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## **Integrative analysis of fitness and metabolic effects of plasmids in *Pseudomonas aeruginosa* PAO1**

San Millan, Alvaro ; Toll-Riera, Macarena ; Qi, Qin ; Betts, Alex ; Hopkinson, Richard J ; McCullagh, James ; MacLean, R Craig

**Abstract:** Horizontal gene transfer (HGT) mediated by the spread of plasmids fuels evolution in prokaryotes. Although plasmids provide bacteria with new adaptive genes, they also produce physiological alterations that often translate into a reduction in bacterial fitness. The fitness costs associated with plasmids represent an important limit to plasmid maintenance in bacterial communities, but their molecular origins remain largely unknown. In this work, we combine phenomics, transcriptomics and metabolomics to study the fitness effects produced by a collection of diverse plasmids in the opportunistic pathogen *Pseudomonas aeruginosa* PAO1. Using this approach, we scan the physiological changes imposed by plasmids and test the generality of some main mechanisms that have been proposed to explain the cost of HGT, including increased biosynthetic burden, reduced translational efficiency, and impaired chromosomal replication. Our results suggest that the fitness effects of plasmids have a complex origin, since none of these mechanisms could individually provide a general explanation for the cost of plasmid carriage. Interestingly, our results also showed that plasmids alter the expression of a common set of metabolic genes in PAO1, and produce convergent changes in host cell metabolism. These surprising results suggest that there is a common metabolic response to plasmids in *P. aeruginosa* PAO1.

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1 **Integrative analysis of fitness and metabolic effects of plasmids in**  
2 ***Pseudomonas aeruginosa* PAO1**

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25 **Conflict of interest**

26 There are no competing financial interests in relation to the work described.

27 **Running title**

28 The effects of plasmids in *Pseudomonas aeruginosa*

29

30 **Abstract**

31 Horizontal gene transfer (HGT) mediated by the spread of plasmids fuels evolution in  
32 prokaryotes. Although plasmids provide bacteria with new adaptive genes, they also  
33 produce physiological alterations that often translate into a reduction in bacterial  
34 fitness. The fitness costs associated with plasmids represent an important limit to  
35 plasmid maintenance in bacterial communities, but their molecular origins remain  
36 largely unknown. In this work we combine phenomics, transcriptomics and  
37 metabolomics to study the fitness effects produced by a collection of diverse  
38 plasmids in the opportunistic pathogen *P. aeruginosa* PAO1. Using this approach, we  
39 scan the physiological changes imposed by plasmids and test the generality of some  
40 main mechanisms that have been proposed to explain the cost of HGT, including  
41 increased biosynthetic burden, reduced translational efficiency, and impaired  
42 chromosomal replication. Our results suggest that the fitness effects of plasmids  
43 have a complex origin, since none of these mechanisms could individually provide a  
44 general explanation for the cost of plasmid carriage. Interestingly, our results also  
45 showed that plasmids alter the expression of a common set of metabolic genes in  
46 PAO1, and produce convergent changes in host cell metabolism. These surprising  
47 results suggest that there is a common metabolic response to plasmids in *P.*  
48 *aeruginosa* PAO1.

49 **Introduction**

50 The movement of genetic information across different bacterial clones is known as  
51 horizontal gene transfer (HGT). HGT acts as a major evolutionary force by enabling

52 bacteria to acquire new genes (Gogarten and Townsend 2005, Wiedenbeck and  
53 Cohan 2011). Plasmids play a crucial role in HGT because they can actively transfer  
54 between bacteria through conjugation, spreading accessory genes (Smalla et al 2015,  
55 Smillie et al 2010, Summers 1996). The most recent and concerning example of how  
56 plasmids contribute to bacterial adaptation is the global dissemination of plasmid-  
57 mediated antibiotic resistance among clinical pathogens, which represents a main  
58 public health emergency worldwide (Carattoli 2013, O'Neill 2016). Despite the  
59 adaptive advantages that plasmids may confer to a recipient bacterium, they also  
60 produce a fitness cost in their host, generating selection against plasmid-carrying  
61 strains (Baltrus 2013, San Millan and MacLean 2017, Vogwill and MacLean 2015).  
62 Although this cost can be ameliorated through compensatory mutations, it represents  
63 one of the main limits for the establishment of the plasmid in a new bacterial  
64 population (Harrison et al 2015, Loftie-Eaton et al 2016, Pena-Miller et al 2015, Porse  
65 et al 2016, San Millan et al 2014a, San Millan et al 2014b, Yano et al 2016).  
66 Therefore, understanding the origins of these costs is key to predicting and,  
67 eventually, preventing the evolution of plasmid-mediated antibiotic resistance.

