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Genome-scale metabolic modelling of responses to polymyxins in Pseudomonas aeruginosa
--Manuscript Draft--

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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 therapies. To this end, a 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 Pseudomonas. It provides a powerful systems pharmacology platform for the elucidation of complex killing mechanisms of antibiotics.

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Response to Reviewers:	Dr Laurie Goodman Editor-in-Chief GigaScience Dear Dr Goodman, RE: Manuscript ID: GIGA-D-17-00272: "Genome-scale metabolic modelling of responses to polymyxins in Pseudomonas aeruginosa" Thank you for the opportunity to revise our manuscript. Please find below a point-by-point response to the reviewers' comments. All major changes have been highlighted in yellow in the 'marked' version of the revised manuscript. The raw data have been submitted to Sequence Read Archive (SRA) and MetaboLights databases and will be made publicly available hopefully by 31 Jan, 2018. We look forward to your correspondence and thank you very much. Best regards, Jian Li Falk Schreiber Monash Biomedicine Discovery Institute, Department of Microbiology, Faculty of Medicine, Nursing and Health Sciences, Monash University, Melbourne 3800, Australia Department of Computer and Information Science, University of Konstanz, Konstanz 78457, Germany Reviewer #1: 1. Introduction section. I would specify that the specific approach of applying gene expression constraints to obtain condition-specific GEMs have been previously used for other MDR bacteria (e.g. A. baumannii doi:10.1038/s41598-017-03416-2). Response: The manuscript has been revised accordingly (Pages 3-4, Lines 74-77). 2. Lines 217-218: I would remove the sentence "Therefore, iPAO1 is a well-defined, metabolism-dedicated model.", in that it is included in the definition of "metabolic reconstruction". The presence of genes associated to non-metabolic COG categories is, in my opinion, due to the presence of misannotated (for what concerns the COG

categories) genes. Honestly, I wouldn't use the distribution of such categories as a measure of a model goodness, especially considering that some genes can be associated to multiple categories. All the other comparisons the authors made already highlighted how this reconstruction is the best one.

Response: The sentence was removed in the revised manuscript as suggested (Page 9, Line 221).

3. Lines 253-258: "this is possibly due to the incorporation of new genes (30.5% increase compared to Opt208964; 27.2% increase compared to iPae1146) whose metabolic functions were previously misannotated." This is not clear... do the authors mean that the addition of new genes brought alternative routes to bypass previously essential gene deletion? This should be rephrased, and, if possible, the proposed explanation should be tested.

Response: The sentence was rephrased as suggested. In the revised manuscript an example was provided to delineate that incorporating isozymes altered previous essentiality prediction results. Please refer Page 11, Lines 256-264 in the revised manuscript.

4. Section "Elucidating the mechanisms of metabolic responses to polymyxin treatment":
In this section the authors use the previously presented model to describe the changes at a systems level of the metabolism in presence of polymyxin treatment. I have two issues concerning this section: The way the authors computed the flux distribution in presence of antibiotics. Given the non-optimal state of such condition, I feel that MOMA is more appropriate. I suggest the authors to test this and compare the results with the current ones.

Response: We respectfully disagree with the reviewer. Minimisation Of Metabolic Adjustment (MOMA) was developed to predict the metabolic flux redistributions in gene knockout mutants. MOMA hypothesises that metabolism of the mutant tends to approximate the wild-type (Segre et al., 2002, Proc Natl Acad Sci. 99(23):15112-7), which is distinct from the antibiotic treatment scenario. For instance, our metabolomics data have demonstrated that polymyxin treatment caused dramatic metabolic changes in bacteria (e.g. Maifiah et al., 2017, Sci Rep, 7: 45527). Therefore, metabolic fluxes with and without antibiotic treatment should not be calculated with MOMA, but FBA (see e.g. Colijn et al., 2009, PLoS Comput Biol, 5(8): e10004). Please refer Pages 17, Lines 414-420 in our revised manuscript.

5. Although a description of the systemic changes induced by antibiotics is important, I think that the authors are missing an important point, that is the condition-specific essential genes. In my opinion this is very important and interesting, also considering that a selling point of the manuscript is that "iPAO1 offers an in silico platform for precision antimicrobial pharmacology therapy".

Response: We appreciate reviewer's suggestion. The methods and results on the condition-specific essential genes were included in the revised manuscript (Page 23, Lines 552-555; Page 11, Lines 264-269).

Reviewer #2:
1. On page 13 line 260 in the section on lipid A modification the authors mention changes in fluxes. They state that fluxes were calculated using FBA. However, in the Methods section I see that the authors used sampling to explore the solution space. The authors must use sampling to compare fluxes between conditions. If the author's used sampling here to the authors must specify so in the main text.

Response: We employed sampling in our original study and have specified the sampling methods in the revised manuscript as suggested. Please refer Page 12, Lines 274, 286-287.

2. Page 14 line 286 - the authors must state how the RNAseq was used to constrain the model. They mention it in the Discussion section (E-FLUX method). However, this must be stated in the Results section as well.

Response: E-Flux method was employed to constrain the model with the RNAseq data, which has been specified in the Methods (Pages 24-25, Lines 586-592) and Results sections as suggested (Page 13, Line 302).

3. Page 14 line 295 - I have a major question about how the authors simulate for growth in CAMHB media? This is an undefined media type and in the Methods section they describe that they set the uptake rates to 1 mmol*gDW*hr^-1 for major carbon sources. The authors must explain why this uptake rate is justified. Did the authors perform any sensitivity analysis on these uptake rates? It's very reasonable to assume that changes in these rates would dramatically affect the fluxes described by the authors in this section. Some justification and or sensitivity analysis must be added here to explain the validity of these uptake rates for growth in this condition.

