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Genome-scale metabolic modelling of responses to polymyxins in Pseudomonas aeruginosa
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Full Title:	Genome-scale metabolic modelling of responses to polymyxins in Pseudomonas aeruginosa
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Abstract:	Background: Pseudomonas aeruginosa often causes multidrug-resistant infections in immunocompromised patients and polymyxins are often used as the last-line therapy. Alarmingly, resistance to polymyxins has been increasingly reported worldwide recently. To rescue this last-resort class of antibiotics, it is necessary to systematically understand how P. aeruginosa alters its metabolism in response to polymyxin treatment, thereby facilitating the development of effective antimicrobial therapies. To this end, genome-scale metabolic model (GSMM) was employed to analyse bacterial metabolic changes at the systems level. Findings: A high-quality GSMM iPAO1 was constructed for P. aeruginosa PAO1 for antimicrobial pharmacological research. Model iPAO1 encompasses an additional periplasmic compartment and contains 3,022 metabolites, 4,265 reactions and 1,458 genes in total. Growth prediction on 190 carbon and 95 nitrogen sources achieved an accuracy of 89.1%, outperforming all reported P. aeruginosa models. Notably, prediction of the essential genes for growth achieved a high accuracy of 87.9%. Metabolic simulation showed that lipid A modifications associated with polymyxin resistance exert a limited impact on bacterial growth and metabolism, but remarkably change the physiochemical properties of the outer membrane. Modelling with transcriptomics constraints revealed a broad range of metabolic responses to polymyxin treatment, including reduced biomass synthesis, upregulated amino acids catabolism, induced flux through the tricarboxylic acid cycle, and increased redox turnover. Conclusions: Overall, iPAO1 represents the most comprehensive GSMM constructed to date for a Gram-negative bacterium. It provides a powerful systems pharmacology platform for the elucidation of complex killing mechanisms of antibiotics.

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Availability of data and materials All datasets and code on which the conclusions of the paper rely must be either included in your submission or deposited in publicly available repositories (where available and ethically appropriate), referencing such data using a unique identifier in the references and in the "Availability of Data and Materials" section of your manuscript. Have you have met the above requirement as detailed in our Minimum Standards Reporting Checklist?	Yes

1 **Genome-scale metabolic modelling of responses to polymyxins in *Pseudomonas***

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24 Part of this work was presented at the 27th European Congress of Clinical Microbiology and

25 Infectious Diseases, 22-25 April 2017, Vienna, Austria.

26 **Abstract**

27 **Background**: *Pseudomonas aeruginosa* often causes multidrug-resistant infections in

28 immunocompromised patients and polymyxins are often used as the last-line therapy.

29 Alarmingly, resistance to polymyxins has been increasingly reported worldwide recently. To

30 rescue this last-resort class of antibiotics, it is necessary to systematically understand how *P.*

31 *aeruginosa* alters its metabolism in response to polymyxin treatment, thereby facilitating the

32 development of effective therapies. To this end, genome-scale metabolic model (GSMM) was

33 employed to analyse bacterial metabolic changes at the systems level.

34 **Findings**: A high-quality GSMM *i*PAO1 was constructed for *P. aeruginosa* PAO1 for

35 antimicrobial pharmacological research. Model *i*PAO1 encompasses an additional periplasmic

36 compartment and contains 3,022 metabolites, 4,265 reactions and 1,458 genes in total. Growth

37 prediction on 190 carbon and 95 nitrogen sources achieved an accuracy of 89.1%,

38 outperforming all reported *P. aeruginosa* models. Notably, prediction of the essential genes for

39 growth achieved a high accuracy of 87.9%. Metabolic simulation showed that lipid A

40 modifications associated with polymyxin resistance exert a limited impact on bacterial growth

41 and metabolism, but remarkably change the physiochemical properties of the outer membrane.

42 Modelling with transcriptomics constraints revealed a broad range of metabolic responses to

43 polymyxin treatment, including reduced biomass synthesis, upregulated amino acids

44 catabolism, induced flux through the tricarboxylic acid cycle, and increased redox turnover.

45 **Conclusions**: Overall, *i*PAO1 represents the most comprehensive GSMM constructed to date

46 for a Gram-negative bacterium. It provides a powerful systems pharmacology platform for the

47 elucidation of complex killing mechanisms of antibiotics.

48 **Keywords:** Genome-scale metabolic model; *Pseudomonas aeruginosa*; polymyxin; lipid A

49 modification; outer membrane

Background

Pseudomonas aeruginosa is a common multidrug-resistant (MDR) pathogen in immune-compromised patients, cystic fibrosis patients and burns victims [1-6]. It possesses a large genome (5.5-7.0 Mb), complex regulatory networks, remarkable metabolic versatility and an extraordinary ability to survive extremely harsh conditions such as prolonged antibiotic exposure [7, 8]. Polymyxins (i.e. polymyxin B and colistin) have been increasingly used as a last-line therapy to treat infections caused by MDR *P. aeruginosa* [9]. Alarmingly, the prevalence of polymyxin resistance in *P. aeruginosa* has increased worldwide over the past few years [3, 10, 11].

The exact mode of action of polymyxins is not clear except the initial electrostatic and hydrophobic interactions with lipid A, a component of the lipopolysaccharide (LPS) in the bacterial outer membrane (OM). Subsequently, cell envelope is disorganised, cellular contents leak, oxidative stress increases, and finally cell death occurs [2, 9, 12, 13]. After polymyxin treatment, *P. aeruginosa* modifies its lipid A structure to attenuate the aforementioned electrostatic interactions [14]. Our recent metabolomics data demonstrated that, apart from lipid A modifications, numerous biochemical pathways are perturbed by polymyxin treatment, indicating that the development of polymyxin resistance by *P. aeruginosa* involves a complicated interplay of multiple cellular processes [15]. There are significant gaps in the knowledge-base of the mechanisms of polymyxin activity and bacterial responses in *P. aeruginosa*, thereby necessitating comprehensive investigations using systems pharmacology approaches.

With the rapid development of genome-scale metabolic models (GSMMs) and the associated flux balance analysis (FBA) methods, systematic investigations into the metabolic changes in response to external nutrient alterations, genetic perturbations, and antibiotic treatments are now becoming feasible [16-24]. Four GSMMs have been constructed for *P. aeruginosa* and

75 they are iMO1056 [25], Opt208964 [26] , iMO1086 [27] and the latest iPae1146 [28]; among

76 which iMO1056 and Opt208964 share the same identifier systems and are fully accessible via

77 Model SEED [29]. The previous applications of these models have included simulating the

78 metabolic dynamics in cystic fibrosis patients [30], elucidating the mechanisms of biofilm

79 formation [31, 32], predicting potential drug targets [33-35] and identifying the key genes

80 controlling virulence factors [28]. As important as they have been, these models have several

81 overarching limitations. Those past models (i) do not include a major cellular component, the

82 periplasmic space; (ii) have poor representation of glycerophospholipid (GPL) biosynthesis;

83 and (iii) lack lipid A modification reactions. Considering the pathogenesis of *P. aeruginosa*,

84 these major limitations significantly compromise the modelling functions. In particularly, the

85 power of the four reported GSMMs to predict metabolic responses to antibiotic treatment is

86 very limited, as periplasmic GPL and LPS biogenesis play critical roles in responses to anti-

87 pseudomonal antibiotics such as polymyxins [15, 36-39].

88 Here we describe *i*PAO1, a newly developed GSMM for *P. aeruginosa* PAO1 based upon

89 Opt208964 [26] and iMO1056 [25] but with intensive manual curation using several major

90 databases and the literature. Most notably, *i*PAO1 is the first GSMM for *P. aeruginosa* where

91 the periplasmic space compartment is incorporated to comprehensively represent cross-

92 membrane transport, GPL metabolism and LPS biosynthesis. To the best of our knowledge

93 *i*PAO1 represents the most comprehensive metabolic reconstruction for *P. aeruginosa* PAO1

94 and the largest manually curated metabolic model for any Gram-negative bacterium thus far.

95 Modelling with *i*PAO1 revealed that the lipid A modifications might exert limited impact on

96 cell growth and metabolism but change the physiochemical properties of bacterial OM.

97 Constrained by gene expression levels, the model was employed to elucidate the metabolic

98 responses to polymyxin B treatment. Together, *i*PAO1 provides a powerful systems platform

99 for antimicrobial pharmacological research to combat the rapidly increasing resistance.

Data Description

The genome sequence and annotation of *P. aeruginosa* PAO1 were obtained from GenBank (Accession NC_002516.2). Models iMO1056 and Opt208964 were retrieved from Model SEED [29]. The gas chromatography–mass spectrometry (GC-MS) metabolomics data were collected from the literature [40]. Metabolites, reactions and pathways were obtained from biochemical databases KEGG (Kyoto Encyclopaedia of Genes and Genomes) [41], MetaCyc [42], TCBD (Transporter Classification Database) [43], TransporterDB [44] and Pseudomonas Genome DB [45]. Growth phenotypes on 190 carbon sources and 95 nitrogen sources were determined using BIOLOG Phenotypic Microarrays. Non-essential gene lists were collected from two previously reported transposon mutant libraries for PAO1 [46, 47]. Lipid A of wild-type *P. aeruginosa* PAK and its polymyxin-resistant mutant PAK*pmrB6* was extracted using mild acid hydrolysis method and the structural analysis of lipid A was conducted using mass spectrometry [39]. RNA was extracted and employed to construct cDNA libraries for RNA-Seq on Illumina MiSeq platform [48]. The raw reads were quality trimmed and aligned to PAO1 reference genome using SubRead [49]. Counts were normalised and the differential gene expression was determined using voom/limma packages with Degust [50]. Whole-cell lipids and intracellular metabolites were extracted using the single-phase Bligh-Dyer method as previously described and analysed by liquid chromatography-mass spectrometry (LC-MS) [14, 39]. Raw metabolomics data were processed with IDEOM software followed by bioinformatic analysis [51].

