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1 **A minimal growth medium for *Pseudomonas azotoformans* associated with insect gut**

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6

7

8 **Abstract**

9 *Pseudomonas* spp. can be found associated with several insect hosts. Some of these strains play
10 a crucial role in helping their hosts overcome biotic challenges such as plant defence effectors.
11 Defined media and optimally minimal media are instrumental in studying the physiology and
12 metabolic pathways of microorganisms. In the case of the insect-associated *Pseudomonas*
13 *azotoformans* isolated from *Galeruca laticollis*' gut, we tested six previously designed
14 defined/minimal media. To determine the optimal medium, we compared the bacterium's
15 growth performances on the different defined/minimal media to its growth performance on a
16 rich medium using the Dynamic Time Warping Algorithm. The growth results at both 25°C
17 and 30°C indicated the SMM as the optimal medium, with bacterial growth performances being
18 the closest to a rich medium. Therefore, this medium would be highly suitable to study the
19 catabolic pathways for the degradation of some xenobiotics in *P. azotoformans*.

20

21 **Introduction**

22 Bacteria of the genus *Pseudomonas* can be found in many habitats, including host-associated
23 ones [1]. Several *Pseudomonas* strains have been found in plants [2–4] and animals [5–7] with
24 a variety of phenotypes ranging from pathogenicity [3,8] to mutualism [5,9,10]. During recent
25 years, some *Pseudomonas* strains were discovered to play a crucial role in helping different
26 insects overcome biotic challenges such as pesticides or plant defence effectors [5,9,11].
27 Although the bacterium's role in these symbioses is established, the molecular and biochemical
28 mechanisms by which the symbionts are able to protect their respective hosts remain unknown.
29 Understanding how *Pseudomonas* species interact with their environment, host-associated or
30 not, is of interest from a fundamental and applied perspective. For example, understanding how
31 these bacteria are able to degrade a wide range of xenobiotics (such as pesticides) could help
32 develop more ecologically-friendly approaches to deal with contaminations due to these
33 xenobiotics. Since such metabolic investigations would ideally be carried out outside of the
34 insect host, the development of suitable bacterial growth media that support good growth and
35 produce consistently high viable cell counts is crucial. Such media are instrumental in studying
36 certain aspects of bacterial metabolism at the biochemical and molecular level (i.e.
37 transcriptomics).

38 However, for physiological and metabolical studies of these strains, defined/minimal media
39 (liquid and agar) that support good growth and gives consistently high viable cell counts are a
40 staple to unveil the mechanisms of action of metabolical processes and clarify the effects of
41 changes in medium composition on cell growth and product formation. Defined media are
42 culture media whose chemical composition is well known. A defined medium with only the
43 ingredients necessary for the growth of a microorganism is called a minimal medium.
44 Nevertheless, the number of ingredients that compose a minimal medium varies enormously

45 and depends mainly on which microorganism is cultivated. Chemically defined minimal media
46 allow for a finer metabolic profiling of the microorganism [12,13].

47 Although several defined or minimal nutritional media have been used for the genus
48 *Pseudomonas*, most of the media were developed for the most common species (e.g., *P.*
49 *syringae*, *P. aeruginosa*, *P. fluorescens*;) [14–16] and the growth performance of other strains
50 on these media have rarely been investigated. Therefore, there is a need to test the already
51 available minimal defined media and possibly develop more suitable ones for other
52 *Pseudomonas* species.

53 Here, we aimed to determine an ideal minimal defined medium to grow a strain of *P.*
54 *azotoformans*, isolated from an insect gut, in the laboratory. To this end, we compared the
55 growth performance of this strain on several media to a rich medium (i.e. Luria Bertani broth).

56

57 **Materials and methods**

58 *Strain:*

59 The bacterial strain was isolated from the gut of the beetle *Galeruca laticollis* plated on LB
60 agar media and incubated at 30°C. The strain was initially identified based on its 16S rRNA
61 gene sequence, and the taxonomic assignment was later confirmed through a phylogenomic
62 analysis (data subject of a separate publication). The strain was identified as *Pseudomonas*
63 *azotoformans* strain GL93.

64 *Culture media and growth conditions:*

65 In addition to LB media, which was used as a growth reference, five minimal media (namely
66 SMM, M9, MOPS, MMV, and MMP) were tested (see supplementary file S1 for details).
67 During the first test, bacteria grown on MMP produced fluorescent siderophores that could

68 potentially interfere with the optical density (OD) readings. In order to minimize the production
69 of these siderophores, the medium was supplemented with calcium chloride (CaCl₂;
70 supplementary figure S1).

71 The bacterium was incubated overnight at 30°C in a fresh LB medium prior to the experiments.
72 Log-phase cells were collected by centrifugation at 2000 g for 10 minutes and washed three
73 times in a sterile NaCl 9‰ solution to remove any remnants of the previous growth medium.
74 Finally, the cells were concentrated to the desired concentration (10⁸ cells/ml), and 1 ml was
75 used for the inoculation of different growth media. To test if the bacterium could grow on the
76 different media, 100 ml of each medium were inoculated with 1 ml of a NaCl 9‰ solution
77 containing 10⁸ bacterial cells. Bacterial growth at 30°C was monitored for seven days, taking
78 OD readings at 24h intervals using an ONDA V-10 plus spectrophotometer.

