Response of pneumococcus to changes in temperature and oxygen varies by serotype, lineage, and site of isolation

Adrienn Tothpal[1,2*], Katherine Desobry[1], Anne L. Wyllie[1], Daniel M. Weinberger[1*]

[1]Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, CT 06510, USA

[2]Institute of Medical Microbiology, Semmelweis University, Budapest 1089, Hungary

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Corresponding authors: tothpal.adrienn@gmail.com, daniel.weinberger@yale.edu

Adrienn Tothpal: Mailing address: Institute of Medical Microbiology, Semmelweis University, Nagyvarad ter 4, Budapest, Hungary HU-1089. Phone: +36 1 459-1500. Fax: +36 1 459-1500

Daniel Martin Weinberger: Mailing address: Yale School of Public Health, 60 College Street, New Haven, CT 06510, USA. Phone: +1 203-737-6004. Fax: +1 203-737-1662

Abstract

Background: Pneumococcus, a commensal bacterium of the nasopharynx, has the potential to cause severe disease. Invasion of different body sites results in exposure to a range of environmental conditions, including different temperature and oxygen levels. The response to these variations could influence virulence and transmissibility.

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 invasive disease (IPD), conjunctivitis, and pneumonia patients. Strains were grown at a range of physiologically-relevant temperatures anaerobically or in ambient air with and without catalase and were monitored by reading the optical density.

Results: There was considerable variability between strains in response to changes in temperature and oxygen. Some of this variability was associated with serotype. Generally, carriage and IPD isolates grew to the maximal density at the temperature of the nasopharynx (~33C), but IPD isolates began growing earliest at internal body temperature. Growth curve characteristics correlated with epidemiological estimates of serotype-specific carriage prevalence and invasiveness.

Discussion: Environmental variability has differential effects on the growth of carriage and disease isolates and of different pneumococcal serotypes, which could influence virulence and transmissibility.

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

Streptococcus pneumoniae (pneumococcus) is a commensal bacterium of the human nasopharynx. The nasopharynx is considered to be the reservoir and source of pneumococcal transmission between individuals [1]. Pneumococcus is an opportunistic pathogen that causes diseases ranging from mild but common illnesses like sinusitis and otitis media to serious illnesses such as pneumonia, sepsis and meningitis [2].

In different anatomical sites within the human host, pneumococci are exposed to variable temperature and oxygen levels. In the nasopharynx, considered its main niche, the average temperature is around 33°C, with some differences between children and adults [3–6]. The core body temperature, which would be encountered during invasion into tissues, is 37°C. The temperature in the lungs is constantly changing based on the temperature of inhaled air but is generally lower than 37°C [7]. During infection by pneumococci or during viral co-infection (such as influenza or RSV), both external and internal temperature increases [8–12]. Oxygen levels also vary within the host. In the nasopharynx, bacteria on top of the mucus layer are exposed to almost ambient air (20% O_2). Pneumococci in biofilms in the nasopharynx encounter lower levels of oxygen [13]. Entering the lower respiratory tract or the middle ear, pneumococci are exposed to micro-aerophilic conditions and to almost anaerobic conditions when present in blood and the cerebrospinal fluid (CSF) [14–16]. Likewise, mucus production during infection (i.e., due to influenza or RSV) can block the air passage and form micro-aerophilic (around 5% O_2) or even anaerobic microenvironments [15,16].

To understand how pneumococci successfully colonize the host and transition from nasopharyngeal colonization to severe disease, it is important to understand how different strains

respond to variations in the microenvironment. Pneumococcus is a diverse pathogen, with >90 serotypes (defined by the capsule polysaccharide) and has tremendous genetic variation that results from recombination. This genetic variability influences the biology and epidemiology of the strains [17]. Serotypes vary in their prevalence among healthy carriers and in the likelihood that they will cause severe disease. Laboratory studies have evaluated variations in growth characteristics by serotype and have identified a relationship between *in vitro* growth characteristics and prevalence of serotypes in the nasopharynx of healthy children [18,19]. However, the effect of variation in temperature and oxygen on the growth of different strains and serotypes has not been systematically explored. The response to such environmental variation could influence the success of a particular serotype in the nasopharynx or the likelihood of causing disease.

The aim of this study was to investigate how environmental variability in temperature and oxygen influences the growth of different strains and serotypes of pneumococci. Using a diverse set of clinical and nasopharyngeal isolates, as well as capsule-switch and capsule-knockout variants generated in the lab, we quantified how the growth of pneumococcus *in vitro* varies under a range of physiologically-relevant temperatures that represent colonization and invasive infections as well in aerobic and anaerobic conditions. We evaluated the relationship between these *in vitro* growth patterns and relevant serotype-specific epidemiological and biological variables.

Methods

Bacterial strains, culture media, and chemicals

Strains

Invasive pneumococcal disease (IPD) isolates were obtained from the isolate bank at the Centers for Disease Control/Active Bacterial Core surveillance; carriage isolates were provided by Ron Dagan (Ben-Gurion University, Israel), Adrienn Tothpal and Eszter Kovacs (Semmelweis University, Hungary [20,21]) and Debby Bogaert and Anne Wyllie (UMC, Utrecht [22]) (**Table 1**). Capsule-switch variants generated on the TIGR4 genetic background and the serotype 6B knockout strain were provided by Marc Lipsitch and generated as previously described [23]. Additional capsule-knockout strains were generated by replacement of the capsule biosynthesis locus with the Sweet Janus cassette [24].

Culture media

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

Growth experiment

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

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. For each growth curve, we estimated the length of the lag phase and the maximum growth rate using the groFit package in R [25]. For each curve we also extracted the maximum OD600 measurement. To quantify variations in 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) [26]. 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 [27]. Correlations of the effect of oxygen (ratio of maximum OD at a particular temperature for a particular isolate grown anaerobically or aerobically with catalase) with previously described epidemiological characteristics (invasiveness, carriage prevalence) were assessed using Spearman's correlations [28]. Associations with the presence of specific capsule components were assessed using Wilcoxon rank sum tests in R.

Principal components analysis and linkage to serotype-specific characteristics

We sought to evaluate the link between growth curve characteristics and previously published serotype-specific characteristics (capsule structure, disease severity, invasiveness, pre-vaccine carriage prevalence) [29–31]. Across all growth curves, isolates, conditions, and time points, there was a large volume of data with many possible variables that could be generated for correlation analyses (e.g., maximum OD600 achieved, OD600 at time point T). To avoid overfitting the data, we first performed principal components analysis. For each of the possible serotype/temperature/oxygen/isolation site combinations, we obtained the mean OD600 value at each time point. We then created an S*C matrix where S represented the number of serotypes and C represented the number of unique temperature/oxygen/isolation site/time point combinations. Therefore, there was a variable for every tested environmental condition, measured at each time point for isolates from different diseases sites, for each serotype. We also created variables for maximum OD600 reached under the different environmental conditions. These analyses were restricted to the carriage and invasive pneumococcal disease (IPD) isolates. With this matrix, missing values occurred (i.e., no serotype 12F carriage isolates were tested, leading to missingness in the summary values). Therefore, we performed a simple mean imputation of the missing values

(calculate mean of the non-missing values for a column and assign this value to the missing values). The variables were then centered and scaled prior to use. Using this matrix with 1470 variables for 51 serotypes, we performed principal components analysis (princomp in R); Six of the principal components (PCs) explained 50% of the variation in the data (PC1: 14%, PC2: 11%, PC3: 9%, PC4: 6%, PC5: 6%, PC6: 6%). These 6 PCs were used in subsequent regression analyses. In a series of linear regressions, we evaluated the association between these 6 PCs and pre-vaccine carriage prevalence (composite of several studies, square root transformed), case-fatality rate, complexity of the capsular polysaccharide (carbons/polysaccharide repeat) and invasiveness (square root transformed). We also used logistic regression to evaluate the association between the 6 PCs and the presence of N-acetylated sugars in the capsule or the presence of GlcA and GalA (both derived from same pathway).