Response: Previous measurements showed that P. aeruginosa cells uptake amino acids at a rate ranging from 0.26 to 1.44 mmol·gDW^-1·h^-1 (J Bacteriol, 105(3): 1039-46; J Bacteriol, 152(2): 636-42). The import of CAMHB ingredients was thus constrained to 1 mmol·gDW^-1·h^-1 without loss of generality. Sensitivity analysis was conducted as suggested (Methods section, Page 25, Lines 592-597 and 600-602) and the results showed that the changes in nutrient uptake bounds did not dramatically affect the key metabolic fluxes. Our sensitivity analysis results have been provided in the Results section (Page 13, Lines 304-305), Additional File 1 and Figure S1.

4. Page 15 line 303 - the authors must state what the "control" condition is. Is this compared to PAO1 growing in CAMHB without polymyxin treatment? Or compared to growth on a different media type, i.e. M9 minimal media + glucose?

Response: The control condition was specified in the revised manuscript as suggested (Page 13 Line 321).

5. The authors state that their model is "the most comprehensive for a gram-negative organism to date". On what basis is this claim made? We would recommend tempering this statement or perhaps limiting it to Pseudomonas models.

Response: The statement was limited to Pseudomonas and was modified in the revised manuscript (Page 2, Line 46; Page 4, Line 98; Page 20, Line 472).

Additional Information:	
Question	Response
Are you submitting this manuscript to a special series or article collection?	No
Experimental design and statistics Full details of the experimental design and statistical methods used should be given in the Methods section, as detailed in our Minimum Standards Reporting Checklist. Information essential to interpreting the data presented should be made available in the figure legends. Have you included all the information requested in your manuscript?	Yes
Resources A description of all resources used,	Yes

including antibodies, cell lines, animals and software tools, with enough information to allow them to be uniquely identified, should be included in the Methods section. Authors are strongly encouraged to cite Research Resource Identifiers (RRIDs) for antibodies, model organisms and tools, where possible. Have you included the information requested as detailed in our Minimum Standards Reporting Checklist?	
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***

2 ***aeruginosa***

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

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

28 **Abstract**

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

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

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

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

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

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

35 was employed to analyse bacterial metabolic changes at the systems level.

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

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

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

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

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

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

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

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

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

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

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

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

48 for *Pseudomonas*. It provides a powerful systems pharmacology platform for the elucidation

49 of complex killing mechanisms of antibiotics.

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

74 flux balance analysis (FBA) methods, systematic investigations into the metabolic changes in

75 response to external nutrient alterations, genetic perturbations, and antibiotic treatments

76 become feasible [16-24]. Several studies employed transcriptomics data as constraints to

77 compute condition-specific metabolic flux changes in response to antibiotic treatments in MDR

78 bacteria, including *Acinetobacter baumannii* [25], *Mycobacterium tuberculosis* [26] and

79 *Yersinia pestis* [27]. For *P. aeruginosa*, four GSMMs have been constructed, iMO1056 [28],

80 Opt208964 [29], iMO1086 [30] and the latest iPae1146 [31]. iMO1056, Opt208964 and

81 iPae1146 employed SEED metabolite and reaction names; iMO1056 and Opt208964 are fully

82 accessible via Model SEED [29, 31, 32]; iMO1086 employed different identifiers (IR/RR plus

83 five digits for reactions and C/EC plus four digits for metabolites) [30]. The previous

84 applications of these models have included simulating the metabolic dynamics in cystic fibrosis

85 patients [33], elucidating the mechanisms of biofilm formation [34, 35], predicting potential

86 drug targets [36-38] and identifying the key genes controlling virulence factors [31]. As

87 important as they have been, these models have several overarching limitations. Those past

88 models (i) do not include a major cellular component, the periplasmic space; (ii) have poor

89 representation of glycerophospholipid (GPL) biosynthesis; and (iii) lack lipid A modification

90 reactions. Considering the pathogenesis of *P. aeruginosa*, these major limitations significantly

91 compromise the modelling functions. In particularly, the power of the four reported GSMMs

92 to predict metabolic responses to antibiotic treatment is very limited, as periplasmic GPL and

93 LPS biogenesis play critical roles in responses to anti-pseudomonal antibiotics such as

94 polymyxins [15, 39-42].

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

96 Opt208964 [29] and iMO1056 [28] but with intensive manual curation using several major

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

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

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

100 *i*PAO1 represents the most comprehensive metabolic reconstruction for *Pseudomonas* thus far.

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

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

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

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

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

106

Data Description

108 The genome sequence and annotation of *P. aeruginosa* PAO1 were obtained from GenBank

109 (Accession NC_002516.2). Models iMO1056 and Opt208964 were retrieved from Model

110 SEED [32]. The gas chromatography–mass spectrometry (GC-MS) metabolomics data were

111 collected from the literature [43]. Metabolites, reactions and pathways were obtained from

112 databases KEGG (Kyoto Encyclopaedia of Genes and Genomes) [44], MetaCyc [45], TCBD

113 (Transporter Classification Database) [46], TransporterDB [47] and Pseudomonas Genome DB

114 [48]. Growth phenotypes on 190 carbon sources and 95 nitrogen sources were determined using

115 BIOLOG Phenotypic Microarrays. Non-essential gene lists were collected from two previously

116 reported transposon mutant libraries for PAO1 [49, 50]. Lipid A of wild-type *P. aeruginosa*

117 PAK and its polymyxin-resistant mutant PAK*pmrB6* was extracted using mild acid hydrolysis

118 method and the structural analysis of lipid A was conducted using mass spectrometry [42].

119 RNA was extracted and employed to construct cDNA libraries for RNA-Seq on Illumina

120 MiSeq platform [51]. The raw reads were quality trimmed and aligned to PAO1 reference

121 genome using SubRead [52]. Counts were normalised and the differential gene expression was

122 determined using voom/limma packages with Degust [53]. Whole-cell lipids and intracellular

123 metabolites were extracted using the single-phase Bligh-Dyer method as previously described

124 and analysed by liquid chromatography-mass spectrometry (LC-MS) [14, 42]. Raw

125 metabolomics data were processed with IDEOM software followed by bioinformatics analysis

126 [54].