79 Three minimal media (SMM, M9 and MMP+CaCl₂) as well as LB proved suitable to grow the
80 *Pseudomonas* strain (i.e. OD_{medium} > OD_{LB}*0.2 after seven days) and were used in a second
81 experiment. A 24-well plate was prepared with 200 µl of a given medium per well, one medium
82 per row (see supplementary figure S2 for an illustration of the experimental design). Five out
83 of the six wells for each medium were inoculated with 10 µl of the bacterial inoculate prepared
84 as outlined above. The last well was used as blank. Bacterial growth was monitored for six
85 days on a BioTek Synergy H1 multi-plate reader with OD readings at 600 nm every 15 minutes,
86 preceded by 5 seconds of orbital shaking to homogenize the cultures. The experiment was
87 carried out twice, once at 30°C and a second time at 25 °C.

88 *Data analyses:*

89 the R (<https://cran.r-project.org>) package `growthcurver`
90 (<https://github.com/sprouffske/growthcurver> [17]) was used to infer the different terms of each

91 logistic curve equation $N_t = \frac{N_0 K}{N_0 + (K - N_0) e^{(-rt)}}$, where N_t is the number of cells (or the

92 absorbance reading) at time t , N_0 is the initial cell count (or absorbance reading), K is the
93 carrying capacity, and r is the growth rate. The Dynamic Time Warping (DTW; [18])
94 Algorithm from the package SimilarityMeasures ([https://cran.r-](https://cran.r-project.org/web/packages/SimilarityMeasures/)
95 [project.org/web/packages/SimilarityMeasures/](https://cran.r-project.org/web/packages/SimilarityMeasures/)) was then used to determine the similarity
96 between the different growth curves. First, we compared the growth curves between the
97 replicates of each growth medium to exclude outliers. To this end, the values generated by
98 pairwise comparisons of all growth curves were aggregated in a similarity distance matrix and
99 visualized after hierarchical clustering. Secondly, we compared the averaged growth curves
100 (i.e., curve resulting from the averaged values of the single curves) of the different media using
101 the same approach used for the intra-medium comparisons.

102 *Metabolic pathway assessment:*

103 The metabolic capacities of *P. azotoformans* were checked using the four complete genomes
104 available on the NCBI database (accession numbers: LT629702, CP041236, CP014546 and
105 CP019856). In particular, the protein sequences of each genome were downloaded and
106 submitted to GhostKOALA (ver 2.2, [19]) for metabolic pathway reconstruction. The pathways
107 related to the central metabolism (e.g., glycolysis, pentose phosphate, TCA cycle, purine and
108 pyrimidine synthesis, amino acid synthesis) were checked visually for completeness.

109

110 **Results and discussion**

111 *Growth performance of P. azotoformans GL93 on different media*

112 *P. azotoformans* was able to grow on all tested media but with very different growth
113 performances (Table 1, Supplementary figure S3). As such, the bacterium reached the
114 stationary phase on LB medium in less than 24h (mean = 18h), but it took longer to reach this
115 phase on other media. It took ca 24h for the bacteria to reach the stationary phase on MMV, ca

116 36h on M9 and more than 48h on the other media. MOPS was the medium with the longest lag
117 phase (mean = 36h). Similarly, the shortest generation time was observed on LB (ca. 1h) and
118 the longest on both MMP and SMM (ca. 20h). However, *P. azotoformans* reached higher
119 bacterial densities in MMP ($OD_{600} = 1.18 \pm 0.04$) and SMM ($OD_{600} = 1.3 \pm 0.01$) than in all
120 other media, including LB ($OD_{600} \approx 1 \pm 0.01$). In the other media, the maximum value of OD_{600}
121 remained inferior to one (namely M9: 0.3 ± 0.01 , MMP + $CaCl_2$: 0.2 ± 0.004 , MMW: $0.12 \pm$
122 0.005 , and MOPS: 0.03 ± 0.004). It is noteworthy that the addition of $CaCl_2$ to the MMP
123 medium reduced the production of siderophores (see Supplementary figure S1), but also had a
124 notable effect on both the optical density (which decreased from an OD_{600} of 1.18 ± 0.04 to 0.2
125 ± 0.004) and on the generation time, lowering it from 20h to 12h. A possible explanation could
126 be that, since the bacterium no longer produced siderophores, more resources could be invested
127 into growth, resulting in a shortening of the time needed for cellular division. Considering the
128 low bacterial density achieved on MMW and MOPS compared to LB (OD_{600} of 0.12 and 0.03,
129 respectively), these two media were not included in subsequent experiments. The low growth
130 performance on these two media could be due to a chemical being present at a highly limiting
131 concentration or a growth-inhibiting factor for our strain.