Analyses

Development of a superior GSMM for *P. aeruginosa* PAO1

124 Initially, a draft model (*i*PAO1_draft1) containing 1,991 reactions, 1,579 metabolites and 1,021

125 genes was created based upon iMO1056 [25] and Opt208964 [26] (**Additional files 2-4**). To

126 obtain a high-quality GSMM, extensive manual curation was conducted. Firstly, *i*PAO1_draft1

127 was complemented using databases and the literature. Specifically, the following additional

128 information was incorporated into the draft model, 285 metabolites and 36 reactions from

129 KEGG [41], 225 metabolites and 50 reactions from MetaCyc [42], and 7 metabolites and 20

130 reactions obtained by previous GC-MS-based quantification [40] (**Additional files 5 and 6**).

131 Secondly, a periplasmic compartment was built to incorporate 698 periplasmic metabolites,

132 509 transport reactions across the inner membrane (IM), 441 transport reactions across the

133 outer membrane (OM), and 403 periplasmic reactions. The resulting intermediate model was

134 designated as *i*PAO1_draft2.

135 Thirdly, the major pathway gaps were filled. GapFind [52] identified 109 dead-end metabolites

136 (**Additional file 7**). The growth phenotypes on 190 carbon and 95 nitrogen nutrients were

137 predicted using *i*PAO1_draft2, and compared with our experimental BIOLOG Phenotypic

138 Microarray (PM) results (**Additional file 8**). As a result, 162 false negative predictions (i.e. the

139 prediction indicated non-growth whereas the BIOLOG experiment demonstrated valid growth

140 on a specific nutrient) were determined, indicating the lack of associated transport or catabolic

141 reactions for these nutrients. To link the dead-end metabolites back to the metabolic network

142 and eliminate inconsistencies with the BIOLOG PM results, several modifications were made

143 including (i) adjustment of the reversibility settings of 180 reactions and changing the

144 directions of 87 reactions (**Additional file 9**); (ii) removal of 14 metabolites and 96 reactions

145 (**Additional files 10 and 11**), which were either duplicated (e.g. β-D-glucose was duplicated

146 with D-glucose) or representing general metabolite classes (e.g. protein, mRNA, DNA); and

147 (iii) addition of 98 boundary reactions, 677 transport reactions, and 252 metabolic reactions

148 (**Additional file 12**). Resolving the false negative predictions of the BIOLOG growth

149 phenotypes substantially improved the model. For example, predictions using *i*PAO1_draft2

150 showed that PAO1 was unable to grow with formic acid as a sole carbon source due to the lack

151 of the corresponding transport reaction. Interrogating the Pseudomonas Genome Database [45]

152 and Pfam [53] identified PA2777, a hypothetical protein in NCBI and UniProt which may

153 encode formic/nitrite transporter (Pfam01226, *P*=7e-34). Subsequent addition of the transport

154 reaction (rxn08526) enabled *in silico* growth of PAO1 on formic acid. Another example is that

155 initially *i*PAO1_draft2 failed to predict utilisation of 1,2-propanediol for growth owing to the

156 exiting gap in dehydrogenation of 1,2-propanediol to lactaldehyde. Using BLASTp with the

157 query sequence of lactaldehyde reductase (*fucO*, *b2799*) from *Escherichia coli* K12 MG1655

158 identified a candidate homologous gene PA1991 (Identity=35%, Eval=2e-75, BLASTp).

159 PA1991 encodes an iron-containing alcohol dehydrogenase and has over 300 orthologues in

160 Gram-negative bacteria which encode lactaldehyde oxidoreductases or 1,2-propanediol

161 dehydrogenases according to OrthoDB [54]. Inactivation of PA1991 resulted in 8-fold

162 prolonged lag phase when *P. aeruginosa* grew on 1,2-propanediol [55]. Therefore, reaction

163 rxn01615 oxidising 1,2-propanediol to lactaldehyde was added into *i*PAO1_draft2. A very

164 large number of such labour intensive manual curations were conducted to improve the model.

165 This enabled *in silico* growth on a number of nutrients from BIOLOG PM, including 4-

166 hydroxyphenylacetate, tyramine, quinic acid, itaconic acid, citramalic acid, L-pyroglutamic

167 acid, carnidine, glycinebetaine, L-methylsuccinate, and D-amino acids (**Additional file 8**).

168 Fourthly, the biogenesis of bacterial envelope was delineated. Cross-linking between amino

169 acids residues among peptidoglycan chains results in a rigid network structure in *P. aeruginosa*

170 [56]. In total, 17 reactions representing peptidoglycan cross-linking and hydrolysis were

171 incorporated by searching for homologues of glycosyltransferases, transpeptidases,

172 carboxypeptidases and endopeptidases in PAO1 [57]. Overall, a detailed peptidoglycan

173 biosynthesis pathway was constructed with 60 reactions. GPL compositions in the bacterial

174 membranes can change in response to antibiotic treatment [36, 58]. Previous studies [59] and

175 our own lipidomics results [14] showed a great diversity in GPL species in *P. aeruginosa*.

176 Overall, 386 unique metabolites (i.e. 66.2% of the 583 metabolites in the GPL metabolism

177 pathway) and 367 reactions (66.7% of the 550 reactions in the GPL metabolism pathway) were

178 incorporated into *i*PAO1_draft2 (**Additional files 1, 13 and 14, Fig 1**). LPS consists of lipid

179 A, core oligosaccharide, and *O*-antigen polysaccharide [37], and plays key roles in the host-

180 pathogen interaction and the resistance to antibiotics such as polymyxins [13, 60]. A detailed

181 synthesis and interconversion network was generated with 432 types of LPS and 1,169

182 reactions (**Fig 2, Additional file 1**). Notably, our GSMM is the most comprehensive to date in

183 lipid A biosynthesis and modifications.

184 The resulting final *i*PAO1 model consisted of 3,022 metabolites, 4,365 reactions and 1,458

185 genes (25.8% of the PAO1 genome, **Additional files 15-17**), representing, respectively, (i)

186 252%, 340% and 40% increase of the components in iMO1056; and (ii) 125%, 171% and 43%

187 increase of the components in Opt208964 (**Table 1**). The significant expansion in *i*PAO1

188 includes cross-membrane transport, GPL/LPS biosynthesis, peptidoglycan biosynthesis, and

189 fatty acid degradation (**Additional files 15-17**). The reactions from *i*PAO1 were categorised

190 into 109 pathways mainly based on classifications in MetaCyc and KEGG. In *i*PAO1,

191 27.9%/43.7%/51.6% metabolites, 20.3%/33.5%/59.5% reactions and 65.3%/17.6%/28.5%

192 genes are originated from iMO1056, Opt208964, and our manual curation, respectively (**Fig

193 3A**).

194 Components in *i*PAO1 were aligned with databases including KEGG [41], MetaCyc [42],

195 PubChem [61], ChemSpider [62], ChEBI [63], Model SEED [29], and BiGG [64] (**Additional

196 files 15 and 16**). Consequently, 1,404 (46.5%), 1,590 (52.6%) and 2,142 (70.9%) metabolites

197 have corresponding identifiers in MetaCyc, KEGG and Model SEED, respectively; 1,556

198 (35.6%), 1,596 (36.6%) and 1,964 (45.0%) reactions were computationally mapped to the

199   reactions from MetaCyc, KEGG and Model SEED, accordingly (**Fig 3B**). A significant portion

200   of mismatches were caused by the incorporation of specific types of metabolites in the GPL

201   metabolism and LPS biosynthesis pathway, which in databases are usually lumped as general

202   compound classes. The properties of metabolites, including mass, charge and formula were

203   included in *i*PAO1. The standard Gibbs free energy change of formation ($\Delta_f G^\circ$), and reaction

204   ($\Delta_r G^\circ$) were obtained from MetaCyc and Model SEED for 1,877 metabolites (62.1%) and 1,355

205   reactions (31.0%) (**Additional files 15 and 16**).

206   A breakdown of genes involved in *i*PAO1 (**Additional file 17**) using the clusters of orthologous

207   groups (COGs) showed remarkable improvement compared to previous reconstructions (**Fig

208   3C**). The largest increase in the coverage compared to iMO1056 is lipid transport and

209   metabolism (24.1%), followed by inorganic ion transport and metabolism (19.3%); whereas

210   compared to Opt208964, the largest increase in the coverage is nucleotide transport and

211   metabolism (57.9%), followed by amino acid transport and metabolism (52.0%). Overall, the

212   transport and metabolism of nucleotides and amino acids showed the highest percent coverage

213   of COG functional categories in *i*PAO1 (72.9% and 65.6%, respectively). Notably, the

214   reactions in categories not apparently related to metabolism were dramatically reduced in

215   *i*PAO1 compared to Opt208964, including translation, ribosomal structure and biogenesis,

216   posttranslational modification, protein turnover, chaperones and signal transduction

217   mechanisms, or undetermined categories including function unknown class. Therefore, *i*PAO1

218   is a well-defined, metabolism-dedicated model.

219   In the new *i*PAO1 model, the pathways GPL metabolism, LPS biosynthesis and transport

220   across OM were ranked the three largest pathways and also contained the highest proportion

221   of curated reactions (**Fig 3D**). Additionally, these three pathways have high reaction-to-gene

222   ratios (13.1-24.2, **Fig 3E**), indicating that enzymes in these pathways are capable of acting on

223   a broad range of substrates. As kinetic parameters are usually not involved in a GSMM,

224 constraint-based analyses (e.g. FBA) of a GSMM do not directly account for enzyme levels,

225 intracellular metabolic concentrations or substrate-level regulation. Accordingly, the affinity

226 difference of various substrates was not considered in our *i*PAO1 modelling effort.