127

128 **Analyses**

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

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

131 genes was created based upon iMO1056 [28] and Opt208964 [29] (**Additional files 2-4**). To

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

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

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

135 KEGG [44], 225 metabolites and 50 reactions from MetaCyc [45], and 7 metabolites and 20

136 reactions obtained by previous GC-MS-based quantification [43] (**Additional files 5 and 6**).

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

138 509 transport reactions across the inner membrane (IM), 441 transport reactions across the

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

140 designated as *i*PAO1_draft2.

141 Thirdly, the major pathway gaps were filled. GapFind [55] identified 109 dead-end metabolites

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

157 of the corresponding transport reaction. Interrogating the Pseudomonas Genome Database [48]

158 and Pfam [56] identified PA2777, a hypothetical protein in NCBI and UniProt which may

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

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

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

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

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

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

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

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

167 dehydrogenases according to OrthoDB [57]. Inactivation of PA1991 resulted in 8-fold

168 prolonged lag phase when *P. aeruginosa* grew on 1,2-propanediol [58]. Therefore, reaction

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

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

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

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

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

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

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

176 [59]. In total, 17 reactions representing peptidoglycan cross-linking and hydrolysis were

177 incorporated by searching for homologues of glycosyltransferases, transpeptidases,

178 carboxypeptidases and endopeptidases in PAO1 [60]. Overall, a detailed peptidoglycan

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

180 membranes can change in response to antibiotic treatment [39, 61]. Previous studies [62] and

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

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

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

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

185 A, core oligosaccharide, and *O*-antigen polysaccharide [40], and plays key roles in the host-

186 pathogen interaction and the resistance to antibiotics such as polymyxins [13, 63]. A detailed

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

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

189 lipid A biosynthesis and modifications.

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

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

192 252%, 340% and 40% increase of the components in iMO1056; and (ii) 125%, 171% and 43%

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

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

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

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

197 27.9%/43.7%/51.6% metabolites, 20.3%/33.5%/59.5% reactions and 65.3%/17.6%/28.5%

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

199 **3A**).

200 Components in *i*PAO1 were aligned with databases including KEGG [44], MetaCyc [45],

201 PubChem [64], ChemSpider [65], ChEBI [66], Model SEED [32], and BiGG [67] (**Additional**

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

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

204 (35.6%), 1,596 (36.6%) and 1,964 (45.0%) reactions were computationally mapped to the

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

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

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

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

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

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

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

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

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

214 **3C**). The largest increase in the coverage compared to iMO1056 is lipid transport and

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

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

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

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

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

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

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

222 posttranslational modification, protein turnover, chaperones and signal transduction

223 mechanisms, or undetermined categories including function unknown class.

224 In *i*PAO1, GPL metabolism, LPS biosynthesis and transport across OM were ranked the three

225 largest pathways and also contained the highest proportion of curated reactions (**Fig 3D**).

226 Additionally, these three pathways have high reaction-to-gene ratios (13.1-24.2, **Fig 3E**),

227 indicating that enzymes in these pathways are capable of acting on a broad range of substrates.

228 As kinetic parameters are usually not involved in a GSMM, constraint-based analyses (e.g.

229 FBA) of a GSMM do not directly account for enzyme levels, intracellular metabolic

230 concentrations or substrate-level regulation. Accordingly, the affinity difference of various

231 substrates was not considered in our *i*PAO1 modelling effort.

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

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

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

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

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

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

238 Growth capability on various nutrients

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

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

241 76 of 95 (80.0%) nitrogen substrates (**Fig 4**). Growth simulation with *i*PAO1 achieved an

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

243 (81.5% for Opt208964 [29], 77.9% for iMO1056 and iMO1086 [30] and 80% for iPae1146

244 [31]. Twenty-one false-positive and 10 false-negative (**Fig 4**, **Additional file 8**) disagreements

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

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

Prediction and validation of gene essentiality

247

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

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

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

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

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

253 genes, amino acid transport and metabolism (352 of 1,315, i.e. 26.7%) represents the largest

254 group, suggesting the existence of large metabolic redundancy.

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

256 transposon mutant libraries [49, 50, 68]. The overall prediction accuracy achieved 87.9%,

257 which is higher than iMO1056 (85.0%) [28] and iMO1086 (84.2%) [30], but slightly lower

258 than Opt208964 (92.9%) [29] and iPae1146 (91.46%) [31]. The higher accuracy in Opt208964

259 is partially due to errors in the annotation of essential genes. For instance, 351 genes in

260 Opt208964 were grouped as experimentally validated essential; however, 145 out of the 351

261 genes are non-essential as their corresponding mutants were found in the transposon mutant

262 library [50]. In iPae1146, removal of 16 isozymes increased the prediction accuracy of essential

263 genes; for example, 3-ketoacyl-ACP reductase (EC 1.1.1.100) reactions in iPae1146 were

264 associated with PA2967 only [31], whereas in *i*PAO1, these reactions were associated with

265 another eight highly conserved isozymes (PA0182, PA1470, PA1827, PA3387, PA4089,

266 PA4389, PA4786, PA5524). Furthermore, condition-specific essential genes were predicted in

267 *i*PAO1 by imposing transcriptomics constraints. Modification of lipid A with 4-amino-4-

268 deoxy-L-arabinose (L-Ara4N) leads to polymyxin resistance in *P. aeruginosa* and deficiency

269 in *arn* genes reverses the susceptibility [69]. Seven additional essential genes (*arnABCDEFT*,

270 PA3552-3558, encoding L-Ara4N biosynthesis) were predicted by *i*PAO1 under polymyxin

271 treatment.

Impact of lipid A modifications on bacterial growth and metabolism

273 *P. aeruginosa* modifies lipid A components in the OM in response to polymyxin treatment [70].

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

275 on our lipidomics data (**Table 2**, [14]), and the metabolic impact of lipid A modifications was

276 predicted by randomly sampling the metabolic solution space with 10,000 points (*cf.* Methods).