132

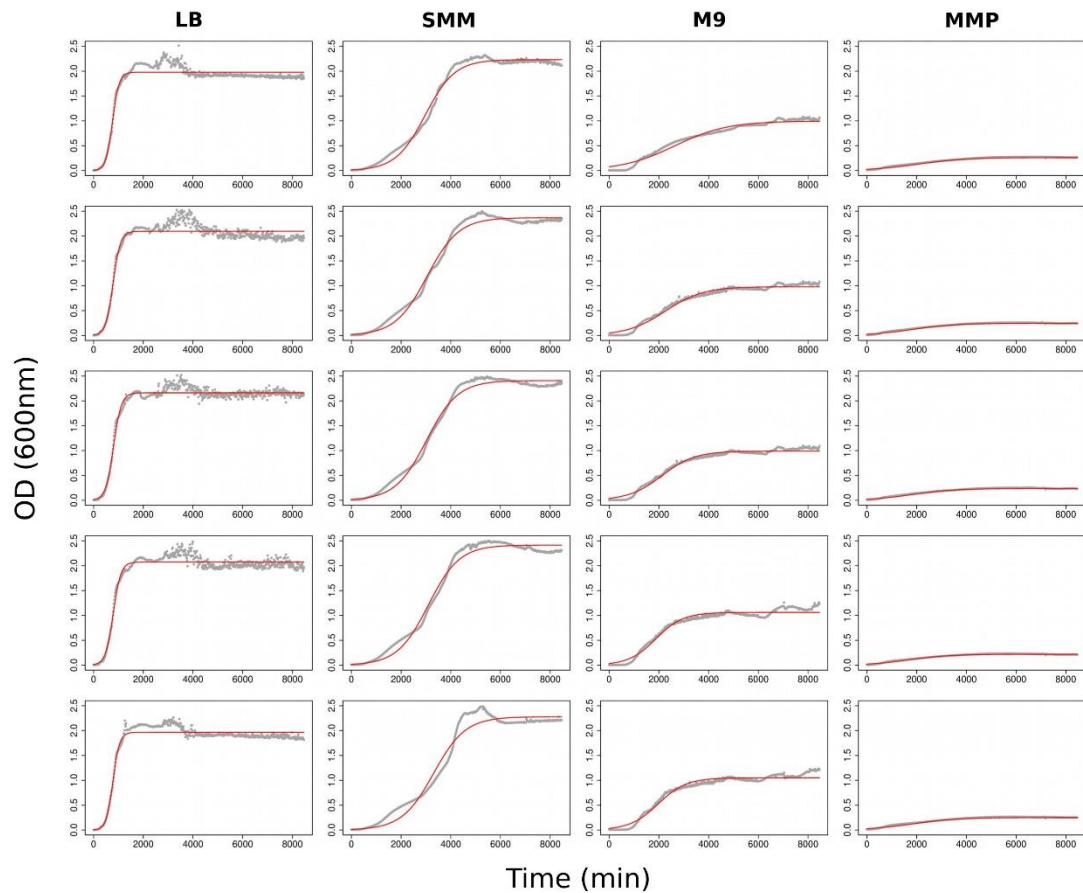
133 **Table 1: Optical density (OD) readings at 600 nm for each medium.**

Medium\time (h)	0	24	48	72	144	168
LB	0.03±0.01	1.19±0.04	0.98±0.04	0.91±0.02	0.97±0.04	0.94±0.02
SMM	0.02±0.02	0.37±0.01	0.53±0.02	0.66±0.02	1.16±0.01	1.3±0.01
M9	0.02±0.02	0.07±0.01	0.42±0.02	0.39±0.03	0.24±0.01	0.3±0.01
MOPS	0	0.02±0	0.03±0.01	0.04±0.01	0.03±0	0.03±0
MMW	0±0	0.16±0	0.18±0	0.15±0	0.1±0	0.12±0
MMP	0	0.32±0.01	0.49±0.02	0.65±0.02	1.08±0.03	1.18±0.04
MMP + $CaCl_2$	0±0	0.12±0	0.18±0	0.2±0.01	0.21±0.01	0.2±0