Data availability

The raw data and R analysis scripts are available in a github repository (https://github.com/weinbergerlab/GrowthVariation).

Results

Growth characteristics

In total, we performed more than 4900 growth curves on 256 different pneumococcal strains, representing 53 different serotypes. We tested aerobic and anaerobic conditions ranging from 30-39°C. There was a positive correlation between growth rate and maximum density achieved (**Supplementary Figure 1**). Most strains reached their maximum density at low temperatures. The length of the lag phase was shortest for carriage isolates at low temperatures (i.e., 33°C) and was shortest for IPD isolates at 37°C. As a representative example, growth curves for serotype 19F isolates from carriage, conjunctivitis, pneumonia, and IPD are shown in **Figure 1**. Growth curves for all isolates and conditions can be explored interactively at https://weinbergerlab.shinyapps.io/ShinyGrowth_v2/.

The effect of temperature and oxygen varies based on site of isolation

The presence of oxygen had different effects on IPD and carriage isolates. On average, IPD isolates grew to a significantly higher density under aerobic+catalase conditions compared with anaerobic conditions (**Figure 2A**). This was true at both low and high temperatures (**Figure 2B, 2C**). In contrast, carriage isolates grew similarly in aerobic+catalase and anaerobic conditions at high temperatures but grew to a significantly higher density in aerobic+catalase at low temperatures. Conjunctivitis and pneumonia isolates also grew similarly in anaerobic and aerobic+catalase conditions, although fewer isolates were tested (**Figure 2**).

When grown in ambient air without catalase, pneumococcus typically produces toxic levels of hydrogen peroxide. As expected, most IPD isolates hardly grew in ambient air due to this toxicity. Unexpectedly, some IPD isolates carriage isolates grew moderately under these conditions (e.g., IPD isolates of serotypes 9N, 12F, 18A,23A, 33F IPD isolate; **Figure 5**). Moreover, carriage isolates grew moderately well in ambient air without catalase, especially at higher temperatures (35-39°C). (**Figure 2C, 3**).

Importance of oxygen varies by serotype

We next investigated whether the effect of oxygen on growth patterns varied by serotype (**Figure 4, 5**). For these analyses, we focused on comparing growth in aerobic+catalase versus anaerobic conditions. Overall, certain serotypes (2, 4, 13, 23B, 35B) grew better under aerobic conditions (with catalase), while other serotypes (6B, 8, 9N, 12F) did not show a difference between aerobic and anaerobic growth (**Figure 4**). The differences between aerobic and anaerobic conditions were more pronounced for the IPD isolates, but the direction of association was generally similar for both IPD and carriage isolates.

The clinical isolates represented diverse genetic backgrounds. We attempted to evaluate the growth characteristics of capsule-knockout strains as well as several capsule-switch variants. While the results were ambiguous, they suggested that the effect of capsule production on growth was more pronounced during anaerobic growth compared with aerobic growth with catalase (**Supplementary Figures 2-5**).

Relationship of growth characteristics to serotype-specific characteristics

Finally, we performed exploratory analyses to evaluate whether the characteristics of the growth curves were related to serotype-specific epidemiological characteristics and biochemical characteristics of the capsule. There was an association between the second principal component (PC2, which explained 11% of the variability in the data) and pre-vaccine carriage prevalence ($p<0.001$) and invasiveness ($p=0.05$) (**Figure 6)**. We therefore evaluated which components of the growth curves contributed most to PC2. PC2 was largely influenced by density at ~5 hours among the carriage isolates at 33C and ~2-3 hours among the IPD isolates at 38°C. These time points corresponded to periods when many of the isolates were in early log-phase growth but some were still in stationary phase. PC2 was also influenced by density at later time points, which largely (inversely) reflects density at early time points.

There was no association between PC1-6 and serotype-specific case fatality ratio, polysaccharide complexity, or specific polysaccharide components. There were also no notable correlations between the effect of oxygen (ratio of maximum OD600 in anaerobic vs aerobic growth with catalase) and the invasiveness of a serotype, case-fatality of the serotype, carriage prevalence of the serotype, or with any capsule components.

Discussion

We provide novel information about the effect of environmental conditions on the growth characteristics of pneumococcal strains. Testing a diverse set of isolates, pneumococci grew to the highest density under conditions that mimic the normal environment of the nasopharynx in terms of temperature and oxygen level. Important differences were observed between carriage and disease isolates and by serotype, possibly reflecting adaptation to the environment from which they were isolated. The hierarchy of strains growing at early time points correlated with several serotype-specific epidemiological and biological characteristics.

As the nasopharynx is the normal habitat of pneumococcus, we hypothesized that the nasopharyngeal temperature would be ideal for growth. Indeed, temperature played an important role in terms of the maximal density achieved and how quickly the isolates started growing (lag phase). Carriage isolates grew to the highest density and had the shortest lag at temperatures resembling those of the nasopharynx. This finding, along with recent work on the effect of lower temperatures on the immune response to pathogens in the upper respiratory tract [32], suggests that the environment of the nasopharynx is optimal for pneumococcal growth and survival. IPD isolates responded differently to environmental variations. While they also reached maximum density at low temperatures, the IPD isolates had the shortest lag phase at 37°C (i.e., core body temperature). Additionally, IPD isolates hardly grew in ambient air without catalase, while carriage isolates grew moderately well, especially at higher temperatures (**Figure 1-4**).

During the invasion process, when the pneumococcus transitions from the nasopharyngeal environment to the internal body environment, it has to adapt to many changes, including nutrient,

temperature and oxygen levels. Temperatures vary from the low-30° range in the upper respiratory tract to 37°C in core body sites and even higher during fever. Likewise, oxygen levels can vary considerably during infection. Increased mucus production (due to co-infection with viruses or other pathogens in the upper respiratory tract) leads to lower oxygen levels and may generate a local hypoxic environment [33–35] . The availability of oxygen is also decreased in pneumonia, empyema, and otitis media. Oxygen levels in the uninflamed middle ear space, for example, resemble that of venous blood, are less than a third that of the airway, and may be further reduced by the presence of effusion [14,15,36]. These gradients of temperature and oxygen could influence which strains succeed, and pneumococcus might adapt to these variations. We found that IPD isolates, but not carriage isolates, started growing more quickly at body temperature (37°C) than at lower temperature but reached a lower final density then the same isolates grown at lower temperatures, and strains generally grew to a higher density with oxygen. However, some isolates appeared to be less susceptible to these variations in oxygen and temperature, potentially influencing the likelihood of a carriage episode leading to infection.

Some of the differences observed in growth phenotypes between carriage and disease isolates could reflect opaque/transparent phase variation [37]. Opaque variants are generally isolated from IPD and have increased capsule production and decreased production of certain surface proteins. Phenotypically, the presence of oxygen accentuates differences in capsule production between opaque and transparent variants [36]. This effect could be mediated via the pathways involved in converting pyruvate to acetyl-CoA, an important biochemical precursor for capsule production for many serotypes [38]. Variations in the use of this pathway between serotypes or the efficiency of

this pathway between lineages could influence some of the patterns that were observed in the growth curves.