277 Overall, 273 fluxes were significantly affected (*Z*-Score, false discovery rate (FDR)

278 <0.01; >0.1 mmol·gDW^{-1}·h^{-1} under at least one condition, **Additional file 20**). The specific

279 growth rate remained unchanged. A 0.026 mmol·gDW^{-1}·h^{-1} flux from glucose via glucose 6-

280 phosphate, uridine diphosphate glucose, and consequently L-Ara4N biosynthesis was

281 identified due to lipid A modifications. The overall fluxes through lipid A deacylation reactions

282 were increased (from 0.007 mmol·gDW^{-1}·h^{-1} to 0.011 mmol·gDW^{-1}·h^{-1}); the generated (*R*)-3-

283 hydroxydecanoate was fuelled into β-oxidation to produce octanoyl-CoA, which was

284 subsequently salvaged for fatty acid biosynthesis.

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

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

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

288 metabolic fluxes were calculated for each of the 1,000 sets of LPS compositions by randomly

289 sampling the solution space with 10,000 points. Across the 1,000 sets of metabolic fluxes

290 (**Additional file 23**), the specific growth rate varied between 0.8812 and 0.8897 mmol·gDW^{-}

291 ^{1}·h^{-1}. Correlative analysis of the apparent overall physiochemical properties of lipid A

292 (**Additional file 22**) with the predicted growth phenotypes showed three interesting findings.

293 Firstly, addition of L-Ara4N reduced the negative charge of lipid A (ρ=1.00), decreased the

294 hydrophobicity of the OM (represented by logP, ρ=-0.59), but required assimilation of more

295 ammonia (represented by ammonia turnover, ρ=0.57). Secondly, hydroxylation on acyl chains

296 of lipid A exerted minor effects over either bacterial growth or physiochemical properties.

297 Thirdly, addition of acyl chains resulted in large lipid A molecules (represented by the atomic

298 counts, $\rho=0.88$), enhanced molecular polarity of lipid A ($\rho=0.87$), increased OM

299 hydrophobicity ($\rho=0.75$), and notably, retarded growth ($\rho=-0.95$), reduced redox and energy

300 turnover ($\rho=-0.98$ for both), and increased requirement of ammonia ($\rho=0.59$) (**Fig 5**). It is

301 evident that none of the three aforementioned modifications produced a dramatic impact on

302 bacterial growth or metabolism (**Additional file 23**).

303 **Elucidating the mechanisms of metabolic responses to polymyxin treatment**

304 RNA-Seq data were utilised as model constraints (**Additional file 24**) with an E-Flux method

305 [71] to calculate the metabolic fluxes in the absence and presence of polymyxin B (*cf*. Methods).

306 The exchange fluxes were constrained based on the maximum uptake rates of the media

307 ingredients (*cf*. Methods and **Additional file 1**). Comparison of the flux distributions revealed

308 that 1,392 reactions were differentially regulated (FDR<0.01, **Additional file 25**). A range of

309 metabolic pathways were significantly disturbed, including central metabolism, amino acid

310 metabolism, purine biosynthesis, fatty acid biosynthesis and metabolism, LPS and GPL

311 biosynthesis and transport reactions. Polymyxin B treatment reduced the growth rate (18.2%),

312 increased oxygen uptake (6.9%) and CO_2 emission (6.0%); however, the respiration quotient

313 remained roughly unchanged (**Table 3**).

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

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

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

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

318 3-phosphoglycerate towards glucose 6-phosphate. The extra flux from 3-phosphoglycerate was

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

1
320 oxidoreductase (rxn01101), 3-phosphoserine:2-oxoglutarate aminotransferase (rxn02914), *O*-

2
3
321 phospho-L-serine phosphohydrolase (rxn00420), and 5,10-methylenetetrahydrofolate:glycine

4
5
322 hydroxymethyltransferase (rxn00692), through which more NADH equivalent was generated

6
7
323 compared to the control (i.e. growth in CAMHB without polymyxin treatment). The resulting

8
9
10
324 one-carbon unit in 5,10-methylenetetrahydrofolate was oxidised to formic acid via 10-

11
12
325 formyltetrahydrofolate amidohydrolase (rxn00691); the generated glycine was fuelled into

13
14
15
326 TCA cycle via glycine:oxygen oxidoreductase (rxn00269) and acetyl-CoA:glyoxylate C-

16
17
327 acetyltransferase (rxn00330). In addition, the metabolic flux via TCA cycle was upregulated

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19
20
328 from citrate to fumarate, with increased NADH production. Within oxidative phosphorylation,

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22
329 the mean fluxes through NADH dehydrogenase (Complex I, rxn10122), cytochrome bc1

23
24
25
330 complex (Complex III, rxn13820), and cytochrome c oxidase (Complex IV, rxn13688)

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27
331 decreased by 6.6%, 7.2% and 7.8%, respectively. The flux via F_0F_1-ATPase (Complex V,

28
29
30
332 rxn10042) was downregulated by 11.1%. The overall fluxes via biosynthesis of

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32
333 macromolecules including LPS, GPL and peptidoglycan decreased due to the significantly

33
34
334 reduced biomass formation. The biosynthesis of spermidine increased by 38.3% in response to

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36
37
335 polymyxin treatment which was also indicated by upregulated expression of *speD* (PA4773;

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39
336 encoding the *S*-adenosyl-L-methionine decarboxylase, $log_2FC=3.62$, FDR<0.01) and *speE*

40
41
42
337 (PA4774; encoding spermidine synthase, $log_2FC = 3.54$, FDR<0.01).

43
44
45
338 Calculating the flux-sum of critical cofactors revealed 13.1% increase of redox turnover and

46
47
339 8.2% decline of energy turnover after 1 mg·L^{-1} polymyxin B treatment for 1 h. Breaking down

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49
50
340 the cofactors showed the turnover of major redox equivalents NADH, NADPH, ubiquinol-8

51
52
341 and FADH2 substantially increased by 12.6%, 13.9%, 3.9% and 35.9%, respectively; whereas

53
54
55
342 the turnover of ATP, the major contributor to energy significantly decreased by 8.52% after 1

56
57
343 mg·L^{-1} polymyxin treatment for 1 h (**Fig 6**, **Additional file 26**). Overall, metabolic flux analysis

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

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65

345 bacterial metabolism due to polymyxin B treatment.