134 The reported values are the average of three readings and the standard error

135

136 Identification of the best minimal medium for *P. azotoformans*



137

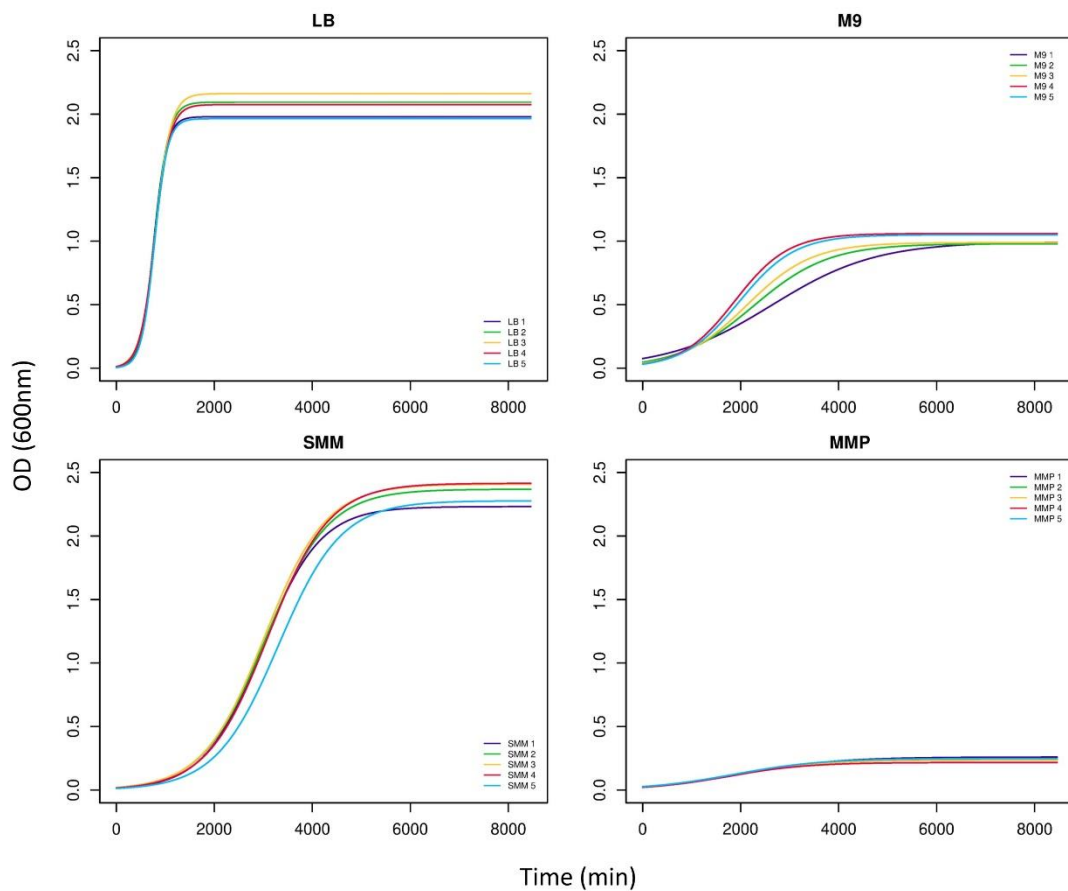
138 **Figure 1:** Growth curves obtained from the absorbance readings of the different media incubated at
139 25°C. The experimental curves (grey dots) correspond to the absorbance values recorded every 15
140 minutes for seven days. Each column corresponds to a medium, and each graph shows the growth
141 performance in a single well (N=5 per medium). The red line represents the theoretical curve fitted over
142 the experimental points.

143

144 The growth of *P. azotoformans* on the selected media (SMM, M9, and MMP + CaCl₂, hereafter
145 MMP) was monitored every 15 minutes at two constant temperatures (i.e., 25°C and 30°C).

146 The data obtained from 5 replicate cultures for each medium at both temperatures was used to
147 infer the theoretical growth curve for each culture (Figs 1, 3). Comparing the growth curves of
148 the five replicate cultures for each medium at both temperatures (Fig. 2, 4) revealed a certain
149 degree of variability between replicates for some conditions. In particular, there was some

150 variability in the exponential growth phase for M9 at both temperatures and for MMP at 30°C
151 (Fig. 2, 4).