227 We employed the biomass formation equation from iMO1056 to construct *i*PAO1 with

228 modifications on LPS and ion species (**Additional file 18**). In addition, to take into account the

229 extra energy consumption caused by charging tRNAs, the original amino acids in the biomass

230 formation reaction were replaced by aminoacyl-tRNA, followed by addition of specific

231 charging reactions to the model. Taken together, *i*PAO1 represents the most comprehensive

232 metabolic reconstruction thus far for *P. aeruginosa* PAO1.

Growth capability on various nutrients

234 Investigation of nutrient utilisation using BIOLOG PM showed PAO1 could utilise a broad

235 range of nutrient sources, indicated by the observed growth on 68 of 190 (35.8%) carbon and

236 76 of 95 (80.0%) nitrogen substrates (**Fig 4**). Growth simulation with *i*PAO1 achieved an

237 overall accuracy of 89.1% (254 of 285), which substantially outperformed previous models

238 (81.5% for Opt208964 [26], 77.9% for iMO1056 and iMO1086 [27] and 80% for iPae1146

239 [28]. Twenty-one false-positive and 10 false-negative (**Fig 4**, **Additional file 8**) disagreements

240 were observed, possibly due to the complexity of regulatory mechanisms and missing

241 annotation of nutrient transport and/or catabolism pathways in PAO1.

Prediction and validation of gene essentiality

243 *In silico* single-gene deletion with *i*PAO1 showed 143 essential genes ($\mu_{mut}<0.01\ \mu_{wt}$), 40 semi-

244 essential genes ($0.01\ \mu_{wt}<\mu_{mut}<0.99\ \mu_{wt}$), and 1,275 non-essential genes ($0.99\ \mu_{wt}<\mu_{mut}<\mu_{wt}$)

245 when growing in Luria-Bertani (LB) media (**Additional file 19**). Among the essential

246 metabolic genes, the largest COG proportion (46 of 143, 32.1%) is cell envelope biogenesis,

247 indicating that there are relatively less alternative reactions in this pathway. For non-essential

248 genes, amino acid transport and metabolism (352 of 1,315, 26.7%) represents the largest group,

249 suggesting the existence of large metabolic redundancy.

250 The predicted gene essentiality was further verified by two independent genome-scale

251 transposon mutant libraries [46, 47, 65]. The overall prediction accuracy achieved 87.9%,

252 which is higher than iMO1056 (85.0%) [25] and iMO1086 (84.2%) [27], but slightly lower

253 than Opt208964 (92.9%) [26] and iPae1146 (91.46%) [28]; this is possibly due to the

254 incorporation of new genes (30.5% increase compared to Opt208964; 27.2% increase

255 compared to iPae1146) whose metabolic functions were previously misannotated.

Impact of lipid A modifications on bacterial growth and metabolism

257 *P. aeruginosa* modifies lipid A components in the OM in response to polymyxin treatment [66].

258 The LPS stoichiometric coefficients in the biomass formula of *i*PAO1 were configured based

259 on our lipidomics data (**Table 2**, [14]), to predict the metabolic impact of lipid A modifications

260 (see Methods Section for details). Overall, 273 fluxes were significantly affected (*Z*-Score,

261 false discovery rate (FDR) <0.01; >0.1 mmol·gDW·h^{-1} under at least one condition, **Additional**

262 **file 20**). The specific growth rate remained unchanged. A 0.026 mmol·gDW·h^{-1} flux from

263 glucose via glucose 6-phosphate, uridine diphosphate glucose, and consequently 4-amino-4-

264 deoxy-L-arabinose (L-Ara4N) biosynthesis was identified due to lipid A modifications. The

265 overall fluxes through lipid A deacylation reactions were increased (from 0.007 mmol·gDW·h^{-1}

266 ^{1} to 0.011 mmol·gDW·h^{-1}); the generated (*R*)-3-hydroxydecanoate was fuelled into β-oxidation

267 to produce octanoyl-CoA, which was subsequently salvaged for fatty acid biosynthesis.

268 To further investigate the impact of lipid A modifications on bacterial growth, 1,000 sets of the

269 compositions of 288 heterogeneous LPS molecules were randomly generated with the total

270 proportion of LPS unchanged in the biomass formation formula (**Additional file 21**). The

271 metabolic fluxes were calculated for each of the 1,000 sets of LPS compositions using FBA.

272 Across the 1,000 sets of metabolic fluxes (**Additional file 23**), the specific growth rate varied

273 between 0.8812 and 0.8897 mmol·gDW·h^{-1}. Correlative analysis of the apparent overall

274 physiochemical properties of lipid A (**Additional file 22**) with the predicted growth phenotypes

275 showed three interesting findings. Firstly, addition of L-Ara4N reduced the negative charge of

276 lipid A (ρ=1.00), decreased the hydrophobicity of the OM (represented by logP, ρ=-0.59), but

277 required assimilation of more ammonia (represented by ammonia turnover, ρ=0.57). Secondly,

278 hydroxylation on acyl chains of lipid A exerted minor effects over either bacterial growth or

279 physiochemical properties. Thirdly, addition of acyl chains resulted in large lipid A molecules

280 (represented by the atomic counts, ρ=0.88), enhanced molecular polarity of lipid A (ρ=0.87),

281 increased OM hydrophobicity (ρ=0.75), and notably, retarded growth (ρ=-0.95), reduced redox

282 and energy turnover (ρ=-0.98 for both), and increased requirement of ammonia (ρ=0.59) (**Fig

283 5**). It is evident that none of the three aforementioned modifications produced a dramatic impact

284 on bacterial growth or metabolism (**Additional file 23**).

Elucidating the mechanisms of metabolic responses to polymyxin treatment

286 RNA-Seq data were utilised as model constraints (**Additional file 24**) to calculate the

287 metabolic fluxes in the absence and presence of polymyxin B (*cf.* Methods). Comparison of

288 the flux distributions revealed that 1,392 reactions were differentially regulated (FDR<0.01,

289 **Additional file 25**). A range of metabolic pathways were significantly disturbed, including

290 central metabolism, amino acid metabolism, purine biosynthesis, fatty acid biosynthesis and

291 metabolism, LPS and GPL biosynthesis and transport reactions. Polymyxin B treatment

292 reduced the growth rate (18.2%), increased oxygen uptake (6.9%) and CO_2 emission (6.0%);

293 however, the respiration quotient remained roughly unchanged (**Table 3**).

294 As the major carbon sources, the amino acids and oligopeptides from cation-adjusted Mueller-

295 Hinton broth (CAMHB) were utilised to generate intermediate metabolites, redox and energy

296 equivalents for biomass formation. In response to polymyxin treatment, the gluconeogenesis

297 pathway was significantly induced from pyruvate to 3-phosphoglycerate, but suppressed from

298 3-phosphoglycerate towards glucose 6-phosphate. The extra flux from 3-phosphoglycerate was

299 shunt to serine and glycine biosynthesis (**Fig 6**) via 3-phospho-D-glycerate:NAD$^+$

300 oxidoreductase (rxn01101), 3-phosphoserine:2-oxoglutarate aminotransferase (rxn02914), *O*-

301 phospho-L-serine phosphohydrolase (rxn00420), and 5,10-methylenetetrahydrofolate:glycine

302 hydroxymethyltransferase (rxn00692), through which more NADH equivalent was generated

303 compared to the control. The resulting one-carbon unit in 5,10-methylenetetrahydrofolate was

304 oxidised to formic acid via 10-formyltetrahydrofolate amidohydrolase (rxn00691); the

305 generated glycine was fuelled into TCA cycle via glycine:oxygen oxidoreductase (rxn00269)

306 and acetyl-CoA:glyoxylate C-acetyltransferase (rxn00330). In addition, the metabolic flux via

307 TCA cycle was upregulated from citrate to fumarate, with increased NADH production. Within

308 oxidative phosphorylation, the mean fluxes through NADH dehydrogenase (Complex I,

309 rxn10122), cytochrome bc1 complex (Complex III, rxn13820), and cytochrome c oxidase

310 (Complex IV, rxn13688) decreased by 6.6%, 7.2% and 7.8%, respectively. The flux via F_0F_1-

311 ATPase (Complex V, rxn10042) was downregulated by 11.1%. The overall fluxes via

312 biosynthesis of macromolecules including LPS, GPL and peptidoglycan decreased due to the

313 significantly reduced biomass formation. The biosynthesis of spermidine increased by 38.3%

314 in response to polymyxin treatment which was also indicated by upregulated expression of

315 *speD* (PA4773; encoding the *S*-adenosyl-L-methionine decarboxylase, log$_2$FC=3.62,

316 FDR<0.01) and *speE* (PA4774; encoding spermidine synthase, log$_2$FC = 3.54, FDR<0.01).

317 Calculating the flux-sum of critical cofactors revealed 13.1% increase of redox turnover and

318 8.2% decline of energy turnover after 1 mg/L polymyxin B treatment for 1 h. Breaking down

319 the cofactors showed the turnover of major redox equivalents NADH, NADPH, ubiquinol-8

320 and FADH2 substantially increased by 12.6%, 13.9%, 3.9% and 35.9%, respectively; whereas

321 the turnover of ATP, the major contributor to energy significantly decreased by 8.52% after 1

322 mg/L polymyxin treatment for 1 h (**Fig 6**, **Additional file 26**). Overall, metabolic flux analysis

323 using *i*PAO1 integrated with our transcriptomics data revealed a significant global impact on

324 bacterial metabolism due to polymyxin B treatment.