Serotype is a major determinant of pneumococcal epidemiology and biology. When comparing growth in aerobic conditions (with catalase) with growth in anaerobic conditions, the benefit of oxygen varied by serotype (**Figure 4, 5**). Serotypes 2, 4, 13, 23B, 35B and 38 grew better with additional catalase than in anaerobic conditions, whereas serotypes 6B, 8, 9N, 12F, 22F and 42 grew similarly in both environments. These patterns did not correlate with the presence of N-acetylated sugars in the capsule, which are derived from a pathway that would be influenced by the presence of oxygen [38], nor did they correlate with invasiveness of the serotype. We tested a relatively small number of isolates per serotype, so it is possible that non-capsular genetic variations or differences in the culture history of the isolates could influence the observed responses to oxygen.

This study had certain limitations. For the growth curves, we used BHI broth which is an artificial growth medium that differs in nutrient composition from the host. We evaluated several minimal media but found that growth was generally poor, making comparisons between strains difficult. While we tested a large number of strains representing many serotypes, some serotypes were only represented by a single isolate (i.e., 11B, 12F, 13). This could limit the generalizability of serotype-specific findings in these instances, making it difficult to make inferences about whether variability was due to serotype, site of isolation, or lineage effects. The strains used in this study were largely a convenience sample from clinical studies. The genetic diversity of pneumococcus makes it difficult to draw conclusions about the cause of differences between strains. The growth curves

with the capsule-knockout strains and capsule-switch variants suggests that the capsule itself could influence these phenotypes. We did not perform any gene expression studies which could be highly influenced by environmental conditions [39]. Further work could explore the genetic basis (both capsular and non-capsular factors) for the differences in growth phenotypes between strains.

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

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Figure legends

Figure 1. Growth patterns of serotype 19F strains isolated from different sites in the body. Growth was measured every 30 minutes over 24 hours at 30-39°C, with ambient air, ambient air with catalase, or under anaerobic conditions. Each line corresponds to a growth curve in a different condition—bluer temperatures correspond to colder conditions, orange colors correspond to warmer conditions. Each column corresponds to an isolate obtained from a different anatomical site.

Figure 2. Effect of oxygen on the maximum density achieved for carriage and clinical samples (A) across all temperatures, (B) at low temperature (30-35°C) and (C) at high temperature (37-39°C). Blue triangles represent aerobic growth with catalase, orange circles represent aerobic without catalase, green squares represent anaerobic growth. The y-axis shows the maximum density achieved for IPD, carriage, pneumonia, and conjunctivitis isolates, after adjusting for serotype and temperature. Mean+/- 95% confidence intervals.

Figure 3. The effect of temperature and oxygen on growth of (A) all isolates, (B) IPD isolate and (C) carriage isolates. The y-axis shows the maximum density at different temperature and oxygen conditions, after adjusting for serotype effects. Mean+/- 95% confidence intervals.

Figure 4. Relative difference in maximum density when grown in aerobic conditions with catalase versus anaerobic conditions. Positive values indicate that a higher density (as measured by optical density) was achieved under aerobic conditions. A value of 0 indicates no

difference. Mean+/- 95% confidence intervals. The reference in the regression is serotype 14, and the temperature was included as a covariate.

Figure 5. The effect of serotype and oxygen on growth of carriage isolates and IPD isolates. The y-axis shows the maximum density achieved for each serotype. Mean+/- 95% confidence intervals. Temperature was included as a covariate.

Figure 6. Correlation between the second principal component and (A) serotype-specific pre-vaccine carriage prevalence in a composite of settings and (B) serotype-specific log-invasiveness. Higher principal component scores correspond to higher density at an early time point. Both of the epidemiologic measures were estimated and described in previous studies. The size of the circle is proportional to the inverse variance of the invasiveness estimate [30].

Supplementary Figure 1. Correlation between maximum density achieved by an isolate and the growth rate across (A) carriage and (B) IPD isolates. Individual dots represent individual isolates. Lines represent LOESS fit. Strains with steep slopes reached a higher maximal OD.

Supplementary Figures 2-5. Growth curves for capsule-switch variants on the TIGR4 background and capsule knockout strains.

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-)

Figure 1

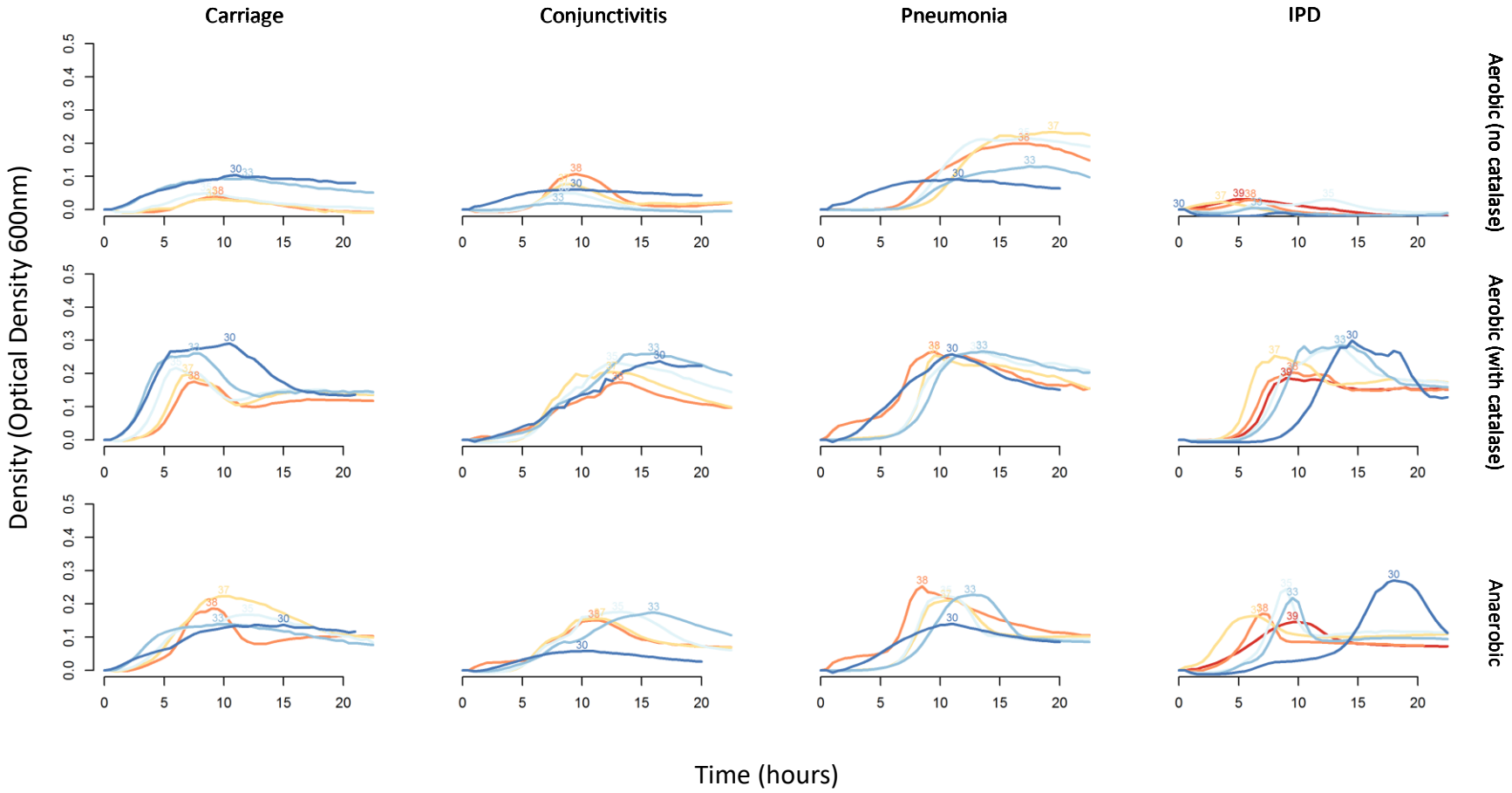

Carriage
Conjunctivitis
Pneumonia
IPD
Aerobic (no catalase)
Aerobic (with catalase)
Anaerobic
Density (Optical Density 600nm)
Time (hours)