68 There are multiple potential sources of costs related to HGT, such as the  
69 sequestration of bacterial replication or expression machinery, the biosynthetic cost  
70 associated with the new plasmid DNA and proteins, and deleterious interactions  
71 between the newly acquired genes and bacterial regulatory networks (Bragg and  
72 Wagner 2009, Cohen et al 2011, Jain et al 1999, Lamberte et al 2017, Lynch and  
73 Marinov 2015, Pál et al 2005, Plotkin and Kudla 2011, Porse et al 2018, Sorek et al  
74 2007). Although it is clear in many cases that these factors will necessarily translate  
75 into an energetic cost for the cell, it is unclear to what extent these translate into  
76 fitness costs. One of the most advocated theories is that a central source of fitness  
77 cost associated with HGT comes from the translation of proteins from newly acquired  
78 genes (Baltrus 2013, Medrano-Soto et al 2004, Tuller et al 2011). This cost is thought

79 to originate from the imbalance between codon usage by the foreign genes and the  
80 available tRNA pool in the recipient bacterium (Plotkin and Kudla 2011), leading to  
81 reduced translation efficiency in the cell (Cortazzo et al 2002, Drummond and Wilke  
82 2008, Komar et al 1999, Kudla et al 2009). Concurring with this idea, computational  
83 studies have shown that codon usage compatibility between newly acquired DNA  
84 and the host chromosome favours HGT (Medrano-Soto et al 2004, Tuller et al 2011).  
85 However, out of the few experimental studies available on the molecular basis of  
86 fitness effects of plasmids (Harrison et al 2015, Loftie-Eaton et al 2017, Porse et al  
87 2016, San Millan et al 2015b, Yano et al 2016), only one reported that translational  
88 inefficiency is responsible for the cost of HGT (Harrison et al 2015). Instead, most  
89 experimental studies have found that plasmids impose a fitness cost by interfering  
90 with chromosomal replication, leading to the induction of the SOS response (Ingmer  
91 et al 2001, Loftie-Eaton et al 2017, San Millan et al 2015b, Shintani et al 2010, Yano  
92 et al 2016).

93 In summary, there is a big gap in our understanding of the origin of the costs of HGT  
94 in general and of plasmids in particular. Here we used a novel integrative approach  
95 to investigate the effects of a diverse collection of plasmids in the opportunistic  
96 pathogen *P. aeruginosa* PAO1. The goal of this comparative approach was to test for  
97 mechanisms that can explain variation in the fitness effects of these plasmids.  
98 Specifically, our aims were: (i) To test some of the main mechanisms that have been  
99 proposed to explain the cost of plasmid acquisition (translation inefficiency, impaired  
100 chromosomal replication and protein biosynthetic costs) and, (ii) To gain a general  
101 understanding on the effects of plasmids in the physiology of *P. aeruginosa* PAO1.  
102 Our results show that plasmids produce a wide range of fitness effects in PAO1.  
103 Although none of the suspected sources of cost could individually explain the fitness  
104 effects of the different plasmids, our transcriptomic and metabolomic results showed  
105 that plasmids tend to alter preferentially the expression of metabolic genes in PAO1,

106 producing a significant and common alteration in the metabolic profiles of the  
107 bacterium.

## 108 **Material and Methods**

109 An extended version of the Material and Methods section, including a comprehensive  
110 description of every method used in this work, as well as a detailed explanation of the  
111 transcriptomic and metabolomic techniques and all the computational analyses, is  
112 provided in the Supplementary Information.

### 113 *Bacterial strains, plasmids and culture conditions*

114 The plasmids used in this study are described in Table 1. *P. aeruginosa* PAO1 was  
115 used as recipient strain. PAO1 WT*plex:lux* (Torres-Barcelo et al 2015) and PAO1  
116 *PlasB::lux* (Popat et al 2012) were also used as recipient strains to investigate the  
117 effects of plasmids on the SOS response and Quorum Sensing system, respectively.  
118 Bacterial strains were cultured in LB broth at 37°C with continuous shaking (225 rpm)  
119 and on LB agar plates at 37°C (Fisher Scientific, NJ, USA). Strains were transformed  
120 by electroporation with the different plasmids as previously described (Choi and  
121 Schweizer 2006), using a Gene Pulser apparatus (Bio-Rad). Transformants were  
122 selected on LB agar plates containing antibiotics as previously described (San Millan  
123 et al 2014a, San Millan et al 2014b).