325

Discussion

327 The emergence of Gram-negative 'superbugs' that are resistant to the last-resort polymyxins

328 highlights the urgent need for novel approaches such as GSMMs to understand the mechanisms

329 of antibacterial activity and resistance. The main utility of GSMMs is their ability to bridge

330 critical gaps between genomics and metabolic phenotypes through the prediction of metabolic

331 responses to antimicrobial treatments at the network level. Here, we report the development,

332 optimisation, validation and application of a high-quality GSMM designated *i*PAO1 for a type

333 strain *P. aeruginosa* PAO1; and importantly, *i*PAO1 was employed to understand the

334 complicated effect of polymyxin treatment on bacterial metabolism. Simulation with *i*PAO1

335 showed that lipid A modifications in response to polymyxin treatment only exert minor effects

336 on bacterial growth and metabolism. Albeit, further calculations that integrate transcriptomics

337 data as model constraints revealed that polymyxin treatment may reduce growth and affect a

338 broad range of pathways.

339 *i*PAO1 represents the most comprehensive metabolic model for *P. aeruginosa* to date and

340 incorporates 1,458 genes, accounting for ~25.8% of the PAO1 genome. Among the four

341 GSMMs developed for *P. aeruginosa* PAO1, iMO1086 and iPae1146 were constructed on the

342 basis of iMO1056 with moderate increase of metabolites, reactions and genes [25, 27, 28];

343 Opt208964 is also in a medium size, which limits modelling capacity [26]. In contrast, *i*PAO1

344 is significantly expanded in model scale, by doubling or even tripling the numbers of

345 metabolites and reactions (**Fig 3A**). *i*PAO1 achieved an unprecedented prediction accuracy of

346 89.1% for growth on various nutrients, outperforming all of the previously reported GSMMs

347 for *P. aeruginosa* [25-28]. The *i*PAO1 model was also employed to predict gene essentiality

348 with a high accuracy of 87.9%. Given the extensive curation and significant expansion, *i*PAO1

349 will serve as the primary reference for future development of metabolic models, particularly

350 for other *P. aeruginosa* strains.

351 Unlike *i*PAO1, none of the previous *P. aeruginosa* GSMMs incorporated the periplasm. As

352 polymyxins initially target LPS in the OM and can cause substantial changes in the cell

353 envelope, the periplasmic space is a major component in *i*PAO1. The periplasmic space of *E.*

354 *coli* is estimated to constitute up to 16% of total cell volume [67]. It contains a thin cell wall

355 composed of peptidoglycan and a variety of ions and proteins, which are involved in transport,

356 folding, cell envelope biogenesis, electron transport and xenobiotic metabolism [68]. *i*PAO1 is

357 the first *P. aeruginosa* GSMM to incorporate the periplasmic compartment, enabling accurate

358 representation of metabolic machinery, especially for those reactions that occur exclusively in

359 this important cellular space and transport of substrates across the IM and OM. Furthermore,

360 *i*PAO1 provides detailed representations of GPL and LPS biosynthesis which allows the

361 precise mapping of GPL and LPS responses from experimental metabolomics and lipidomics

362 data (**Figs 1 and 2**).

363 In response to polymyxin treatment, Gram-negative bacteria modify their lipid A with cationic

364 moieties (i.e. phosphoethanolamine and L-Ara4N) that act to repel the like-charge of the

365 polymyxin molecule [37]. Based on our simulations (**Additional file 20**), we purport that such

366 lipid A modifications exerted a limited impact on cellular metabolism and growth. Most of the

367 flux changes were insignificant; the remaining significant flux changes mainly resulted from

368 futile cycles containing sets of reactions using redox equivalents, whereas the net carbon flow

369 remained unchanged. Simulation using randomised lipid A compositions further consolidated

370 our hypothesis that lipid A modifications cause only moderate variations of bacterial growth

371 and metabolism (**Fig 5, Additional file 23**). Notwithstanding, our simulation results revealed

372 that lipid A modifications result in substantial physiochemical changes in the OM of *P.*

373 *aeruginosa*, including (i) neutralising the surface negative charge by addition of positively

374 changed L-Ara4N; and (ii) altering the polarity and hydrophobicity by acylation and

375 deacylation. The general mode of action of polymyxin involves the initial electrostatic

376 interaction between the cationic side chains of the polymyxin molecule with the anionic lipid

377 A head groups [60]. These events are subsequently followed by hydrophobic interactions

378 between the *N*-terminal fatty acyl chain and position 6/7 hydrophobic side chains of the

379 polymyxin with the hydrophobic fatty acyls of lipid A [60]. Therefore, in concept both the

380 addition of L-Ara4N and deacylation of lipid A should contribute to polymyxin resistance.

381 Indeed, our recent transcriptomic and neutron reflectometry studies discovered that deletion of

382 the corresponding gene *pagL* (PA4661) resulted in an increased susceptibility to polymyxins,

383 in a polymyxin-resistant mutant PAK*pmrB6* derived from *P. aeruginosa* PAK [14, 69],

384 demonstrating that the lipid A deacylation also plays a key role in the response of *P. aeruginosa*

385 to polymyxin treatment.

386 Our recent transcriptomics and metabolomics studies discovered that polymyxin treatment

387 leads to remarkable growth reduction and metabolic perturbations in Gram-negative bacteria

388 [38, 39, 70-72]. The integration of transcriptomics results into GSMMs allow for more accurate

389 predictions of metabolic responses to either environmental (i.e. antibiotic treatment) or genetic

390 perturbations (i.e. mutations) [73]. In the present study, we employed the E-Flux method to

391 integrate transcriptomics data as flux constraints [74]. E-Flux can map continuous gene

392 expression levels to the metabolic network and uses the transcript abundance to determine the

393 degree to which a reaction is active or inactive [74]. Therefore, E-Flux provides a more

394 physiologically relevant description of the continuous nature of the reaction activity and avoids

395 to use any artificial thresholds to binarise gene expression data [75]. Comparison of the

396 calculated flux distributions revealed that a broad range of metabolic perturbations occur in

397 response to polymyxin treatment (**Fig 6**), ranging from central carbon metabolism to oxidative

398 phosphorylation and amino acid metabolism. Reduced growth, increased redox turnover and

399 decreased energy turnover due to polymyxin treatment were evident (**Fig 6**), indicating that

400 bacterial cells regulated their metabolism to produce more redox power to cope with the

401 oxidative stress. This is consistent with previous findings that showed bactericidal antibiotics

402 induced lethal oxidative damages via generating highly deleterious free radicals with

403 subsequent culmination of cellular death [76]. In addition, our simulations revealed that

404 polymyxin treatment induced an uptake of L-alanine, which was catabolised to generate more

405 NADH (**Fig 7**). This indicates that rich media (e.g. CAMHB) may provide abundant amino

406 acids and peptides that can be utilised by bacterial cells to generate sufficient redox equivalents

407 to cope with the oxidative damage caused by polymyxin treatment. Furthermore, our

408 simulation results also showed an upregulated metabolic flux towards L-spermidine

409 biosynthesis upon polymyxin B treatment (rxn00127 and rxn01406, **Additional file 25**).

410 Previous studies showed that polyamines (e.g. spermidine) could protect *P. aeruginosa* from

411 antimicrobial peptide killing [77]. It is assumed that the cationic spermidine could interact with

412 the anionic LPS, mask the negative cell surface, and reduce the electrostatic interactions

413 between polymyxin B and bacterial OM. Therefore, the enhanced biosynthesis of spermidine

414 might increase its abundance at the cell surface and contribute to polymyxin resistance.

415 The constructed *i*PAO1 provides a detailed presentation of LPS biogenesis (**Fig 2**), in particular

416 lipid A modifications. Further integration with specific regulatory modules will enable

417 dynamic simulation of metabolic responses to polymyxin treatment. Previous studies revealed

418 that various two-component regulatory systems (2CSs), including PhoPQ, PmrAB, ParRS,

419 CprRS and ColRS, play key roles in regulating polymyxin resistance [78-82]. Among them,

420 the PmrAB and PhoPQ systems are able to sense the depletion of external cations (e.g. Mg^{2+}

421 and Ca^{2+}) and upregulate the expression of the *arnBCADTEF-ugd* operon which is responsible

422 for the modification of lipid A with L-Ara4N [83]. Moreover, the fatty acylation of lipid A by

423 PagP is under the control of PhoPQ [84, 85]. ParRS and CprRS are independent 2CSs that

424 mediate the upregulation of *pmrAB*, *arnBCADTEF-ugd* operon, *pagL* and adaptive resistance

425 in response to polymyxin treatment [78, 86]. In overview, lipid A modifications due to

426 polymyxin treatment are strictly controlled by very complex regulatory networks involving

427 signal sensors, transcriptional regulators, and metabolic enzymes. Therefore, future studies are

428 warranted to integrate these regulatory modules into the GSMM to enable simulating bacterial

429 response dynamics to polymyxin treatment and analysing adaptive resistance mechanisms in

430 *P. aeruginosa*.

431 Overall, we have constructed, optimised and validated a high-quality genome-scale metabolic

432 model *i*PAO1 for *P. aeruginosa* PAO1. This comprehensive model incorporates metabolic

433 pathways, particularly the biogenesis of membrane components, and enables delineating the

434 complex metabolic responses to antibiotics. *i*PAO1 provides a valuable systems tool for

435 quantitative simulation of bacterial metabolic responses to antibiotics, elucidation of the

436 molecular mechanisms of antimicrobial killing and resistance, and facilitation of designing

437 rational antimicrobial combination therapy. To the best of our knowledge, this study is the first

438 to integrate antimicrobial pharmacology, computational biology, metabolic network and

439 systems pharmacology to analyse large-scale datasets, in order to better understand the

440 dynamic and complex nature of polymyxin killing and resistance. Combined with antibiotic

441 pharmacokinetics and pharmacodynamics, *i*PAO1 offers an *in silico* platform for precision

442 antimicrobial pharmacology therapy.