Figure 2

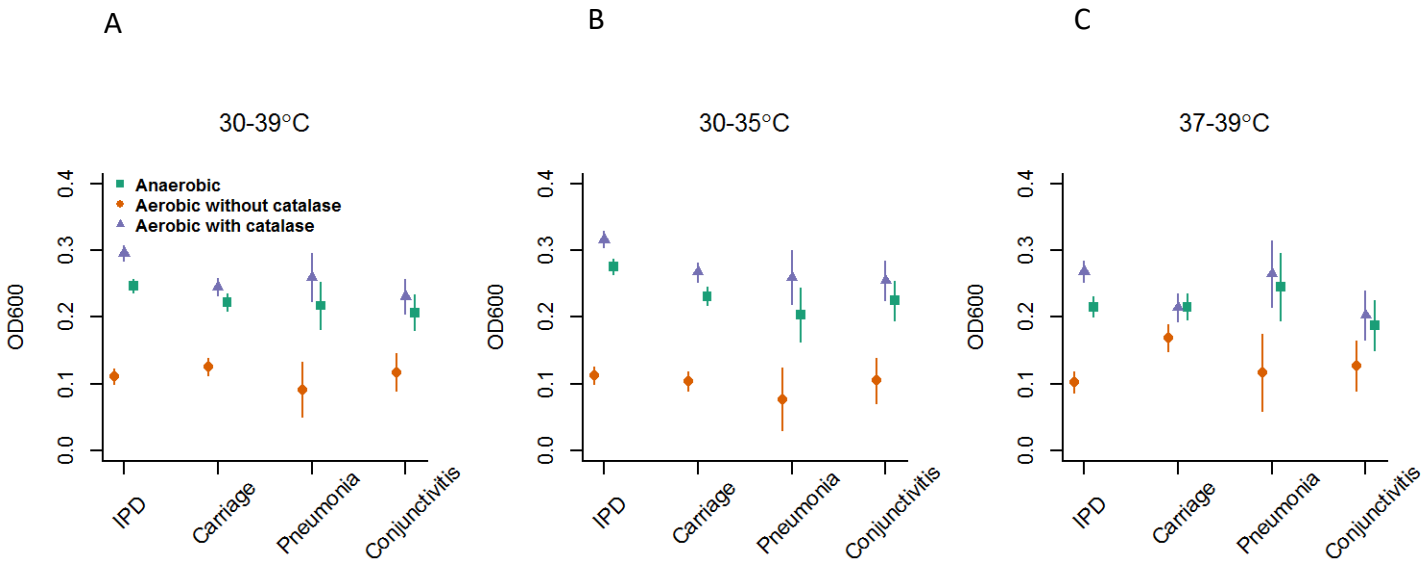

A
B
C
30-39°C
30-35°C
37-39°C
Anaerobic
Aerobic without catalase
Aerobic with catalase
OD600
0.0
0.1
0.2
0.3
0.4
IPD
Carriage
Pneumonia
Conjunctivitis

Figure 3

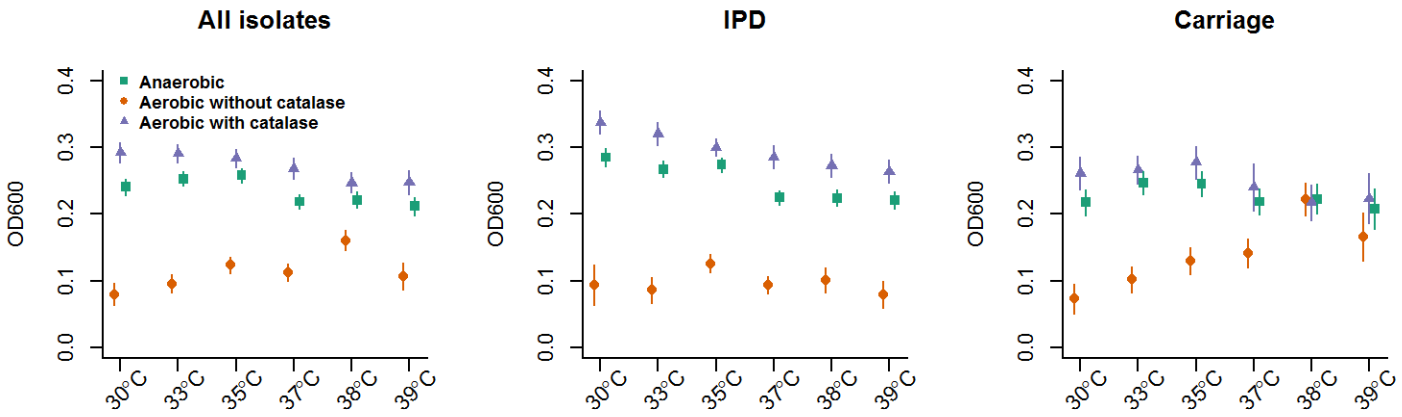
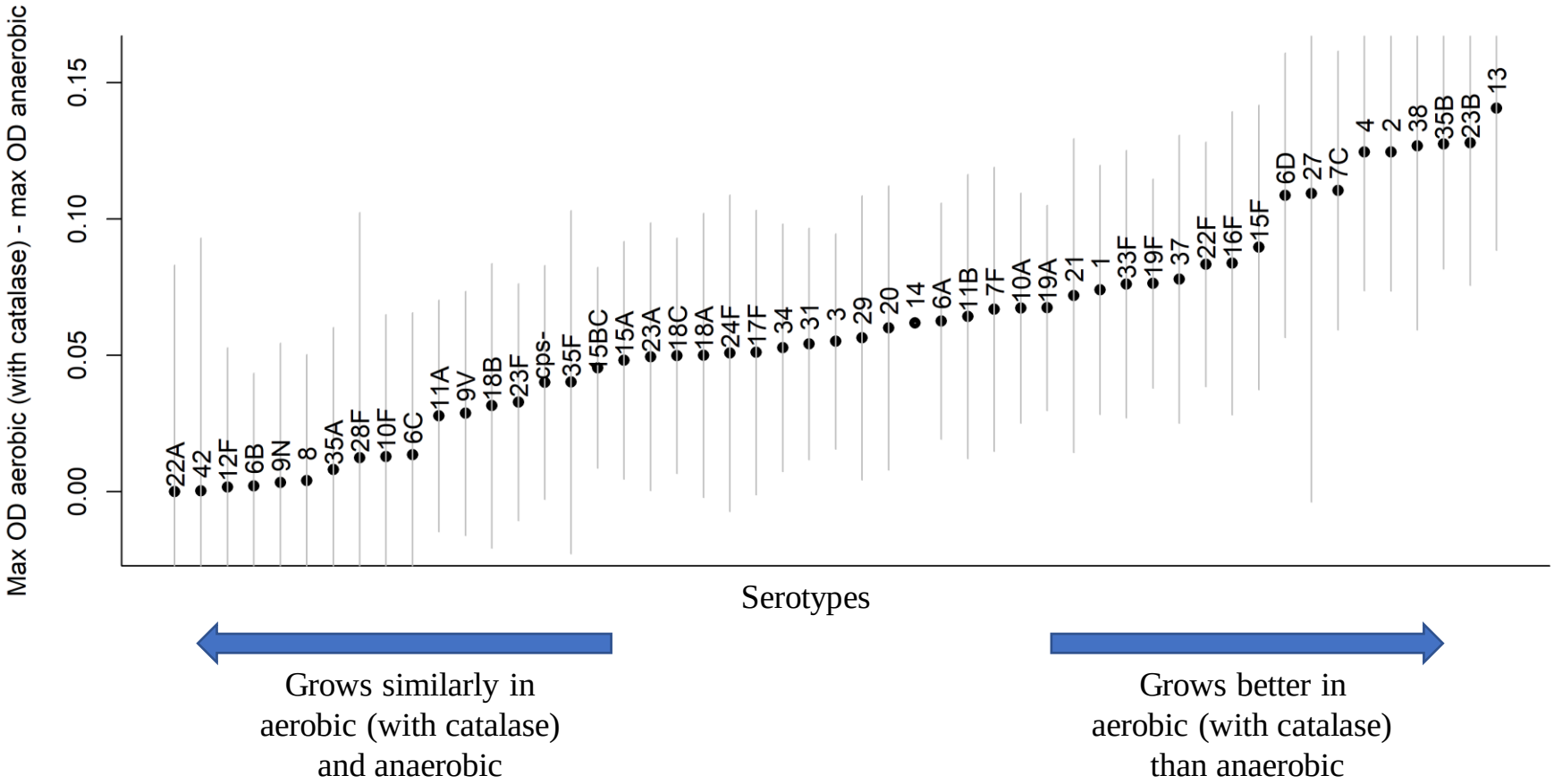