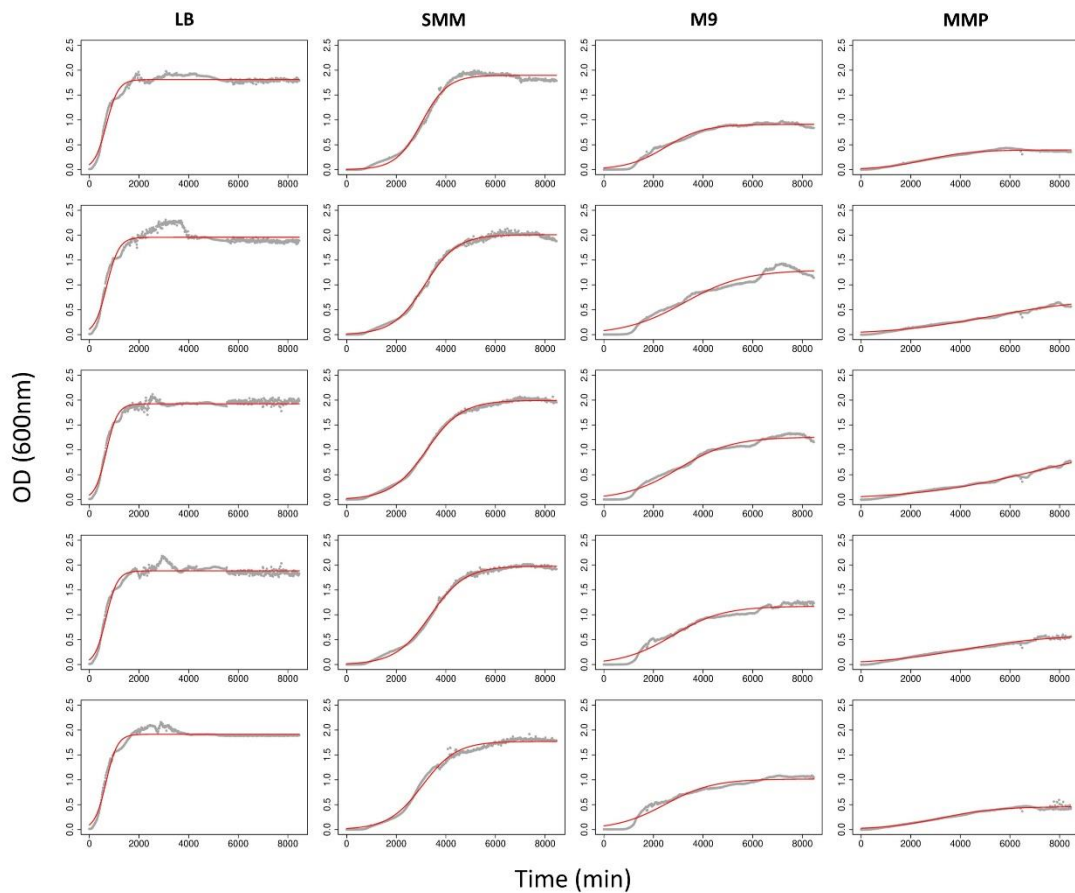
152

153 **Figure 2:** The theoretical growth curves of the 5 replicate cultures per medium at 25°C.

154

155 In order to determine which replicates represented significant deviations from the general trend
156 of each medium, we applied the DTW algorithm on a pairwise distance matrix between the
157 replicates. Using this approach, several replicates were indeed determined to be outliers: 1 LB
158 replicate, 1 SMM replicate and 2 M9 replicates at 25°C (Supplementary Figure S4), as well as
159 1 SMM and 1 M9 replicate at 30°C (Supplementary Figure S6). No outliers were detected
160 among the different replicates of MMP at both temperatures. The replicates determined to be
161 outliers were removed and the remaining replicates were used to calculate the average

162 theoretical growth curve for each medium at both temperatures (Supplementary Figures S5,
163 S7).



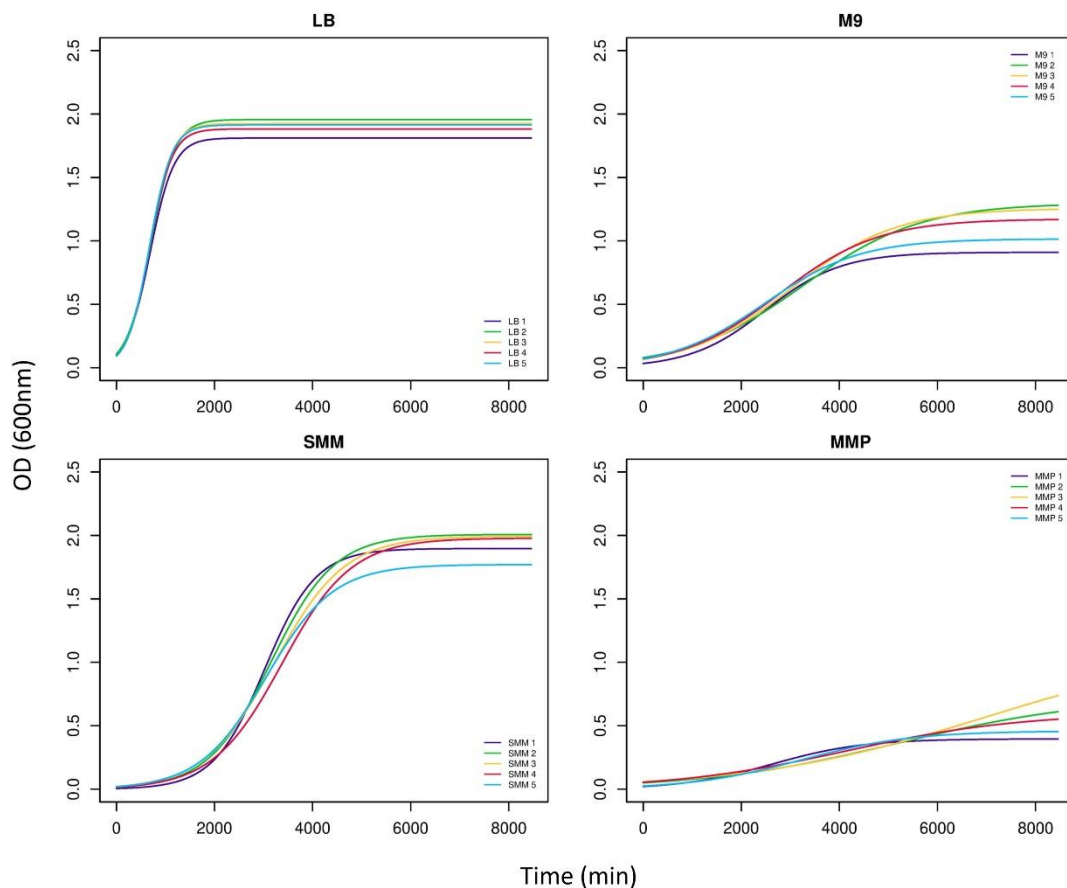
164
165 **Figure 3:** Growth curves obtained from the absorbance readings of the different media incubated at
166 30°C. The experimental curves (grey dots) correspond to the absorbance values recorded every 15
167 minutes for seven days. Each column corresponds to a medium, and each graph describes the growth
168 performance in a single well (N=5 per medium). The red line represents the theoretical curve fitted over
169 the experimental points.