346

Discussion

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

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

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

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

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

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

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

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

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

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

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

359 broad range of pathways.

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

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

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

363 basis of iMO1056 with moderate increase of metabolites, reactions and genes [28, 30, 31];

364 Opt208964 is also in a medium size, which limits modelling capacity [29]. In contrast, *i*PAO1

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

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

367 89.1% for growth on various nutrients, outperforming all of the previously reported GSMMs

368 for *P. aeruginosa* [28-31]. The *i*PAO1 model was also employed to predict gene essentiality

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

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

371 for other *P. aeruginosa* strains.

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

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

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

375 *coli* is estimated to constitute up to 16% of total cell volume [72]. It contains a thin cell wall

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

377 folding, cell envelope biogenesis, electron transport and xenobiotic metabolism [73]. *i*PAO1 is

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

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

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

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

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

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

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

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

386 polymyxin molecule [40]. Based on our simulations (**Additional file 20**), we purport that such

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

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

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

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

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

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

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

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

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

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

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

A head groups [63]. These events are subsequently followed by hydrophobic interactions

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

polymyxin with the hydrophobic fatty acyls of lipid A [63]. Therefore, in concept both the

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

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

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

in a polymyxin-resistant mutant PAK*pmrB6* derived from *P. aeruginosa* PAK [14, 74],

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

to polymyxin treatment.

Our recent transcriptomics and metabolomics studies discovered that polymyxin treatment

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

[41, 42, 75-77]. The integration of transcriptomics results into GSMMs allow for more accurate

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

perturbations (i.e. mutations) [78]. In the present study, we employed the E-Flux method to

integrate transcriptomics data as flux constraints [26]. E-Flux can map continuous gene

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

degree to which a reaction is active or inactive [26]. Therefore, E-Flux provides a more

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

to use any artificial thresholds to binarise gene expression data [79]. In the present study,

metabolic fluxes with and without antibiotic treatment were not be calculated with

Minimization Of Metabolic Adjustment (MOMA), as MOMA was developed to predict the

419 metabolic flux redistributions in gene knockout mutants [80]. MOMA hypothesises that

420 metabolism of the mutant tends to approximate the wild-type [80], which is distinct from the

421 antibiotic treatment scenario. For instance, our metabolomics data have demonstrated that the

422 antibiotic treatment caused dramatic metabolic changes in bacteria [41].

423 Comparison of the calculated flux distributions revealed that a broad range of metabolic

424 perturbations occur in response to polymyxin treatment (**Fig 6**), ranging from central carbon

425 metabolism to oxidative phosphorylation and amino acid metabolism. Reduced growth,

426 increased redox turnover and decreased energy turnover due to polymyxin treatment were

427 evident (**Fig 6**), indicating that bacterial cells regulated their metabolism to produce more redox

428 power to cope with the oxidative stress. This is consistent with previous findings that showed

429 bactericidal antibiotics induced lethal oxidative damages via generating highly deleterious free

430 radicals with subsequent culmination of cellular death [81]. In addition, our simulations

431 revealed that polymyxin treatment induced an uptake of L-alanine, which was catabolised to

432 generate more NADH (**Fig 7**). This indicates that rich media (e.g. CAMHB) may provide

433 abundant amino acids and peptides that can be utilised by bacterial cells to generate sufficient

434 redox equivalents to cope with the oxidative damage caused by polymyxin treatment.

435 Furthermore, our simulation results also showed an upregulated metabolic flux towards L-

436 spermidine biosynthesis upon polymyxin B treatment (rxn00127 and rxn01406, **Additional

437 file 25**). Previous studies showed that polyamines (e.g. spermidine) could protect *P. aeruginosa*

438 from antimicrobial peptide killing [82]. It is assumed that the cationic spermidine could interact

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

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

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

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

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

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

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

446 CprRS and ColRS, play key roles in regulating polymyxin resistance [69, 83-86]. Among them,

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

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

449 for the modification of lipid A with L-Ara4N [87]. Moreover, the fatty acylation of lipid A by

450 PagP is under the control of PhoPQ [88, 89]. ParRS and CprRS are independent 2CSs that

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

452 in response to polymyxin treatment [83, 90]. In overview, lipid A modifications due to

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

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

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

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

457 *P. aeruginosa*.

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

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

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

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

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

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

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

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

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

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

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

469 polymyxin chemotherapy.

470

Potential implications

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

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

474 polymyxin systems pharmacology. As the largest curated GSMM thus far for *Pseudomonas*,

475 *i*PAO1 represents all aspects of the cellular metabolism and may serve as the platform for

476 integrative analysis of multi-omics data. Simulation with transcriptomics constraints in this

477 study revealed metabolic flux changes in amino acid catabolism, tricarboxylic acid cycle, and

478 redox turnover caused by polymyxin treatment. Correlative analysis of metabolomics and

479 transcriptomics with the constraint-based modelling is necessary for delineating the regulatory

480 effects on metabolism. The methodology of using GSMMs to analyse multi-level omics data

481 is applicable to other areas beyond antimicrobial pharmacology. Further integration with

482 antimicrobial pharmacokinetics and pharmacodynamics will not only provide better

483 pharmacological understanding, but also empower the model to quantitatively predict the

484 bacterial responses to antimicrobial therapy in the context of complex interplay of signalling,

485 transcriptional regulation and metabolism. In summary, our GSMM approach provides a

486 powerful systems tool to elucidate the complex mode of action of antibiotics and will paradigm

487 shift antimicrobial pharmacology.

488

Methods

Strain, media and BIOLOG experiments

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

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

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

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

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

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

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

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

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

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

501 indicated the utilisation of nutrients.

Development of a GSMM for *P. aeruginosa* PAO1

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

504 systems from Model SEED [32], iMO1056 [28] and Opt20896434 [29] were merged.