All isolates
IPD
Carriage
Anaerobic
Aerobic without catalase
Aerobic with catalase
OD600
0.0
0.1
0.2
0.3
0.4
30°C
33°C
35°C
37°C
38°C
39°C

Figure 4



Grows similarly in aerobic (with catalase) and anaerobic

Grows better in aerobic (with catalase) than anaerobic

Figure 5

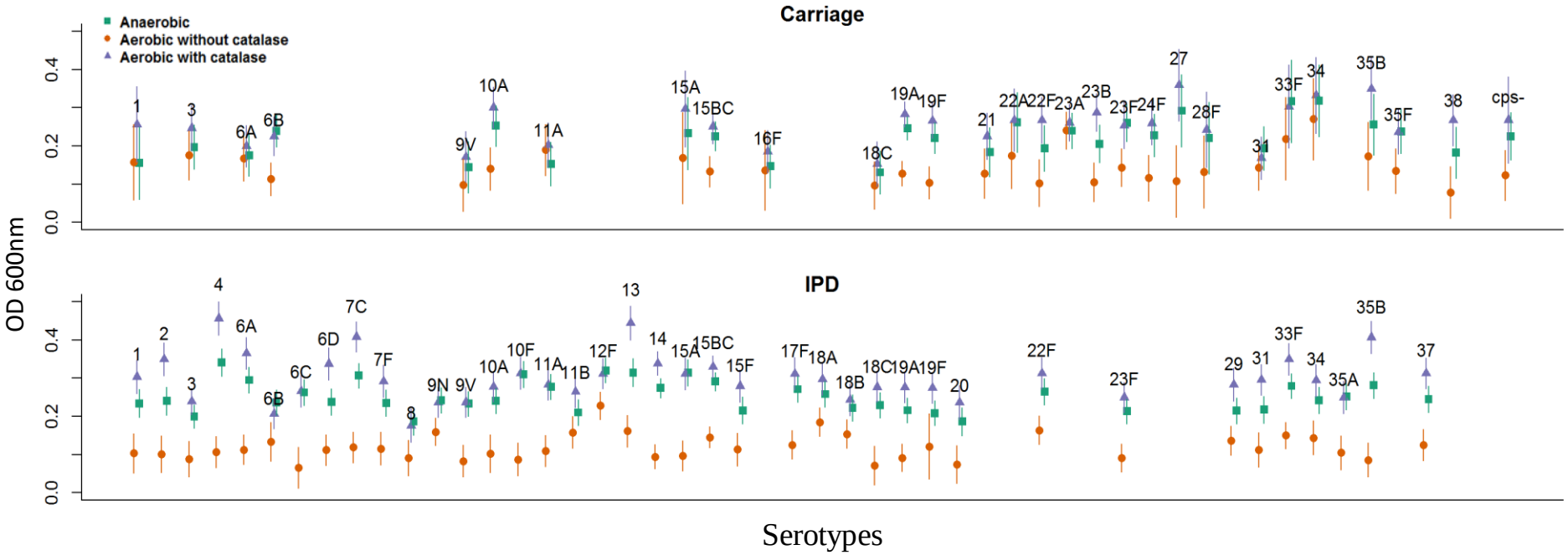
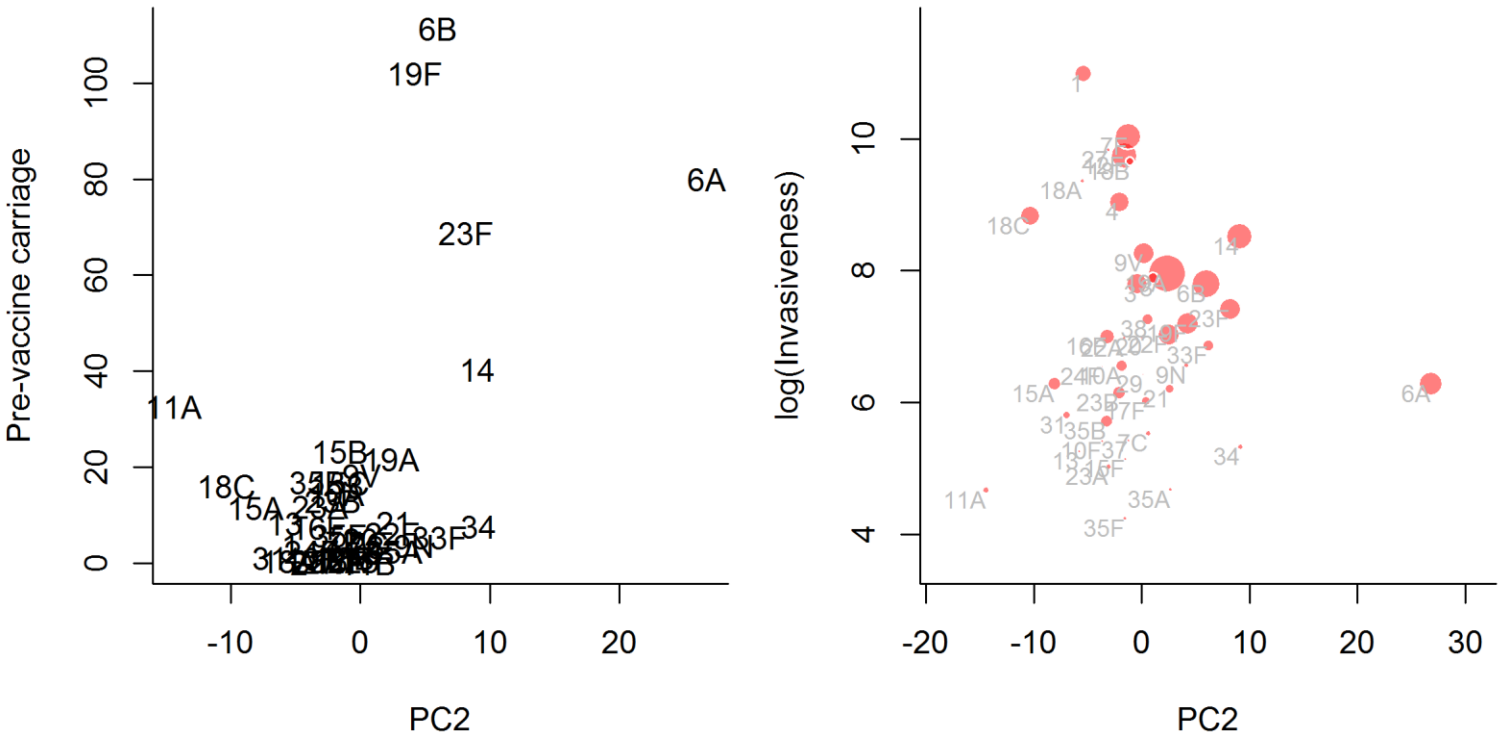
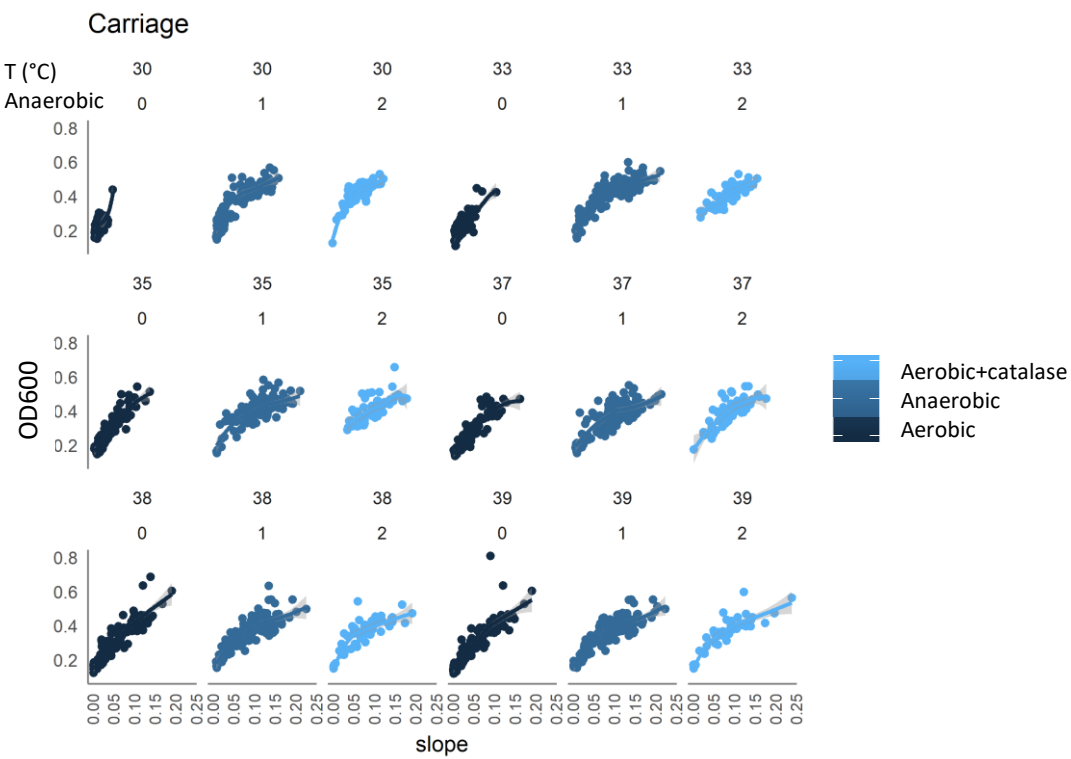