170

171 Comparing the average growth curves of the three minimal media to that of the rich LB medium
172 revealed that the SMM medium performed best and even surpassed the LB medium in terms
173 of bacterial density (i.e. higher OD₆₀₀) in the stationary phase (Fig. 5A, C). In contrast, the
174 other two minimal media M9 and MMP could not sustain the same growth and vitality
175 performance (Fig. 5). MMP was the most different from bacterial growth on the LB medium.

176 This was also evident from the inferred generation time at each medium based on the average
177 absorbance values: generation time at 25°C was 99 min, 432 min, 560 min and 609 min on LB,
178 SMM, M9 and MMP, respectively (Supplementary figure S5; tables 2, 3).

179



180

181 **Figure 4:** The theoretical growth curves of the 5 replicate cultures per medium at 30°C.

182

183 When comparing the growth performances on the different media at 25° and 30°C, it emerged
184 that *Pseudomonas* generally grew more slowly at 30°C than at 25°C. Hence, generation time
185 at 30°C was 162 min, 446 min, 684 min and 1100 min on LB, SMM, M9 and MMP,
186 respectively (Supplementary figure S7; tables 2, 3). SMM represents the exception to this trend,
187 since bacteria growing on SMM had similar growth rates at both temperatures (average

188 generation time at 25°C: 432 min vs average generation time at 30°C: 446). Despite growing
 189 at a similar rate, bacterial density remained lower at 30°C than at 25°C (Fig. 5 A.C).

190 **Table 2: Logistic curve parameters estimated for each sample both at 30°C and 25°C**