505 Databases including KEGG [44], MetaCyc [45], Pseudomonas Genome DB [48] and the

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

507 metabolites and reactions were kept consistent with Model SEED [29], and cross-referred to

508 MetaCyc, KEGG, PubChem [64], ChEBI [66], ChemSpider [65] and BiGG [67]. The PAO1

509 genome annotation from Pseudomonas Genome DB [48] was employed to construct 'gene to

510 protein to reaction' (GPR) associations [91]. A periplasm compartment was incorporated into

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

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

513 [92]. Transport reactions were generated to enable material exchange across membranes

514 according to TCBD [46] and TransporterDB [47]. The model was constructed using the

515 Systems Biology Markup Language (SBML) [93, 94]. VANTED [95] was employed for

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

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

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

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

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

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

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

523 The Gapfind function from the COBRA toolbox [96] was employed to identify the isolated and

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

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

526 by homologous enzymes (E-value$<1\times10^{-5}$, identity$\geq35\%$, coverage$\geq50\%$) were added to the

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

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

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

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

531 was created using the one from iMO1086 [30], with slight modifications on compositions of

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

533 associated maintenance was from iMO1086 [30].

Growth prediction in BIOLOG media

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

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

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

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

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

$$s.t. \quad \mathbf{Sv} = 0$$

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

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

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

544 modelling procedures were performed with the COBRA toolbox [96] in MATLAB. The

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

546 the prediction accuracy using Fisher's exact test.

Gene essentiality prediction

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

549 models were then used to predict the specific growth rate in LB broth [32] using FBA. Genes

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

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

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

553 allele mutant library [50, 68] and (ii) mini-Tn5 insertion mutant library [49], were employed

554 to assess the overall prediction accuracy with Fisher's exact test. To determine polymyxin-

555 specific essential genes, transcriptomic constrains were imposed (below) before conducting *in*

556 *silico* single-gene deletion simulations. The calculated essential genes identified in polymyxin

557 treatment alone but not in the control were considered as polymyxin-specific.

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

modifications

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

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

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

563 **2**) [14]. For PAKpmrB6, a missense mutation (L243Q) in *pmrB* resulted in constitutive

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

565 polymyxin, including *arn* operon and *pagL* [42, 97]. Aerobic growth was simulated on minimal

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

567   sampled with 10,000 random points using the ll-ACHRB algorithm [98]. Flux samples of the

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

569   were identified using a *Z*-score based approach [71].

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

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

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

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

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

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

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

$$F = \sum_{j=1}^{n} w_j f_j$$

578   where w_j represents the stoichiometric coefficient of the *j*-th of 288 heterogeneous LPS

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

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

581   alterations.

582   **Predict metabolic responses to polymyxin treatment by constraining fluxes with**

583   **transcriptomics data**

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

585   employed as flux constraints for modelling [51]. For each gene under every condition, the

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

587   edgeR package [99], and normalised to constrain flux upper bounds (b_i) using the E-Flux

588   algorithm [26]. Specifically, for each reaction catalysed by a single enzyme, the upper flux

589 bound was set to the determined RPKM value under the respective condition. For a reaction

590 catalysed by an enzyme complex, the upper bound was set to the minimum RPKM value of the

591 associated genes. For a reaction catalysed by isozymes, the upper bound was set to the sum of

592 RPKM values of the associated genes. The maximum of upper bounds was then normalised to

593 10,000 mmol·gDW^{-1}·h^{-1}. The lower bounds a_i were set to 0 for irreversible and $-b_i$

594 mmol·gDW^{-1}·h^{-1} for reversible reactions, respectively. CAMHB was used in the RNA-Seq

595 experiment and it is known as an undefined medium containing mainly amino acids and

596 oligopeptides [100]. The maximum uptake rates of amino acids in *P. aeruginosa* vary between

597 0.26-1.44 mmol·gDW^{-1}·h^{-1} [101-103]. Therefore, the upper bounds (b_i^{CAMHB}) of uptake rates

598 of amino acids, vitamins and dipeptides in *i*PAO1 were constrained to 1 mmol·gDW^{-1}·h^{-1}

599 without loss of generality. For each condition, the solution space was sampled with 10,000

600 points using ll-ACHRB as above. Statistical significance of differential flux distributions was

601 computed using the *Z*-score method above. The turnover rate for key metabolites was

602 calculated by summing up all influxes or effluxes [104]. To assess the impact of changing

603 nutrient uptake bounds, sensitivity analysis was conducted by randomly sampling solution

604 space as above while varying b_i^{CAMHB} from 0.26 to 1.44 mmol·gDW^{-1}·h^{-1}.

605

Availability of supporting data and materials

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

608 database under the BioProject accession number PRJNA414673. The metabolomics and

609 lipidomics data have been deposited in the Metabolight database with the accession number

610 MTBLS630. Supporting data, also including the scripts used in this project, are available via

611 the *GigaScience* repository GigaDB[106].

612

Additional files

Additional file 1 (additionalFile1.docx): Manual curation of GPL biosynthesis, LPS biosynthesis and modification pathways, and sensitivity analysis of nutrient uptake bounds.

Additional file 2 (additionalFile2.xlsx): Metabolites in the constructed draft model *i*PAO1_draft1.

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

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

Additional file 5 (additionalFile5.xlsx). Supplemented metabolites according to previous GC-MS based metabolomics data.

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

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

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

Additional file 9 (additionalFile9.xlsx): Reactions with changed reversibility and directionality during manual curation.

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

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

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

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

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

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

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

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

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

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

641 the information derived from two transposon insertion mutant libraries.

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

643 modifications using lipidomics data as stoichiometric constraints.

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

645 species.

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

647 molecules.

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

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

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

651 RNA-Seq data.

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

653 treatment using RNA-Seq data as flux constraints.

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

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

656 1.