Carriage
IPD
Anaerobic
Aerobic without catalase
Aerobic with catalase
OD 600nm
0.0
0.2
0.4
Serotypes

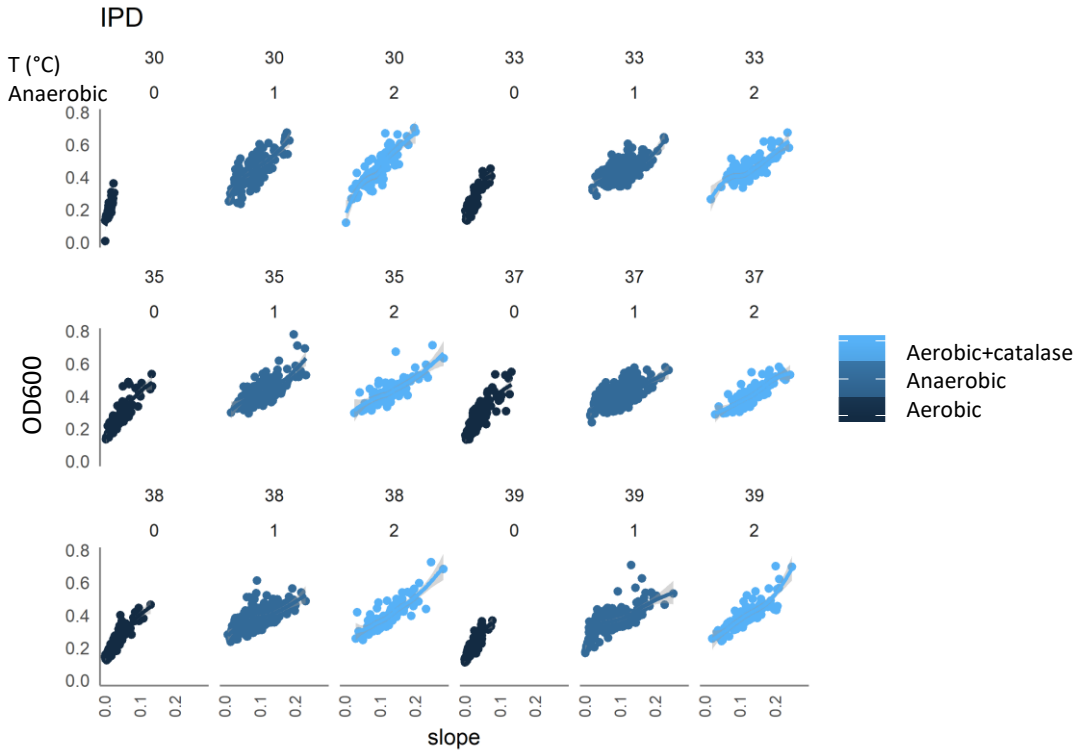
Figure 6