### 124 *Competitive fitness assays*

125 The fitness of each plasmid-carrying PAO1 clone was determined relative to a PAO1-  
126 GFP plasmid-free control strain. The GFP label did not produce a significant  
127 reduction in fitness in PAO1 (San Millan et al 2014b). Competition experiments were  
128 performed as previously described (San Millan et al 2014b). The fitness of the strain  
129 carrying the plasmid relative to the PAO1-GFP strain was determined using the  
130 formula (Lenski et al 1991):

$$W_{p+} = \ln(N_{final,p+}/N_{initial,p+}) / \ln(N_{final,p-}/N_{initial,p-})$$

131 where  $W_{p+}$  is the relative fitness of the plasmid-bearing clone,  $N_{initial,p+}$  and  $N_{final,p+}$  are  
132 the numbers of cells of the plasmid-carrying clone before and after the competition,  
133 and  $N_{initial,p-}$  and  $N_{final,p-}$  are the numbers of PAO1-GFP cells before and after the  
134 competition. As a control group, PAO1 and PAO1-GFP were competed in every  
135 experiment. We performed six biological replicates for each competition.

### 136 *Statistical analyses*

137 All statistical analyses and production of graphics were performed using R (R Core  
138 Team, 2014).

### 139 *Data availability*

140 The reads generated in this study have been deposited in the European Nucleotide  
141 Archive database with the accession number PRJEB24427  
142 (<http://www.ebi.ac.uk/ena/data/view/PRJEB24427>).

## 143 **Results**

### 144 *Fitness effects of plasmids in different environments*

145 We measured the fitness effects of a collection of six antibiotic resistance plasmids  
146 with different replication types, from different origins (clinical and environmental) and  
147 with different sizes, in *P. aeruginosa* PAO1 (Table 1). First, we performed  
148 competition experiments in LB broth to determine the relative fitness of each of the  
149 plasmid-carrying strains compared to plasmid-free PAO1 (Figure 1A). The plasmids  
150 produced a variety of fitness effects, from a significant advantage to different degrees  
151 of cost. These results matched those of previous studies investigating the fitness  
152 effect of these plasmids in PAO1 (San Millan et al 2014a, San Millan et al 2014b).

153 One common unanswered question in the field is how much the environment impacts  
154 the fitness effects of plasmids (genotype-by-environment interactions). To study the  
155 fitness effects of the plasmids in a range of different environments, we used Biolog  
156 EcoPlates, which provide 31 different carbon substrates. We observed measurable  
157 growth in 10 of the carbon sources, and we determined the growth rate of PAO1 and  
158 each plasmid-carrying strain in these environments as well as in LB. In Figure 1B we  
159 compare the growth rates of plasmid-free and plasmid-carrying strains in the different  
160 environments. To obtain a general idea of the fitness effects of the plasmids across  
161 environments, we regressed the growth rate of the plasmid-bearing clone against the  
162 growth rate of PAO1 in the different environments. The slope of this regression  
163 measures the deviation in fitness caused by plasmid acquisition and, as such, this  
164 metric provides a measure of the fitness effect of the plasmid across environments.  
165 Interestingly, the slope of this regression correlated very strongly with competitive  
166 fitness in LB, implying that plasmids tend to entail similar costs across different  
167 nutrient environments (Pearson's test,  $r = 0.982$ ,  $P < 0.001$ ,  $t = 10.36$ ,  $df = 4$ ). Moreover,  
168 we observed a positive correlation between the initial growth rate of the plasmid-free  
169 strain and the reduction in growth rate imposed by the plasmids across environments  
170 (Pearson's test,  $r = 0.296$ ,  $P = 0.016$ ,  $t = 2.47$ ,  $df = 64$ ), suggesting that plasmids  
171 produced a larger cost under conditions promoting fast bacterial growth.

#### 172 *Plasmid genes are highly expressed*

173 To better understand the origin of the fitness effects of plasmids, we performed  
174 transcriptomic analyses of five of the plasmid-carrying (pAMBL1, pAMBL2, pAKD1,  
175 Rms149 and pBS228) and the plasmid-free PAO1 using RNA-Seq (Supplementary  
176 Tables S1 and S2); we have previously used this approach to successfully  
177 characterize the origin of the cost produced by the remaining plasmid, pNUK73 (San  
178 Millan et al 2015b). We analysed the transcription profiles of the five plasmids in  
179 PAO1 (see methods, Figure 2, Supplementary Table S1). In four cases, plasmid