443

444 **Potential implications**

445 The generated collection of transcriptomics metabolomics, lipidomics and lipid A profiling

446 data provides comprehensive datasets of *P. aeruginosa* for future integrative analysis of

447 polymyxin systems pharmacology. As the largest curated GSMM thus far for any Gram-

448 negative bacteria, *i*PAO1 represents all aspects of the cellular metabolism and may serve as the

449 platform for integrative analysis of multi-omics data. Simulation with transcriptomics

450 constraints in this study revealed metabolic flux changes in amino acid catabolism,

451 tricarboxylic acid cycle, and redox turnover caused by polymyxin treatment. Correlative

452 analysis of metabolomics and transcriptomics with the constraint-based modelling is necessary

453 for delineating the regulatory effects on metabolism. The methodology of using GSMMs to

454 analyse multi-level omics data is applicable to other areas beyond antimicrobial pharmacology.

455 Further integration with antimicrobial pharmacokinetics and pharmacodynamics will not only

456 provide better pharmacological understanding, but also empower the model to quantitatively

457 predict the bacterial responses to antimicrobial therapy in the context of complex interplay of

458 signalling, transcriptional regulation and metabolism. In summary, our GSMM approach

459 provides a powerful systems tool to elucidate the complex mode of action of antibiotics and

460 will paradigm shift antimicrobial pharmacology.

461

Methods

Strain, media and BIOLOG experiments

464 *P. aeruginosa* PAO1 was cultured in Luria-Bertani (LB) media and subcultured on nutrient

465 agar. Cells were swapped into sterile capped tube containing 16 mL IF-0 solution (Cell

466 Biosciences, West Heidelberg, Australia) till the turbidity achieved 42% transmittance in a

467 Turbidimeter (Pacificlab, Blackburn, Australia). The cell suspension was then diluted 5 times

468 with IF-0 solution and dye (Cell Biosciences) to final 85% transmittance. BIOLOG PM 1-3

469 (Cell Biosciences, Heidelberg, Australia) were used to investigate the carbon and nitrogen

470 utilisation with two independent biological replicates. Sodium succinate was used as the carbon

471 source for examining nitrogen utilization. Growth was detected after 24-h incubation at 37°C,

472 using an Infinite M200 microplate reader (Tecan, Mannedorf, Switzerland) at 595 nm.

473 Readings that were ≥1.5-fold of the negative control (i.e. growth media without bacteria)

474 indicated the utilisation of nutrients.

Development of a GSMM for *P. aeruginosa* PAO1

476 To expedite the model development, two curated models for PAO1 with the same identifier

477 systems from Model SEED [29], iMO1056 [25] and Opt20896434 [26] were merged.

478 Databases including KEGG [41], MetaCyc [42], Pseudomonas Genome DB [45] and the

479 literature were employed to complete the model with missing components. The identifiers of

480 metabolites and reactions were kept consistent with Model SEED [26], and cross-referred to

481 MetaCyc, KEGG, PubChem [61], ChEBI [63], ChemSpider [62] and BiGG [64]. The PAO1

482 genome annotation from Pseudomonas Genome DB [45] was employed to construct 'gene to

483 protein to reaction' (GPR) associations [87]. A periplasm compartment was incorporated into

484 the model. Reactions and metabolites were then assigned to cytoplasm, periplasm and external

485 environment according to the localisation prediction of metabolic enzymes by PSORTb 3.0

486 [88]. Transport reactions were generated to enable material exchange across membranes

487 according to TCBD [43] and TransporterDB [44]. The model was constructed using the

488 Systems Biology Markup Language (SBML) [89, 90]. VANTED [91] was employed for

489 visualisation and analysis of the metabolic network. For each metabolite in the model, specific

490 features including compartment localisation, mass, charge, formula, formation free energy,

491 database identifiers and source were added (**Additional file 14**). Each reaction entered into the

492 model was checked with elementary and charge balance. Reversibility was determined first

493 from the primary literature for each particular enzyme or reaction, if available. Further curation

494 on reaction reversibility and directions was conducted based on change of free energy and

495 knowledge about the physiological direction of a reaction in a pathway.

496 The Gapfind function from the COBRA toolbox [92] was employed to identify the isolated and

497 dead-end metabolites in the model. Candidate reactions from KEGG, MetaCyc and BiGG were

498 manually inspected for relevance and homology evidence using BLASTp; reactions catalysed

499 by homologous enzymes (E-value$<1\times10^{-5}$, identity$\geq$35%, coverage$\geq$50%) were added to the

500 model to eliminate the gaps. Mispredictions of BIOLOG growth phenotypes were employed to

501 refine the draft model (*i*PAO1_draft2). Further curation was performed to represent the

502 complex biosynthesis pathways of macromolecules (e.g. peptidoglycan, GPL and LPS).

503 The biomass formation equation consisting of necessary building blocks for bacterial growth

504 was created using the one from iMO1086 [27], with slight modifications on compositions of

505 ions, peptidoglycans, GPL and LPS (**Additional file 17**). The growth and non-growth

506 associated maintenance was from iMO1086 [27].

Growth prediction in BIOLOG media

508 *i*PAO1 was employed to predict the growth phenotypes on chemically-defined media with 190

509 carbon and 95 nitrogen sources (**Additional file 18**) using the FBA method [24]. The objective

510 function of biomass formation was maximised with the specific nutrient uptake rate set at 10

511 mmol·gDW·h^{-1} under aerobic condition.

512
$$\max \quad v_{\text{biomass}}$$

513
$$s.t. \quad \mathbf{S}\mathbf{v} = 0$$

514
$$a_i \leq v_i \leq b_i, i = 1,2,\cdots,n$$

515 where v_{biomass} denotes the biomass formation flux, **S** represents the stoichiometric matrix and

516 each metabolic flux v_i was constrained by lower and upper bound a_i and b_i, respectively. All

517 modelling procedures were performed with the COBRA toolbox [92] in MATLAB. The

518 calculated specific growth rates $v_{biomass}$ were then compared to the BIOLOG PM data to assess

519 the prediction accuracy using Fisher's exact test.

Gene essentiality prediction

521 *In silico* single-gene deletion was performed using the CORBRA toolbox and the mutant

522 models were then used to predict the specific growth rate in LB broth [29] using FBA. Genes

523 with 99% reduction of the specific growth rate relative to the wild type were defined as essential

524 for cell growth; otherwise, they were considered as semi-essential (1-99% reduction) and non-

525 essential (<1% reduction). Two existing PAO1 transposon insertion mutant libraries, (i) two-

526 allele mutant library [47, 65] and (ii) mini-Tn5 insertion mutant library [46], were employed

527 to assess the overall prediction accuracy with Fisher's exact test.

Simulation of bacterial growth and metabolic phenotype changes in response to lipid A

modifications

530 The LPS stoichiometric coefficients in the biomass formula under the control and lipid A

531 modification conditions were set according to the measured lipid A compositions in the wild-

532 type *P. aeruginosa* PAK and its polymyxin-resistant mutant PAK*pmrB6*, respectively (**Table**

533 **2**) [14]. For PAKpmrB6, a missense mutation (L243Q) in *pmrB* resulted in constitutive

534 activation of the PmrAB system and induced expression of the regulated genes regardless of

535 polymyxin, including *arn* operon and *pagL* [39, 93]. Aerobic growth was simulated on minimal

536 media with glucose uptake at 10 mmol·gDW^{-1}·L^{-1}. For each simulation, the solution space was

537 sampled with 10,000 random points using the ll-ACHRB algorithm [94]. Flux samples of the

538 control and lipid A modification were then compared. Significantly perturbed metabolic fluxes

539 were identified using a Z-score based approach [95].

540 To further analyse the metabolic impact of lipid A modifications, the proportions of all types

541 of LPS in the biomass formula were randomly assigned and the process was repeated 1,000

542 times. For each repetition, the specific growth rates were calculated and solution space was

543 sampled using the methods above. For each type of lipid A, specific physiochemical properties

544 (*f*) including total atom number, partition coefficient (logP), average charge and molecular

545 polarity were predicted at pH 7 using the cxcalc tool from ChemAxon (Budapest, Hungary).

546 The overall apparent properties *F* of the OM were estimated by calculating the weighted sum.

547
$$F = \sum_{i=1}^{n} w_i f_i$$

548 where w_i represents the stoichiometric coefficient of the *i*-th of 288 heterogeneous LPS

549 molecules in the biomass formula. Pairwise correlation analysis was conducted between lipid

550 A modifications, physiochemical properties changes, bacterial growth and metabolism

551 alterations.

552 Predict metabolic responses to polymyxin treatment by constraining fluxes with

553 transcriptomics data

554 The RNA-Seq data from 1-h 1 mg·L^{-1} polymyxin B treatment experiment using PAO1 were

555 employed as flux constraints for modelling [48]. For each gene under every condition, the

556 RPKM (Reads Per Kilobase Million) value was calculated from the aligned reads using the

557 edgeR package [96], and normalised to constrain flux upper bounds using the E-Flux algorithm

558 [74]. Since CAMHB was used in the RNA-Seq experiment, the maximum uptake rates of

559 amino acids, vitamins and dipeptides in *i*PAO1 were set to 1 mmol·gDW·h^{-1} considering that

560 these nutrients in CAMHB provided the major carbon sources for bacterial growth. For each

561 condition, the solution space was sampled with 10,000 points using ll-ACHRB as above.

562 Statistical significance of differential flux distributions was computed using the *Z*-score method

563 above. The turnover rate for key metabolites was calculated by summing up all influxes or

564 effluxes [97].

565

Availability of supporting data and materials

567 The raw RNA-Seq data have been deposited in the NCBI Sequence Read Archive (SRA)

568 database under the BioProject accession number (to be updated). The metabolomics and

569 lipidomics data have been deposited in Metabolight database with the accession number (to be

570 updated).