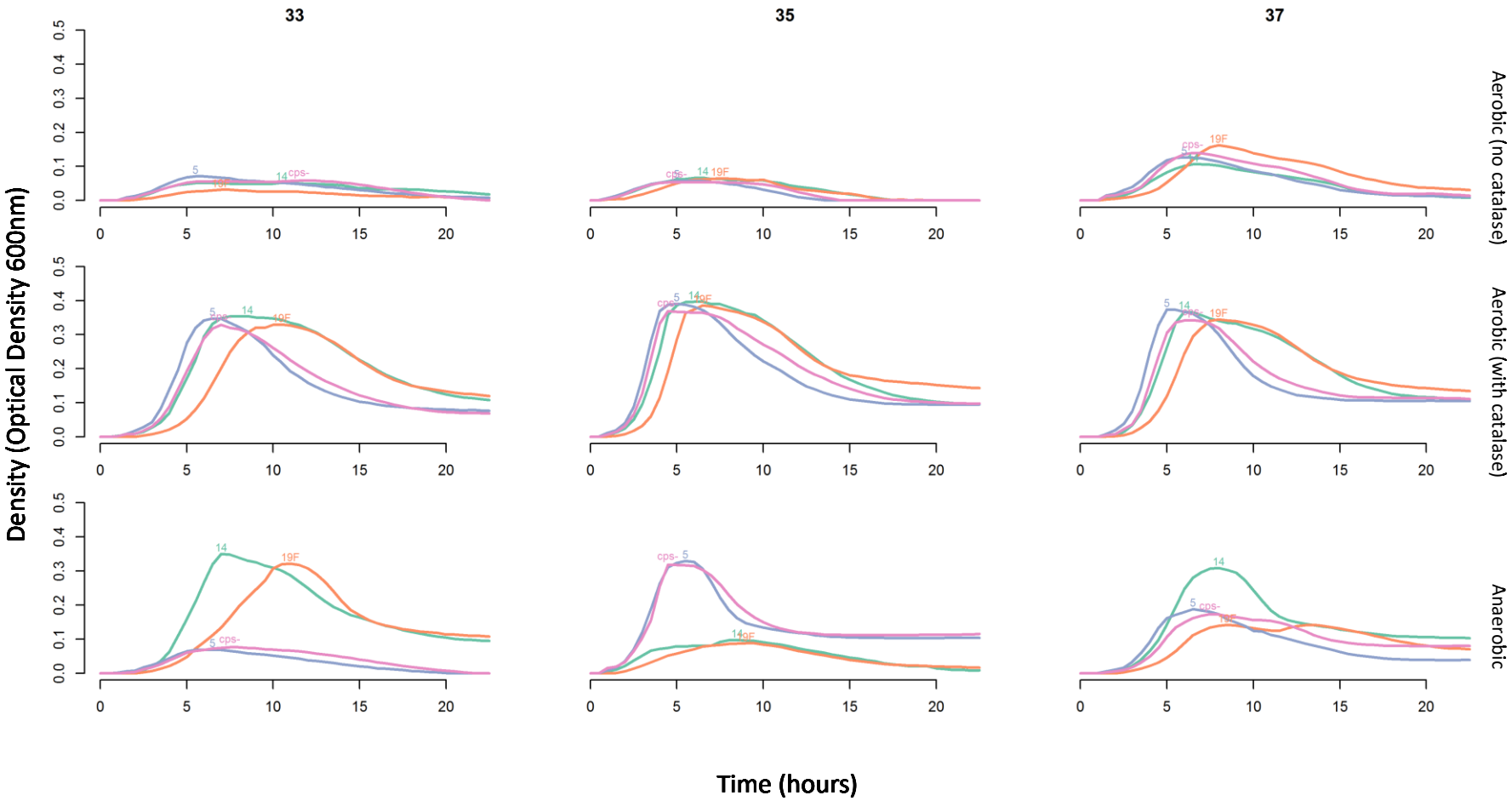
Supplementary figure 1A



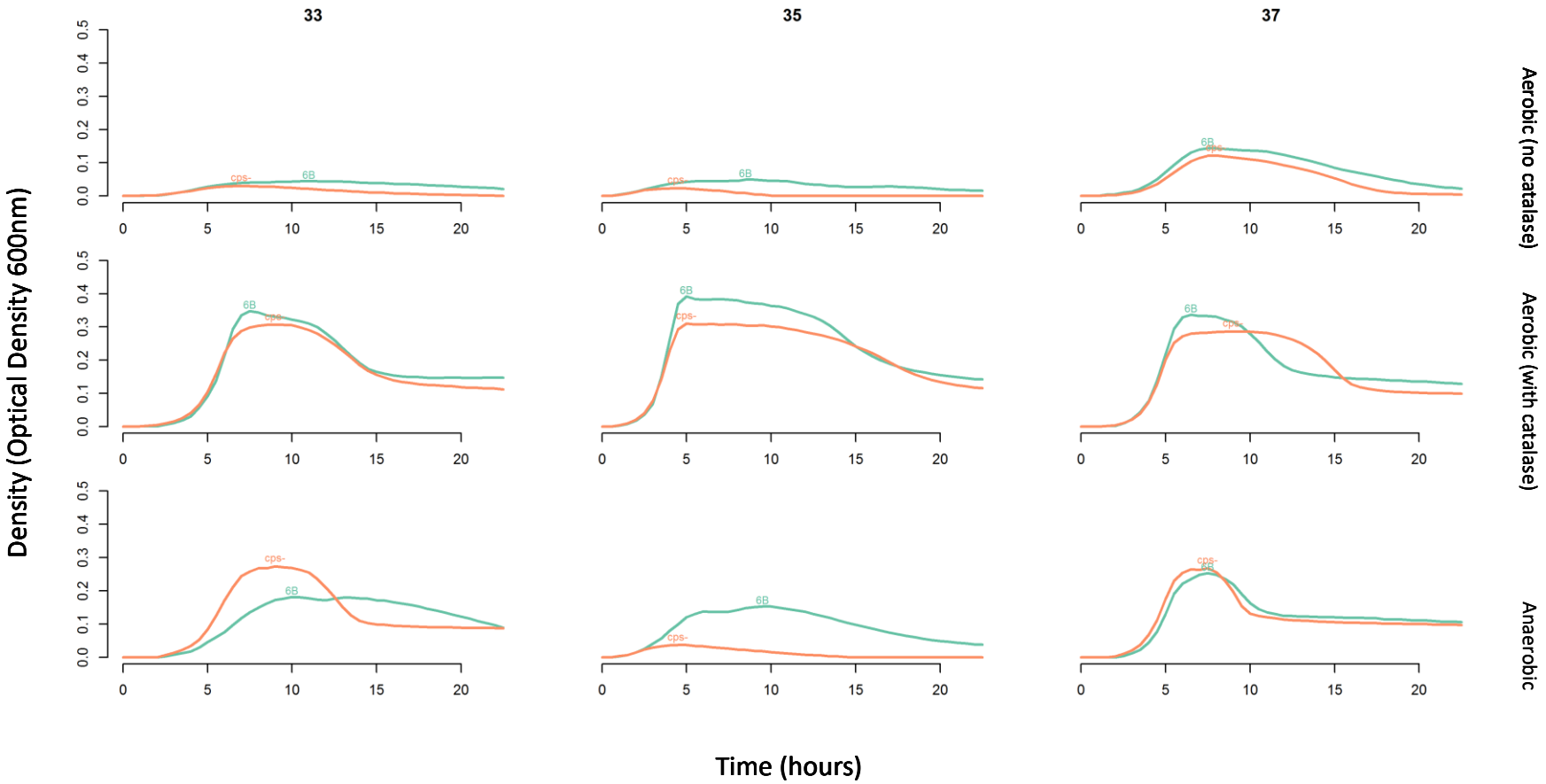
Supplementary figure 1B



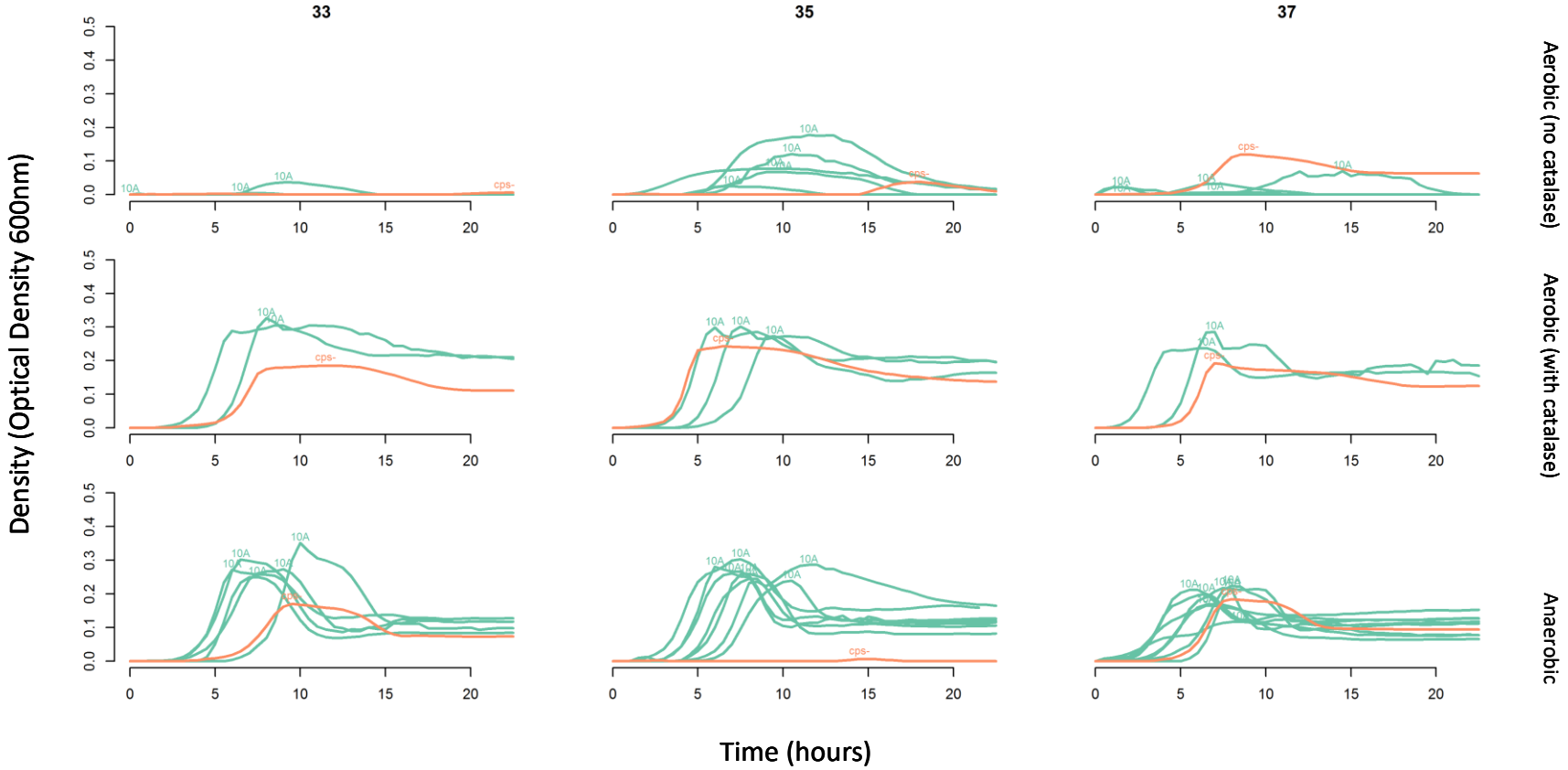