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List of abbreviations

2CS: two-component regulatory system; CAMHB: cation-adjusted Mueller-Hinton broth; COG: clusters of orthologous groups; FBA: flux balance analysis; FDR: false discovery rate; GC-MS: gas chromatography-mass spectrometry; GPL: Glycerolphospholipid; GPR: gene to protein to reaction; GSMM: genome-scale metabolic models; IM: inner membrane; KEGG: Kyoto Encyclopaedia of Genes and Genomes; L-Ara4N: 4-Amino-4-deoxy-L-arabinose; LB: Luria-Bertani; LC-MS: liquid chromatography-mass spectrometry; LPS: lipopolysaccharide; MDR: Multidrug-resistant; OM: outer membrane; PM: Phenotypic Microarray; RPKM: Reads Per Kilobase Million; SBML: Systems Biology Markup Language; SRA: Sequence Read Archive; TCDB: Transporter Classification Database.

668

Competing interests

The authors declare no competing interest for this work.

671

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682

Authors' contributions

684 J.L. and F.S. conceived the project and Y.Z. developed the GSMM and conducted most

685 analysis. T.C. and M.K. validated the model. J.Z., J.Lu and B.S. curated the model. T.V., T.L.

686 and J.S. helped supervise the project. M.H. and M.H.M.M. provided the lipidomics and

687 transcriptomics data, respectively.

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993

Figure legends

995 **Figure 1**. The curated GPL biosynthesis in *i*PAO1. [c], intracellular metabolites; [p], periplasmic metabolites; [e], external metabolites. Blue arrows indicate transport reactions. Full names of metabolite classes are listed in **Additional file 27.**

998 **Figure 2.** LPS biosynthesis and modification in *i*PAO1. (A) VANTED diagram showing the biosynthesis of different LPS molecules. (B) LPS biosynthesis pathway; lipid A and LPS are indicated in the same colour as in (A).

1001 **Figure 3.** Constitutional genes, reactions and metabolites in *i*PAO1. (A) Sources of *i*PAO1 components. (B) Radar map showing the percentages of metabolites and reactions with valid database identifiers. (C) The COG functional classification of the involved genes in iMO1056, Opt208964 and *i*PAO1. Percentages given in the middle indicate the coverages of COG groups. The proportions of the curated reactions (D), reaction-to-gene ratio (E) and predicted

1006 subcellular localisations of the involved proteins (F) are shown for each pathway or COG group.

1007 In panel D, red bars indicate the curated reactions; whereas blue bars indicate the reactions

1008 from previous model. In panel D and E, pathways with the highest curation proportion or

1009 reaction-to-gene ratio are highlighted in red.

1010 **Figure 4.** Comparison of the BIOLOG result (left columns) and model prediction (right

1011 columns). Blue indicates growth; whereas yellow indicates no growth.

1012 **Figure 5.** Simulation of the impact of lipid A modifications on bacterial growth, metabolism

1013 and OM physiochemical properties. The significant correlation ($P<0.05$) of paired items is

1014 indicated in red.

1015 **Figure 6.** Polymyxin B induced metabolic perturbations. The distributions of metabolic fluxes

1016 and metabolite turnover rates are shown in subgraphs with red indicating control and blue

1017 indicating polymyxin B treatment.

1018 **Figure S1**. Sensitivity analysis of the mean metabolic fluxes (A) and metabolite turnover rates

1019 to the variation of nutrient uptake upper bounds. Red indicates the control and blue indicates

1020 polymyxin B treatment.

1021

1022

1023 **Tables and their legends**

1024 **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 |

1025   [a] Pathway information is not available in iMO1056 from the Model SEED database.

1026

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

1028

1029

Table 3. Specific growth rate, significantly altered major exchange fluxes (>1 mmol·gDW^{-1}·h^{-1}), respiration quotient and the fluxes through F_0F_1-ATPase calculated using the RNA-Seq data [51] as flux constraints.

Exchange flux (mmol·gDW^{-1}·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·gDW^{-1}·h^{-1})	-188.6±52.4	-167.6±48.4	29.62	0.00

[a] FDR was calculated using the Benjamini-Hochberg method [105].

Figure 1

LPS

A-band

B-band

Uncapped

FA[e]

Outer membrane

FA[p]

FA[p]

CLPN[p] FA[p]

FA[p]

FA[p]

1AG3P[p]

1AGPC[p]

PA[p]

1AGPG[p]

1AGPE[p]

FA[p] PA[p]

PC[p] FA[p]

PG[p] FA[p]

PE[p] FA[p]

2AG3P[p]

12DGR[p]

2AGPC[p] PGP[p]

2AGPG[p]

2AGPE[p]

Inner membrane

FA[c]

AcCoA[c] ⤙∙∙∙∙ FACoA[c] ⟶ FA[c] ⟵ 2AG3P[c]

12DGR[c]

2AGPC[c]

2AGPG[c]

2AGPE[c]

FA[c]

FA[c]

FA[c]

1AG3P[c] ⟵ FAP[c]

PC[c]

PG[c]

PE[c]

AcACP[c] ⤙∙∙∙∙ FAACP[c]

PA[c]

APG[c]

PGP[c] APG[c] PS[c]

APG[c]

CDP-DAG[c]

Figure 2

A

- Lipid A
- Lipid A + core (glycoform 1)
- Lipid A + core (glycoform 2)
- LPS (common antigen)
- LPS (O5-specific antigen)
- LPS (uncapped)

B

- 144 LPS (common antigen)
- 144 LPS (O5-specific antigen)
- 144 LPS (uncapped)

Acylation (PagP)
Deacylation (PagL)

Outer Membrane

Common antigen (A-band)
O5-specific antigen (B-band)

36 lipid A + core (glycoform 1)

36 lipid A + core (glycoform 2)

ArnT
L-Ara4N
L-Ara4N
ArnT

Inner Membrane

MsbA
MsbA

9 types of lipid A (addition of secondary C12 or 2-OH-C12)

Outer core (glycoform 2)

Outer core (glycoform 1)