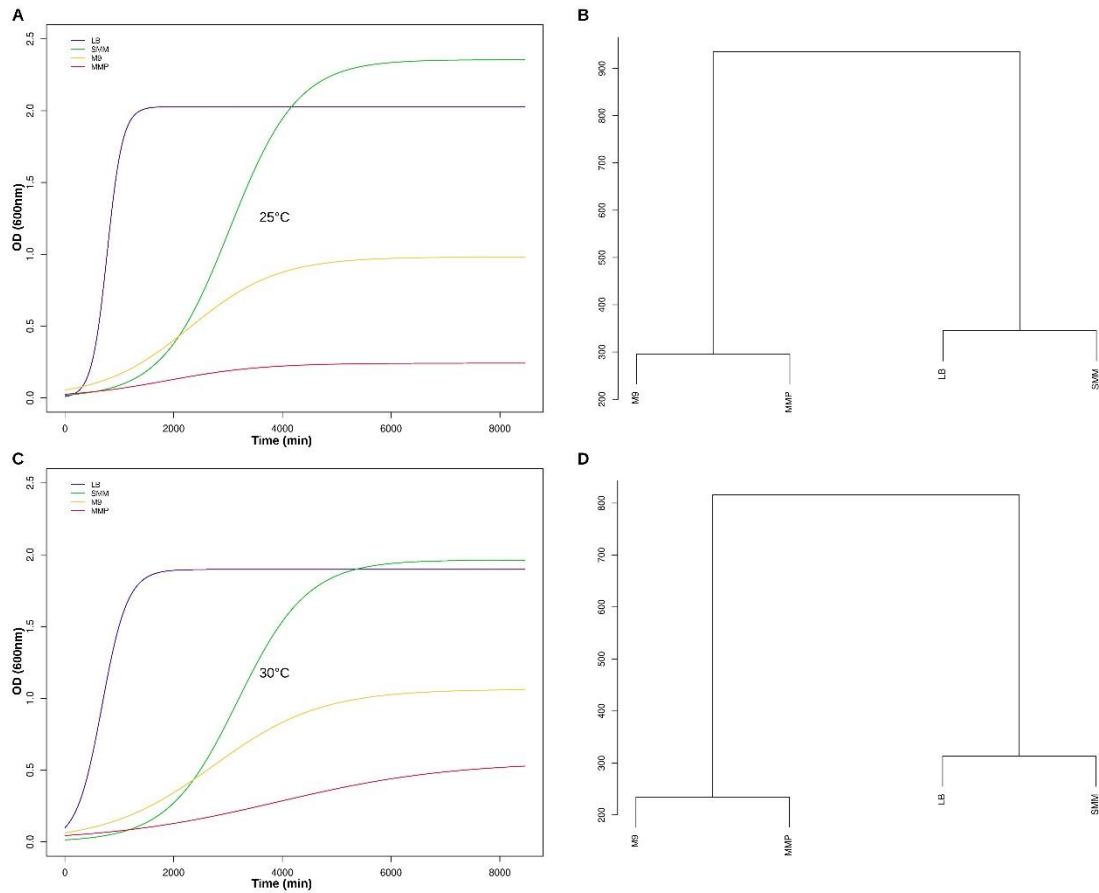
Sample	30°C				25°C			
	K	N0	r	Generation time	K	N0	r	Generation time
LB1	1.811	0.101	0.004	168	1.979	0.007	0.007	93
LB2	1.956	0.109	0.004	171	2.094	0.011	0.007	103
LB3	1.924	0.091	0.004	157	2.161	0.014	0.006	110
LB4	1.882	0.094	0.004	159	2.075	0.013	0.006	107
LB5	1.914	0.094	0.004	158	1.964	0.006	0.007	93
SMM1	1.896	0.006	0.002	363	2.232	0.015	0.002	412
SMM2	2.008	0.015	0.001	448	2.368	0.02	0.002	444
SMM3	1.994	0.022	0.001	497	2.408	0.02	0.002	436
SMM4	1.978	0.017	0.001	490	2.415	0.017	0.002	433
SMM5	1.770	0.020	0.001	474	2.277	0.013	0.002	445
M91	0.911	0.035	0.001	536	0.996	0.076	0.001	736
M92	1.298	0.080	0.001	831	0.98	0.048	0.001	530
M93	1.257	0.069	0.001	739	0.989	0.039	0.002	462
M94	1.172	0.070	0.001	700	1.061	0.033	0.002	379
M95	1.016	0.072	0.001	670	1.05	0.032	0.002	395
MMP1*	0.397	0.022	0.001	643	0.261	0.027	0.001	663
MMP2*	0.733	0.050	0.001	1391	0.243	0.026	0.001	599
MMP3*	1.144	0.059	0.001	1670	0.236	0.024	0.001	592
MMP4*	0.601	0.055	0.001	1247	0.218	0.023	0.001	561
MMP5*	0.459	0.025	0.001	784	0.25	0.028	0.001	626

191 * The medium contains CaCl₂ at the concentration reported in the supplementary file

192 **Table 3: Logistic curve parameters estimated for the averaged curve for each medium both at**
 193 **30°C and 25°C**

sample	30°C				25°C			
	K	N0	r	Generation time	K	N0	r	Generation time
LB	1.897	0.098	0.004	163	2.028	0.009	0.007	99
SMM	1.963	0.014	0.002	446	2.355	0.018	0.002	432
M9	1.063	0.062	0.001	684	0.981	0.054	0.001	560
MMP*	0.557	0.043	0.001	1100	0.241	0.025	0.001	610

194 * The medium contains CaCl₂ at the concentration reported in the supplementary file



195

196 **Figure 5:** Comparison of the growth performance of *P. azotoformans* on the different media at 25°C
197 (A, B) and 30°C (C, D). A and C: Averaged theoretical growth curves on the different media. B and D:
198 Dendrograms of the curves' similarity using the DTW metric.

199

200 Taken together, *P. azotoformans* grown on SMM performed best compared to the other
201 minimal media (shorter generation time and higher bacterial density). However, it was not
202 comparable to growth on a rich medium.

203 Based on the metabolism of *P. azotoformans* inferred from the available genomes on the NCBI
204 database (Table 4), we can hypothesize that the higher growth performance on the SMM
205 medium could be due to minimal composition. SMM contains the necessary glucose as a
206 carbon source, glutamine as a nitrogen source, K_2HPO_4 as a phosphate source and $MgSO_4$ as
207 a sulfur source. These four ingredients are sufficient in *P. azotoformans* for the biosynthesis of

208 all the needed building blocks for metabolism and growth. The addition of other nutrients, such
209 as for the other media tested, will probably trigger other, possibly secondary, metabolic
210 pathways. These active metabolic pathways could divert resources (e.g., ATP, metabolites)
211 from the central metabolism, thus slowing the growth and multiplication of the bacterial cells.
212 This may result in slower performances when *P. azotoformans* is incubated in more complex
213 media.

214

215 **Conclusion**

216 An optimal medium allows for good microbial growth, which can be summarised as short
217 generation time and high cell concentration in solution. In light of the results achieved here,
218 the SMM medium is the most suitable medium for studying the metabolism of *P. azotoformans*.
219 SMM outperformed the other two media (i.e., M9 and MMP), and the bacterial growth
220 performances were consistently closer to those on LB. Furthermore, while the optimal growth
221 temperature is 25°C, the SMM medium was the least susceptible to performance variations at
222 a suboptimal growth temperature.