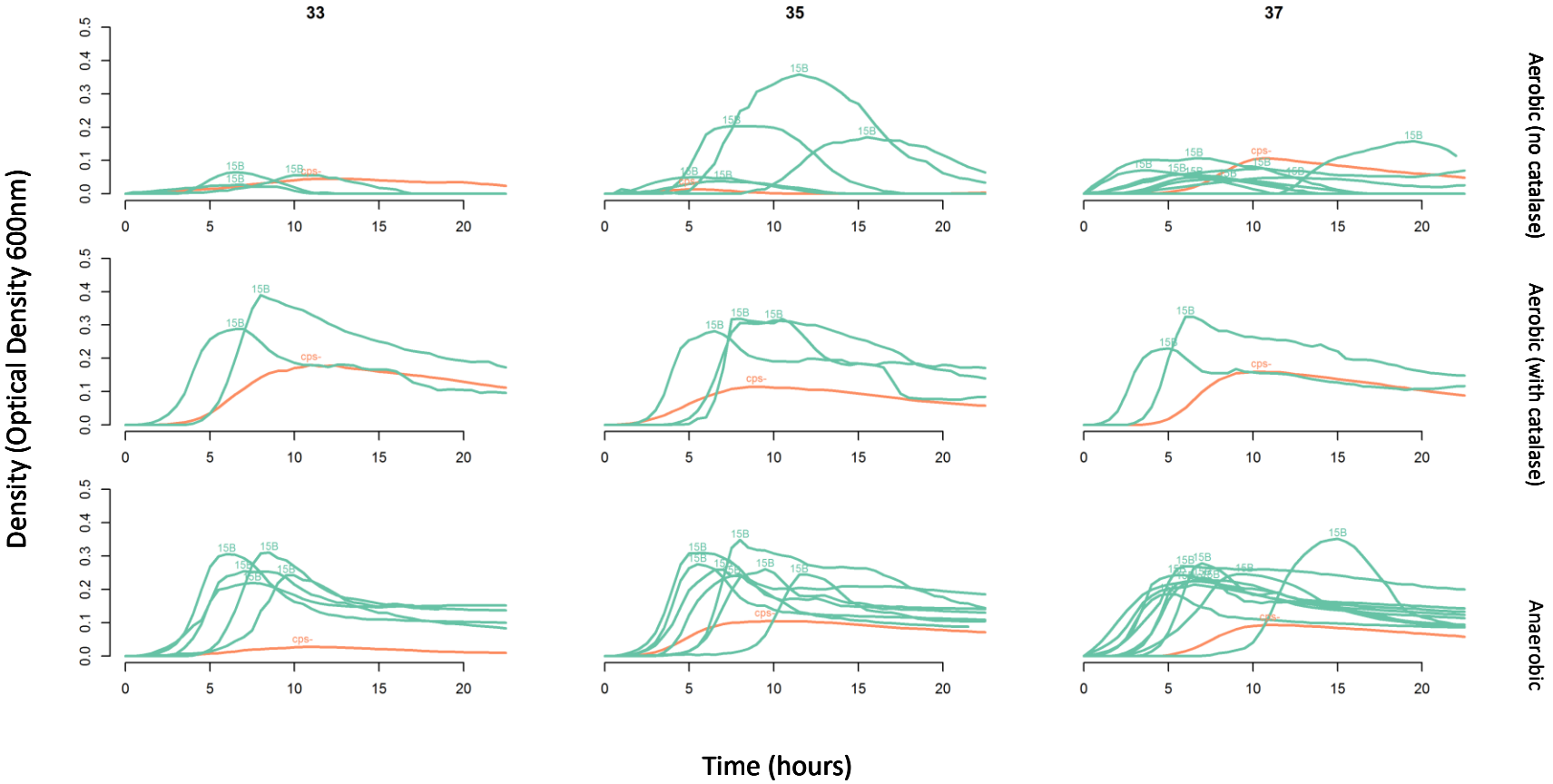
Supplementary figure 2



Supplementary figure 3



Supplementary figure 4



Supplementary figure 5