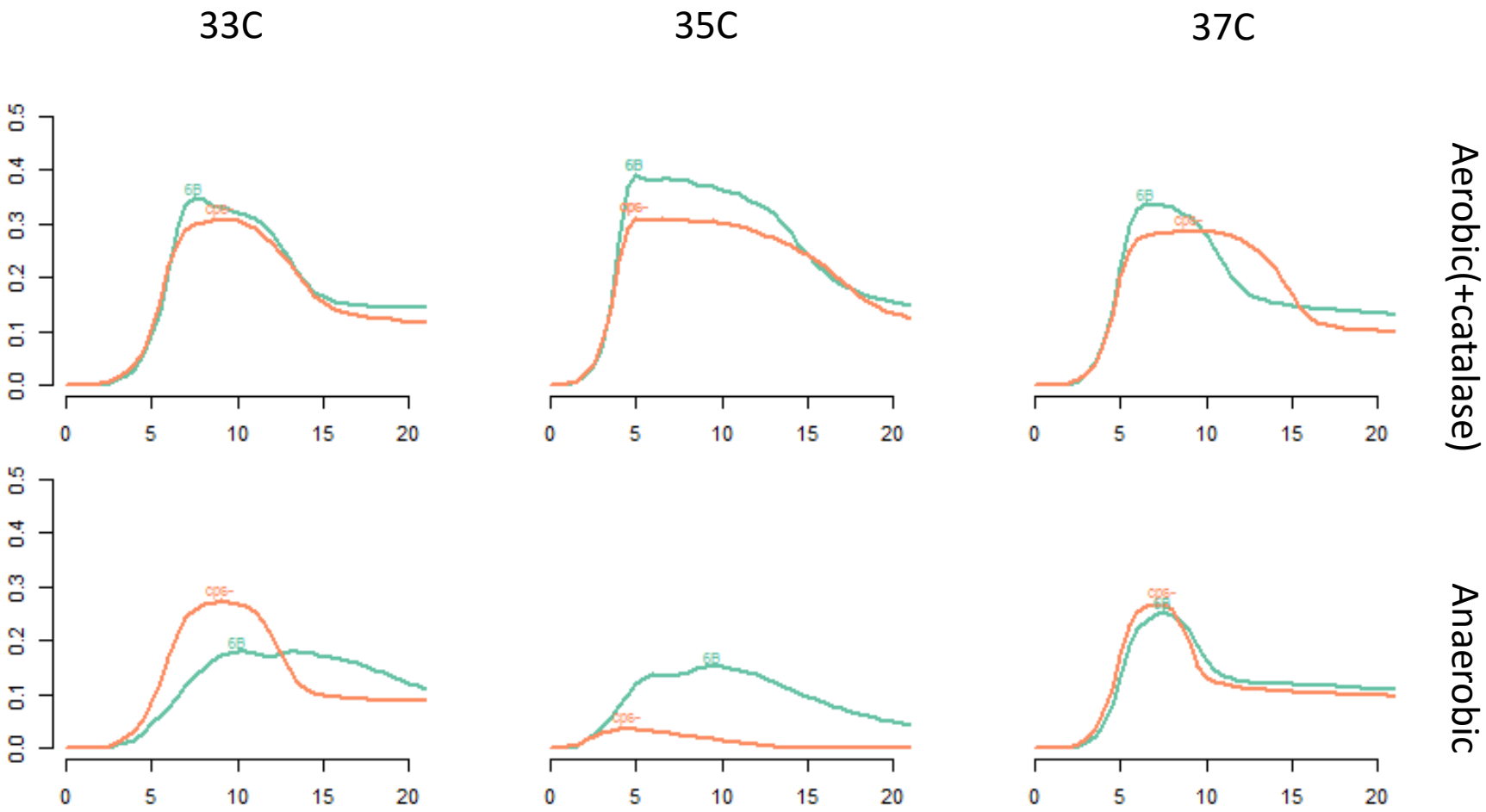

33C
35C
37C
Aerobic(+catalase)
Anaerobic
10A
cpe-
0.0
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20

Figure S6


33C
35C
37C
Aerobic(+catalase)
Anaerobic
6B
cps-
0.0
0.1
0.2
0.3
0.4
0.5
0
5
10
15
20