571

Additional files

573 Additional file 1 (additionalFile1.docx): Manual curation of GPL biosynthesis, LPS

574 biosynthesis and modification pathways.

575 Additional file 2 (additionalFile2.xlsx): Metabolites in the constructed draft model

576 *i*PAO1_draft1.

577 Additional file 3 (additionalFile3.xlsx): Reactions in the constructed draft model *i*PAO1_draft1.

578 Additional file 4 (additionalFile4.xlsx): Genes in the constructed draft model *i*PAO1_draft1.

579 Additional file 5 (additionalFile5.xlsx). Supplemented metabolites according to previous GC-

580 MS based metabolomics data.

581 Additional file 6 (additionalFile6.xlsx): Supplemented reactions according to previous GS-MS

582 based metabolomics data.

583 Additional file 7 (additionalFile7.xlsx): Root gap metabolites identified using GapFind from

584 the COBRA toolbox.

585 Additional file 8 (additionalFile8.xlsx): Comparison of the predicted growth phenotypes with

586   the BIOLOG PM results.

587   Additional file 9 (additionalFile9.xlsx): Reactions with changed reversibility and directionality

588   during manual curation.

589   Additional file 10 (additionalFile10.xlsx): Deleted metabolites during manual curation.

590   Additional file 11 (additionalFile11.xlsx): Deleted reactions during manual curation.

591   Additional file 12 (additionalFile12.xlsx): Added reactions during manual curation.

592   Additional file 13 (additionalFile13.xlsx): Added intermediate metabolites in GPL biosynthesis

593   pathway.

594   Additional file 14 (additionalFile14.xlsx): Added reactions in GPL biosynthesis pathway.

595   Additional file 15 (additionalFile15.xlsx): Metabolites in the constructed model *i*PAO1.

596   Additional file 16 (additionalFile16.xlsx): Reactions in the constructed model *i*PAO1.

597   Additional file 17 (additionalFile17.xlsx): Genes in the constructed model *i*PAO1.

598   Additional file 18 (additionalFile18.xlsx): Biomass formation formula.

599   Additional file 19 (additionalFile19.xlsx): Comparison of the predicted gene essentiality with

600   the information derived from two transposon insertion mutant libraries.

601   Additional file 20 (additionalFile20.xlsx): Metabolic flux changes in response to lipid A

602   modifications using lipidomics data as stoichiometric constraints.

603   Additional file 21 (additionalFile21.xlsx): Randomised stoichiometric coefficients of LPS

604   species.

605   Additional file 22 (additionalFile22.xlsx): Predicted physiochemical properties of lipid A

606   molecules.

607   Additional file 23 (additionalFile23.xlsx): Metabolic flux changes in response to lipid A

608 modifications with randomly assigned lipid A compositions as stoichiometric constraints.

609 Additional file 24 (additionalFile24.xlsx): Metabolic flux constraints calculated based on

610 RNA-Seq data.

611 Additional file 25 (additionalFile25.xlsx): Metabolic flux changes in response to polymyxin

612 treatment using RNA-Seq data as flux constraints.

613 Additional file 26 (additionalFile26.xlsx): Metabolite turnover rates.

614 Additional file 27 (additionalFile27.xlsx): Full names of the metabolite abbreviations in Figure

615 1.

616

617 **List of abbreviations**

618 2CS: two-component regulatory system; CAMHB: cation-adjusted Mueller-Hinton broth;

619 COG: clusters of orthologous groups; FBA: flux balance analysis; FDR: false discovery rate;

620 GC-MS: gas chromatography-mass spectrometry; GPL: Glycerolphospholipid; GPR: gene to

621 protein to reaction; GSMM: genome-scale metabolic models; IM: Inner membrane; KEGG:

622 Kyoto Encyclopaedia of Genes and Genomes; L-Ara4N: 4-Amino-4-deoxy-L-arabinose; LB:

623 Luria-Bertani; LC-MS: liquid chromatography-mass spectrometry; LPS: lipopolysaccharide;

624 MDR: Multidrug-resistant; OM: outer membrane; PM: Phenotypic Microarray; RPKM: Reads

625 Per Kilobase Million; SBML: Systems Biology Markup Language; SRA: Sequence Read

626 Archive; TCDB: Transporter Classification Database.

627

628 **Competing interests**

629 The authors declare no competing interest for this work.

630

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Authors' contributions

J.L. and F.S. conceived the project and Y.Z. developed the GSMM and conducted most analysis. T.C. and M.K. validated the model. J.Z., J.Lu and B.S. curated the model. T.V., T.L. and J.S. helped supervise the project. M.H. and M.H.M.M. provided the lipidomics and transcriptomics data, respectively.

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934

Figure legends

936　**Figure 1**. The curated GPL biosynthesis in *i*PAO1. [c], intracellular metabolites; [p],

937　periplasmic metabolites; [e], external metabolites. Blue arrows indicate transport reactions.

938　Full names of metabolite classes are listed in **Additional file 27.**

939　**Figure 2.** LPS biosynthesis and modification in *i*PAO1. (A) VANTED diagram showing the

940　biosynthesis of different LPS molecules. (B) LPS biosynthesis pathway; lipid A and LPS are

941　indicated in the same colour as in (A).

942　**Figure 3.** Constitutional genes, reactions and metabolites in *i*PAO1. (A) Sources of *i*PAO1

943　components. (B) Radar map showing the percentages of metabolites and reactions with valid

944　database identifiers. (C) The COG functional classification of the involved genes in iMO1056,

945　Opt208964 and *i*PAO1. Percentages given in the middle indicate the coverages of COG groups.

946　The proportions of the curated reactions (D), reaction-to-gene ratio (E) and predicted

947　subcellular localisations of the involved proteins (F) are shown for each pathway or COG group.

948　In panel D, red bars indicate the curated reactions; whereas blue bars indicate the reactions

949　from previous model. In panel D and E, pathways with the highest curation proportion or

950　reaction-to-gene ratio are highlighted in red.

951　**Figure 4.** Comparison of the BIOLOG result (left columns) and model prediction (right

952　columns). Blue indicates growth; whereas yellow indicates no growth.

953 **Figure 5.** Simulation of the impact of lipid A modifications on bacterial growth, metabolism

954 and OM physiochemical properties. The significant correlation ($P<0.05$) of paired items is

955 indicated in red.

956 **Figure 6.** Polymyxin B induced metabolic perturbations. The distributions of metabolic fluxes

957 and metabolite turnover rates are shown in subgraphs with red indicating control and blue

958 indicating polymyxin B treatment.

959

960 **Tables and their legends**

961 **Table 1.** Components in model iMO1056, Opt208964 and *i*PAO1.

	*i*PAO1	iMO1056	Opt208964
Genes	1,458	1,042	1,021
Reactions	4,365	992	1,609
Cytoplasmic metabolic reactions	1,716	730	1,132
Periplasmic metabolic reactions	403	0	0
External metabolic reactions	40	0	0
Transport reactions	960	150	253
Transport across IM	519	0	0
Transport across OM	441	0	0
Transport from cytoplasm to extracellular space	0	150	253
Boundary reactions	352	112	223

Reactions without associated genes	628	159	374
Sink reactions	0	0	1
Metabolites	3,022	858	1,344
Cytosol	1,519	746	1,121
Periplasm	698	0	0
Extracellular space	805	112	223
Pathways	109	-[a]	117

962   [a] Pathway information is not available in iMO1056 from the Model SEED database.

963   **Table 2.** Lipid A composition (%) in the outer leaflet of the OM in PAO1 [14].

Lipid A species	Control	Polymyxin B treated
Hexa-lipid A	42.5±0.46	11.7±1.13
Penta-lipid A	57.5±0.46	67.7±3.16
L-Aminoarabinosylated hexa-LA	0	1.24±0.31
L-Aminoarabinosylated penta-LA	0	19.4±3.44
Total	100	100

964

965   **Table 3.** Specific growth rate, significantly altered major exchange fluxes (>1 mmol·gDW·h^{-1}),

966   respiration quotient and the fluxes through F_0F_1-ATPase calculated using the RNA-Seq data

967   [48] as flux constraints.

Exchange flux (mmol·gDW·h^{-1})	Control	Polymyxin B treatment	Z-score	FDR[a]

Specific growth rate (h^{-1})	0.82±0.00	0.67±0.00	10,201.3	0.00
H_2O	46.9±21.8	53.0±19.0	20.37	0.00
O_2	-106.0±23.0	-113.4±19.8	24.30	0.00
CO_2	109.2±22.6	115.8±19.3	22.62	0.00
NH_4^+	36.6±9.29	38.0±8.77	10.94	0.00
Glycine	2.15±4.76	1.92±4.46	3.05	0.00
L-Alanine	1.21±5.01	-0.52±2.20	31.77	0.00
Succinate	2.08±4.19	2.52±4.42	7.27	0.00
H^+	-41.5±14.1	-40.4±11.9	6.44	0.00
Methanethiol	1.53±0.82	1.34±1.11	12.62	0.00
H_2S	1.66±1.74	1.41±2.18	9.29	0.00
Respiration Quotient (RQ)	1.03±0.10	1.02±0.10	7.63	0.00
ATPase ($mmol \cdot gDW \cdot h^{-1}$)	-188.6±52.4	-167.6±48.4	29.62	0.00

[a] FDR was calculated using the Benjamini-Hochberg method [98].