Figure 3A-C

Figure 4

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

Figure 6

Figure S1

Additional file 1

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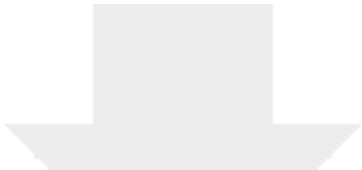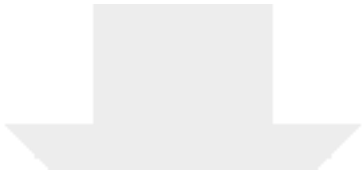additionalFile1.docx

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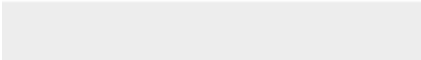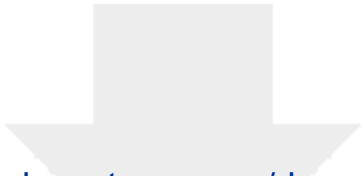**Supplementary Material**

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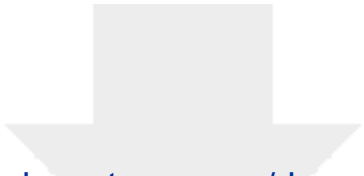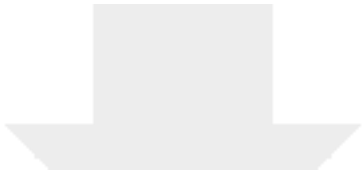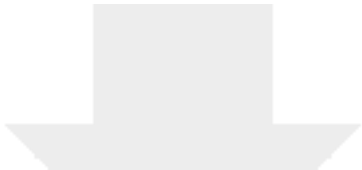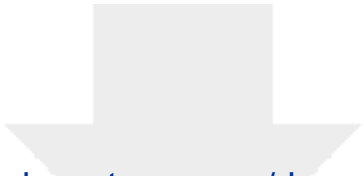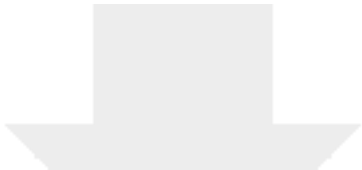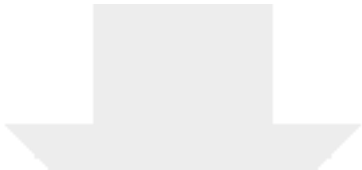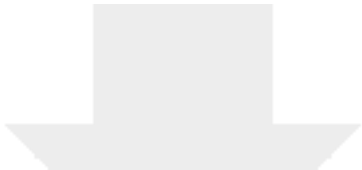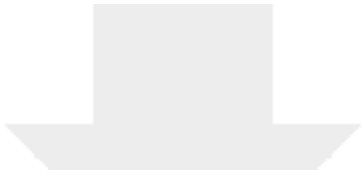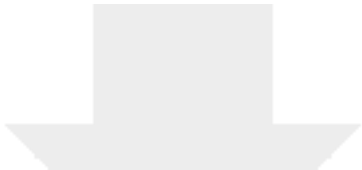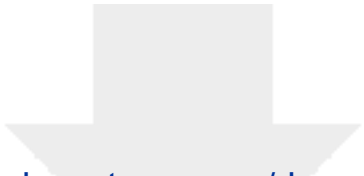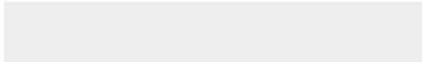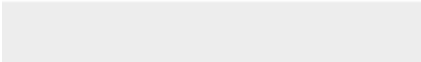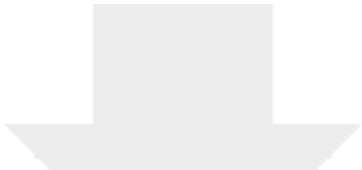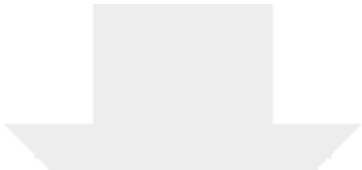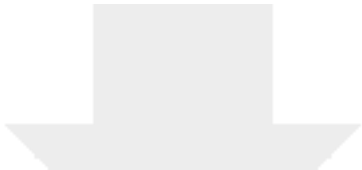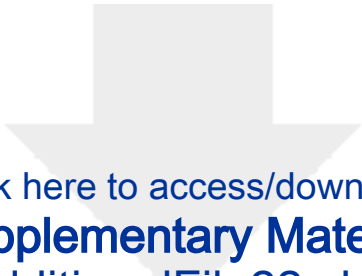**Supplementary Material**
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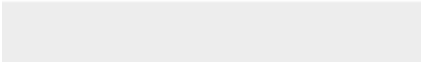**Supplementary Material**
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Additional file 7

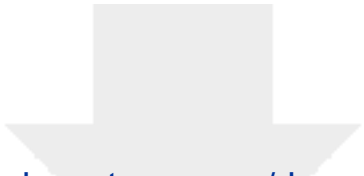Click here to access/download
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Additional file 13

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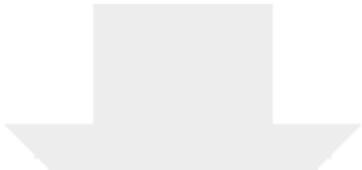**Supplementary Material**
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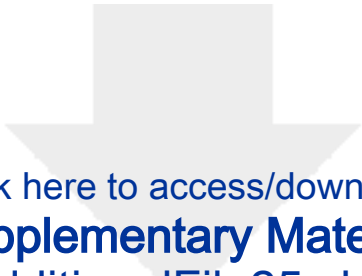Click here to access/download
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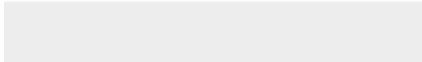**Supplementary Material**
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Additional file 23

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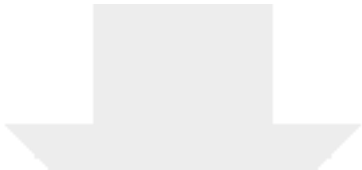**Supplementary Material**
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Additional file 24

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Additional file 25

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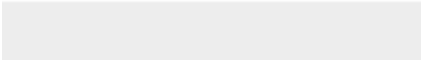
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MONASH University

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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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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