223 The better growth performance on the SMM medium could be partly explained by the fact that
224 the medium contains the necessary nutrients sufficient for the biosynthesis of all the needed
225 building blocks for metabolism and growth. In addition, *P. azotoformans* is a phylogenetic
226 closer to *P. fluorescens* [20], which SMM was originally designed, than other *Pseudomonas*
227 species. In contrast, most of the other minimal media tested here had been designed for *P.*
228 *aeruginosa*, except for the more generic MMP. The phylogenetic proximity with *P. fluorescens*
229 may suggest similar metabolic needs between the two species. Thus, a medium developed for
230 one of the two species could offer better growth performances for the second species than
231 media developed for more distant relatives.

232 **Table 4: Level of completeness of the different metabolic pathways that are part of the central**
 233 **metabolism in four P. azotoformans strains**

Strain (accession number)	LMG21611 (LT629702)	P45A (CP041236)	S4 (CP014546)	F77 (CP019856)
glycolysis	complete	complete	complete	complete
pentose phosphate	complete	complete	complete	complete
TCA	complete	complete	complete	complete
oxidative phosphorylation	complete	complete	complete	complete
fatty acids synthesis	complete	complete	complete	complete
glycerophospholipid synthesis	complete	complete	complete	complete
purine synthesis	complete	complete	complete	complete
pyrimidine synthesis	complete	complete	complete	complete
serine synthesis	complete	complete	complete	complete
cysteine synthesis	complete	complete	complete	complete
phenylalanine synthesis	complete	complete	complete	complete
tryptophan synthesis	complete	complete	complete	complete
alanine synthesis	complete	complete	complete	complete
aspartate synthesis	complete	complete	complete	complete
asparagine synthesis	complete	complete	complete	complete
glutamine synthesis	complete	complete	complete	complete
glutamate synthesis	complete	complete	complete	complete
threonine synthesis	complete	complete	complete	complete
glycine synthesis	complete	complete	complete	complete
valine synthesis	complete	complete	complete	complete
leucine synthesis	complete	complete	complete	complete
isoleucine synthesis	complete	complete	complete	complete
lysine synthesis	complete	complete	incomplete	complete
arginine synthesis	complete	complete	complete	complete
tyrosine synthesis	complete	complete	complete	complete
histidine synthesis	complete	complete	complete	complete
methionine synthesis	complete	complete	complete	complete
Proline synthesis	complete	complete	complete	complete
Thiamine synthesis	complete	complete	complete	complete
riboflavin synthesis	complete	complete	complete	complete
vitamin B6 synthesis	complete	complete	complete	complete
NAD ⁺ /NADP ⁺ synthesis	complete	complete	incomplete	complete
Coenzyme A synthesis	complete	complete	incomplete	complete
biotin synthesis	incomplete	incomplete	incomplete	incomplete
folate synthesis	complete	complete	complete	complete

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300

301 **Supplementary materials**

302 **Supplementary file S1:** Composition of the different defined media used in this study.

303 **Supplementary figure S1:** The effect of adding CaCl₂ to the MMP medium on the production
304 of siderophores. A: *Pseudomonas* grown in MMP. The yellow colour indicates the production
305 of siderophores. B: *Pseudomonas* grown in MMP + CaCl₂. The transparent medium indicates
306 that no siderophores were produced.

307 **Supplementary figure S2:** The experimental setup with the media disposition on the plate for
308 the second part of the experiment (i.e., continuous long time monitoring using a plate reader).

309 **Supplementary figure S3:** Comparison of the growth performance of *P. azotoformans* on the
310 six different media at 30°C. The curves represent the averaged theoretical growth curves for
311 each medium.

312 **Supplementary figure S4:** Dendrograms showing the similarity between the theoretical
313 growth curves of the five replicates for each medium at 25°C. The dotted red line delineates
314 outliers based on the DTW metric (75% similarity threshold).

315 **Supplementary figure S5:** Averaged growth curve for each medium at 25°C after excluding
316 outliers. The experimental curves (grey dots) correspond to the absorbance values recorded
317 every 15 minutes for seven days. The red line represents the theoretical curve fitted over the
318 experimental points. The inserts show the generation time inferred from the averaged
319 absorbance values.

320 **Supplementary figure S6:** Dendrograms showing the similarity between the theoretical
321 growth curves of the five replicates for each medium at 30°C. The dotted red line delineates
322 outliers based on the DTW metric (75% similarity threshold).

323 **Supplementary figure S7:** Averaged growth curve for each medium at 30°C after excluding
324 outliers. The experimental curves (grey dots) correspond to the absorbance values recorded
325 every 15 minutes for seven days. The red line represents the theoretical curve fitted over the
326 experimental points. The inserts show the generation time inferred from the averaged
327 absorbance values.