968

969

Figure 1

Figure 2

Figure 3A-C

Figure 3D-F

PM1 (Carbon)	Biolog	iPAO1
L-Arabinose	×	×
N-Acetyl-D-Glucosamine	√	√
D-Saccharic Acid	×	×
Succinic Acid	√	√
D-Galactose	×	×
L-Aspartic Acid	√	√
L-Proline	√	√
D-Alanine	√	√
D-Trehalose	×	√
D-Mannose	×	×
Dulcitol	×	×
D-Serine	×	√
D-Sorbitol	×	×
Glycerol	√	√
L-Fucose	×	×
D-Glucuronic Acid	×	×
D-Gluconic Acid	√	√
D,L-a-Glycerol Phosphate	×	√
D-Xylose	√	×
L-Lactic Acid	√	√
Formic Acid	√	√
D-Mannitol	√	√
L-Glutamic Acid	√	√
D-Glucose-6-Phosphate	×	√
D-Galactonic Acid-g-Lactone	×	×
D,L-Malic Acid	√	√
D-Ribose	√	√
Tween 20	√	×
L-Rhamnose	×	×
D-Fructose	√	√
Acetic Acid	√	√
D-(+)-Glucose	√	√
Maltose	×	×
D-Melibiose	×	×
Thymidine	×	×
L-Asparagine	√	√
D-Aspartic Acid	×	×
D-Glucosaminic Acid	×	×
1,2-Propanediol	√	√
Tween 40	√	×
a-Keto-Gutaric Acid	√	√
a-Ketobutyric Acid	√	√
a-Methyl-D-Galactoside	×	×
a-D-Lactose	×	×
Lactulose	×	×
Sucrose	×	×
Uridine	×	√
L-Glutamine	√	√
m-Tartaric Acid	×	×
D-Glucose-1-Phosphate	×	√
D-Fructose-6-Phosphate	×	×
Tween 80	√	×
a-Hydroxy Glutaric Acid-g-Lactone	×	×
D,L-a-Hydroxy-Butyric Acid	√	√
b-Methyl-D-Glucoside	×	×
Adonitol	×	×
Maltotriose	×	×
2'-Deoxy Adenosine	×	×
Adenosine	√	√
Glycyl-L-Aspartic Acid	×	√
Citric Acid	√	√
m-Inositol	×	×
D-Threonine	×	×
Fumaric Acid	√	√
Bromo Succinic Acid	√	×
Propionic Acid	√	√
Mucic Acid	×	×
Glycolic Acid	×	×
Glyoxylic Acid	×	×
D-Cellobiose	×	×
Inosine	√	√
Glycyl-L-Glutamic Acid	√	√
Tricarballylic Acid	×	×
L-Serine	√	√
L-Threonine	×	√
L-Alanine	√	√
Ala-Gly	×	√
Acetoacetic Acid	√	√
N-Acetyl-D-Mannosamine	×	×
Mono Methyl Succinate	√	√
Methyl Pyruvate	√	×
D-Malic Acid	×	×
L-Malic Acid	√	√
Glycyl-L-Proline	√	√
p-Hydroxy Phenyl Acetic Acid	√	√
M-Hydroxy Phenyl Acetic Acid	×	×
Tyramine	√	√
D-Psicose	×	×
L-Lyxose	×	×
Glucuronamide	√	√
Pyruvic Acid	√	√
L-Galactonic Acid-g-Lactone	×	×
D-Galacturonic Acid	×	×
b-Phenylethylamine	×	×
2-Aminoethanol	√	√

PM2 (Carbon)	Biolog	iPAO1
Chondroitin Sulfate C	×	×
a-Cyclodextrin	×	×
b-Cyclodextrin	×	×
g-Cyclodextrin	×	×
Dextrin	×	×
Gelatin	√	×
Glycogen	×	×
Inulin	×	√
Laminarin	×	×
Mannan	×	×
Pectin	×	×
N-Acetyl-D-Galactosamine	×	×
N-Acetyl-Neuraminic Acid	×	×
b-D-Allose	×	×
D-Amygdalin	×	×
D-Arabinose	×	×
D-Arabitol	×	×
L-Arabitol	×	×
Arbutin	×	×
2-Deoxy-D-Ribose	×	×
i-Erythritol	×	×
D-Fucose	×	×
3-0-b-D-Galacto-pyranosyl-D-Arabinose	×	×
Gentiobiose	×	×
L-Glucose	×	×
Lactitol	×	×
D-Melezitose	×	×
Maltitol	×	×
a-Methyl-D-Galactoside	×	×
b-Methyl-D-Galactoside	×	×
3-Methyl Glucose	×	×
b-Methyl-D-Glucuronic Acid	×	×
a-Methyl-D-Mannoside	×	×
b-Methyl-D-Xyloside	×	×
Palatinose	×	×
D-Raffinose	×	×
Salicin	×	×
Sedoheptulosan	×	×
L-Sorbose	×	×
Stachyose	×	×
D-Tagatose	×	×
Turanose	×	×
Xylitol	×	×
N-Acetyl-D-glucosaminitol	×	×
g-Amino Butyric Acid	√	√
d-Amino Valeric Acid	√	√
Butyric Acid	√	√
Capric Acid	×	√
Caproic Acid	√	√
Citraconic Acid	×	×
Citramalic Acid	√	√
D-Glucosamine	×	×
2-Hydroxybenzoic acid	×	×
4-Hydroxy Benzoic Acid Sodium	√	√
b-Hydroxy Butyric Acid	√	√
g-Hydroxy Butyric Acid	×	√
2-Oxovaleric Acid	×	×
Itaconic Acid	√	√
5-Keto-D-Gluconic Acid	×	×
D-Lactic Acid Methyl Ester	×	×
Malonic Acid	√	√
Melibionic Acid	×	×
Oxolic Acid	×	×
Oxalomalic Acid	×	×
Quinic Acid	√	√
D-Ribono-1,4-Lactone	×	×
Sebacic Acid	×	×
Sorbic acid	√	√
Succinamic Acid	√	√
D-Tartaric Acid	√	×
L-Tartaric Acid	×	×
Acetamide	√	√
L-Alaninamide	√	√
N-Acetyl-L-Glutamic Acid	√	√
L-Arginine	√	√
Glycine	√	√
L-Histidine	√	√
L-Homoserine	×	√
Hydroxy-L-Proline	√	√
L-Isoleucine	√	√
L-Leucine	√	√
L-Lysine	×	√
L-Methionine	×	√
L-Ornithine	√	√
L-Phenylalanine	×	√
L-Pyroglutamic Acid	√	√
L-Valine	√	√
D,L-Carnitine	√	√
Sec-Butylamine	×	×
D.L-Octopamine	√	√
Putrescine	×	×
Dihydroxy Acetone	×	×
2,3-Butanediol	√	√
Diacetyl	×	×
3-Hydroxy 2-Butanone	×	×

PM3 (Nitrogen)	Biolog	iPAO1
Ammonia	√	√
Nitrite	√	√
Sodium Nitrate	√	√
Urea	√	√
Biuret	×	×
L-Alanine	√	√
L-Arginine	√	√
L-Asparagine	√	√
L-Aspartic Acid	√	√
L-Cysteine	√	√
L-Glutamic Acid	√	√
L-Glutamine	√	√
Glycine	√	√
L-Histidine	√	√
L-Isoleucine	√	√
L-Leucine	√	√
L-Lysine	√	√
L-Methionine	√	√
L-Phenylalanine	√	√
L-Proline	√	√
L-Serine	√	√
L-Threonine	√	√
L-Tryptophan	√	√
L-Tyrosine	√	√
L-Valine	√	√
D-Alanine	√	√
D-Asparagine	√	√
D-Aspartic Acid	×	×
D-Glutamic Acid	√	√
D-Lysine	√	√
D-Serine	√	√
D-Valine	√	√
L-Citrulline	√	√
L-Homoserine	×	√
L-Ornithine	√	√
N-Acetyl-L-Glutamic Acid	√	√
N-Phthaloyl-L-Glutamic Acid	√	×
L-Pyroglutamic Acid	√	√
Hydroxylamine	√	√
Methylamine	×	×
N-Amylamine	×	×
N-Butylamine	×	×
Ethylamine	√	√
Ethanolamine	√	×
Ethylenediamine	×	×
Putrescine	√	√
Agmatine	√	√
Histamine	√	√
b-Phenylethylamine	√	√
Tyramine	√	√
Acetamide	√	√
Formamide	×	×
Glucuronamide	√	√
D,L-Lactamide	√	√
D-Glucosamine	×	×
D-Galactosamine	×	×
D-Mannosamine	×	×
N-Acetyl-D-Glucosamine	√	√
N-Acetyl-D-Galactosamine	√	√
N-Acetyl-D-Mannosamine	×	×
Adenine	√	√
Adenosine	√	√
Cytidine	√	√
Cytosine	√	√
Guanine	√	√
Guanosine	√	√
Thymine	√	√
Thymidine	×	×
Uracil	√	√
Uridine	√	√
Inosine	√	√
Xanthine	√	√
Xanthosine	√	√
Uric Acid	√	√
Alloxan	√	×
Allantoin	√	√
Parabanic Acid	√	×
D,L-a-Amino-N-Butyric Acid	×	×
g-Amino Butyric Acid	√	√
e-Amino-N-Caproic Acid	×	×
D,L-a-Amino- Caprylic Acid	×	×
d-Amino-N-Valeric Acid	√	√
a-Amino-N-Valeric Acid	√	×
Ala-Asp	√	√
Ala-Gln	√	√
Ala-Glu	√	√
Ala-Gly	√	√
Ala-His	√	√
Ala-Leu	√	√
Ala-Thr	√	√
Gly-Asn	√	√
Gly-Gln	√	√
Gly-Glu	√	√
Gly-Met	×	√
Met-Ala	√	√

Figure 5

Figure 5

Figure 6

Gluconeogenesis

Glucose 6-phosphate

Fructose 6-phosphate

rxn00549

Glyceraldehyde 3-phosphate

NADH
1,3-Biphosphoglycerate

ATP
3-Phosphoglycerate

rxn01106

GTP
ATP

Pyruvate

rxn00420

rxn00330

NADH

Acetyl-CoA

Glyoxalate

Serine/glycine biosynthesis

Malate

rxn00256

rxn00974

rxn00199

Acetyl-CoA

Oxaloacetate

Citrate

NADH

Tricarboxylic acid
cycle

Isocitrate

Fumarate

NADH
α-Ketoglutarate

QH$_2$

NADH

Succinate

Succinyl-CoA

rxn08094

Oxidative phosphorylation

Periplasm

NADH H$^+$ Ubiquinol-8   H$^+$ Cytochrome c2   H$^+$ O$_2$

Inner Membrane

NAD$^+$ Ubiquinone-8   Cytochrome c3   H$_2$O

ADP   ATP
H$^+$

Cytoplasm

Complex I
rxn10122

Complex III
rxn13820

Complex IV
rxn13688

F$_0$F$_1$-ATPase
rxn10042

Turnover rate of cofactors

ATP
cpd00002[c]

NADH
cpd00004[c]

NADPH
cpd00005[c]

Ubiquinol-8
cpd15561[c]

FADH2
cpd00982[c]

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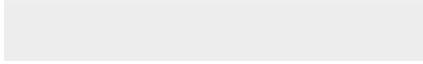**Supplementary Material**
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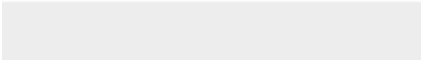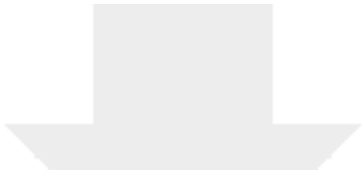
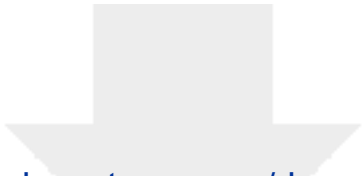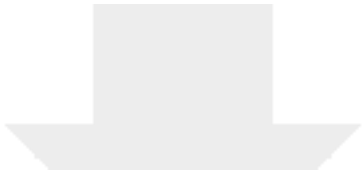Additional file 4

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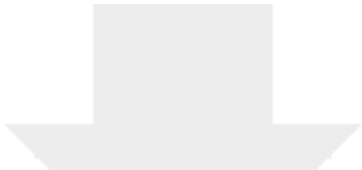
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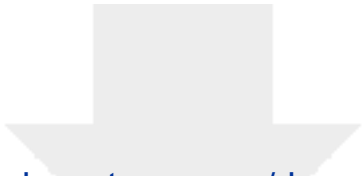**Supplementary Material**
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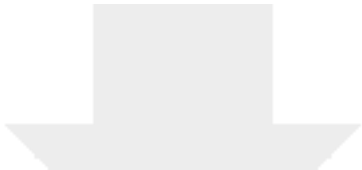
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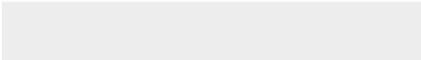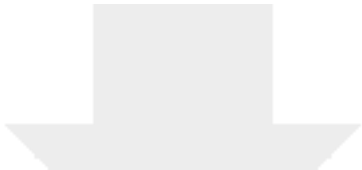
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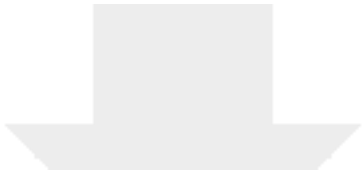
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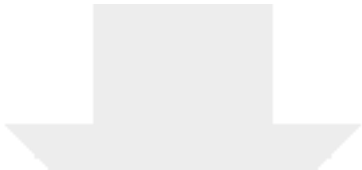
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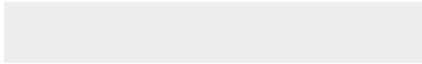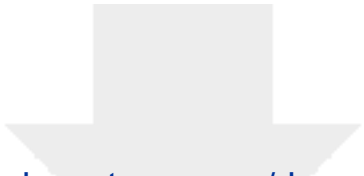
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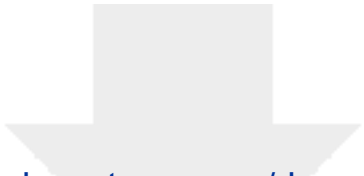
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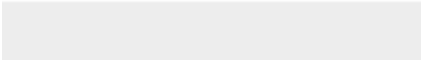**Supplementary Material**
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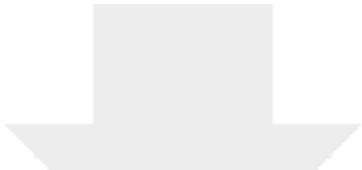
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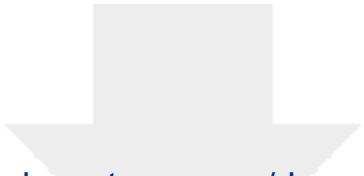
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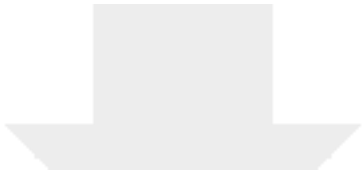additionalFile16.xlsx
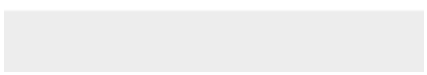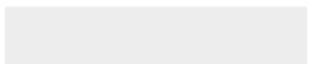
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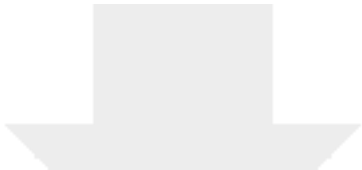**Supplementary Material**
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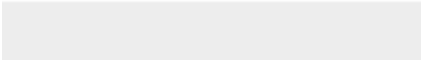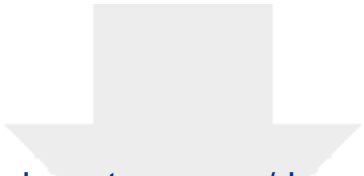
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MONASH University

Professor Jian Li
Head, Laboratory of Antimicrobial Systems Pharmacology
Monash Biomedicine Discovery Institute

Dr Laurie Goodman
Editor-in-Chief
GigaScience

16 October 2017

Dear Dr Goodman,

We are pleased to submit our manuscript entitled "*Genome-scale metabolic modelling of responses to polymyxins in Pseudomonas aeruginosa*" for your consideration as an original Research Article in *GigaScience*.

Antimicrobial resistance has become one of the greatest threats to global health today. Multidrug-resistant (MDR) *P. aeruginosa* has been categorised by the World Health Organization as a "Critical" Gram-negative 'superbug' against which no new antibiotics will be available in the near future. Polymyxins are 'old' antibiotics firstly discovered in 1947, but have been abandoned since the 1970s. Over the last decade polymyxins have been revived as the last-line therapy against Gram-negative 'superbugs', including *P. aeruginosa*, which are resistant to all other antibiotics. However, the mechanism of their antibacterial activity remains largely unknown.

Here we report the construction of a superior genome-scale metabolic model (GSMM) *i*PAO1 for *P. aeruginosa* PAO1 which represents *the largest genome-scale metabolic model thus far for any Gram-negative bacteria*. *i*PAO1 provides a powerful systems pharmacology tool to elucidate the complex mode of action of antibiotics and shift the paradigm of the "one-gene, one-receptor, one-mechanism" approach. It is able to quantitatively simulate complex bacterial cellular responses in response to antibiotic treatments.

To date, there are four curated GSMMs for *P. aeruginosa*, iMO1056 (developed in 2008), Opt20896429 (2010), iMO1086 and iPae1146 (two minor updated versions of iMO1056 developed in 2011 and 2017, respectively), and all are for the strain PAO1. *Unfortunately, none of these four GSMMs incorporates the periplasmic space, and glycerolphosholipid (GPL) and lipopolysaccharide (LPS) biosynthesis is very poorly represented.* These shortcomings significantly limit their usefulness for antimicrobial pharmacology. Growth prediction with *i*PAO1 on 190 carbon and 95 nitrogen nutrients outperformed all the previous models with an accuracy of 89.1%. Prediction of the essential genes for growth on rich media achieved a high accuracy of 87.9%. Specifically, the significant advantages of our *i*PAO1 include: (1) incorporation of the periplasmic space; (2) addition of detailed GPL and LPS biosynthesis pathways supported by our own metabolomics and lipidomics data; and (3) significant expansion of the modelling scale with a high prediction accuracy. For the first time, metabolic simulation using *i*PAO1 showed that lipid A modifications exert limited impacts on bacterial growth and metabolism, but remarkably change the physiochemical properties of bacterial outer membrane. Modelling with transcriptomics constraints revealed a broad range of metabolic responses to polymyxin treatment, including reduced biomass formation, upregulated amino acids catabolism, induced tricarboxylic acid cycle, and increased redox turnover. Overall,

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Professor Jian Li
Head, Laboratory of Antimicrobial Systems Pharmacology
Monash Biomedicine Discovery Institute

our GSMM approach has a significant potential in accelerating antimicrobial pharmacological discovery against Gram-negative 'superbugs'.

To the best of our knowledge, this study is the first to integrate antimicrobial pharmacology, computational biology, metabolic network and systems pharmacology to analyse large-scale datasets, in order to better understand the dynamic and complex nature of polymyxin killing and resistance. We believe this manuscript perfectly matches the theme of *GigaScience* and will be of broad interest to microbiologists, bioinformaticians and antimicrobial researchers.

We confirm that our submission comprises original and unpublished material which is not currently under consideration for publication elsewhere, and has been approved by all authors. Thank you for considering our work for publication in *GigaScience.* We look forward to your correspondence.

Yours sincerely,

Jian Li   PhD   Falk Schreiber   PhD

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