180 genes showed higher levels of expression than the chromosomal genes of their hosts  
181 (correcting for plasmid copy number (San Millan et al 2014a), Kolmogorov-Smirnov  
182 test, two sided,  $P < 0.0005$ ). The only plasmid that did not show increased gene  
183 expression was pAKD1, which is the only conjugative plasmid in the collection.  
184 Conjugative plasmids usually control the expression of the conjugative machinery  
185 tightly, presumably because expression of conjugative genes carries a large cost  
186 (Fernandez-Lopez et al 2014). pAKD1 carries more than 20 genes involved in  
187 conjugation, which were tightly repressed (Supplementary Table S1). To compare  
188 the expression of genes with detectable levels of transcription in pAKD1 and PAO1  
189 chromosome, we removed non-expressed chromosomal and plasmid genes from the  
190 analysis. After this correction, pAKD1 genes also showed a higher level of  
191 expression than those from the chromosome (Kolmogorov-Smirnov test, two sided,  
192  $P < 0.0005$ ).

193 Interestingly, antibiotic resistance genes were among the genes with highest level of  
194 expression in the plasmids (Figure 2, Supplementary Table S1). Most of the plasmid-  
195 carried antibiotic resistance genes were located in integrons, which are genetic  
196 platforms able to capture promoterless genes called cassettes and express them in a  
197 decreasing gradient from a single strong promoter (Escudero et al 2015). Integrons  
198 are highly prevalent on plasmids (mobile integrons), and they are usually associated  
199 with antibiotic resistance genes (Partridge et al 2009). Our results showed that  
200 integrons drive high-level expression of plasmid resistance genes. Despite the high  
201 expression level of plasmid genes, the RNA reads mapping to plasmids only  
202 represented between 1.75% and 2.85% of the total number of reads in the cell (Table  
203 S3), and we did not find a negative correlation between the amount of reads mapping  
204 to plasmids and the relative fitness of the plasmid-carrying PAO1 (Pearson's test,  $r =$   
205  $0.408$ ,  $P = 0.495$ ,  $t = 0.775$ ,  $df = 3$ ). This result suggests that the energetic cost  
206 associated with RNA synthesis alone cannot explain the cost imposed by plasmids.

207 *Plasmids alter the expression of metabolic genes in PAO1*

208 We compared the expression of chromosomal genes in the different plasmid-carrying  
209 strains to that of plasmid-free PAO1. Plasmids altered the expression (over- or  
210 under-expression) of chromosomal genes [hereafter, differentially expressed (DE)  
211 genes]; from 34 genes in PAO1/pAKD1 to 228 in PAO1/pAMBL1 (Figure 3,  
212 Supplementary Table S2, Supplementary Figure S2). Interestingly, we observed that  
213 even though plasmids produced very different effects on the transcription profile of  
214 PAO1, there was a significant subset of genes that were DE in common in the  
215 different plasmid-carrying PAO1 (Supplementary Figure S3). For example, there  
216 were 38 DE genes in common in at least three plasmid-carrying strains  
217 (Supplementary Table S4), while chance alone would predict less than one (Fisher's  
218 test,  $P < 0.0001$ ,  $df = 1$ ). Notably, when we compared these results with our previous  
219 work involving pNUK73, we found that 29 out of those 38 genes were also DE in  
220 PAO1/pNUK73 (Supplementary Table S4) (San Millan et al 2015b).

221 To better understand the effects of plasmids on PAO1, we performed a functional  
222 enrichment analysis of the DE genes in the chromosomes of the plasmid-carrying  
223 clones (Figure 3, Supplementary Table S5). PAO1/pAKD1 did not show an  
224 enrichment of genes DE in any particular category. pAMBL1 and pAMBL2  
225 preferentially altered the expression of metabolic genes in the chromosomes of their  
226 hosts. Rms149 altered the expression of metabolic genes and genes related to  
227 ribosomes and translation. Finally, DE genes in PAO1/pBS2388 were enriched in  
228 protein secretion systems, which are key virulence factors in *P. aeruginosa*. When  
229 we analysed all the DE genes in all the strains combined, there was again an  
230 overrepresentation of genes involved in metabolism. Moreover, the 38 DE genes in  
231 common in at least three plasmid-carrying strains were also enriched in metabolic  
232 functions (Supplementary Table S5). Together, these results suggest two  
233 conclusions: (i) plasmids tend to alter the expression of metabolic genes in the

234 chromosome of PAO1 and, (ii) there is a group of genes, enriched in metabolic  
235 functions, which are DE in response to the presence of different plasmids.

236 To dissect the results obtained in the functional enrichment analysis, we scanned the  
237 profiles of expression of the metabolic genes that were DE in the plasmid-carrying  
238 strains (Bartell et al 2017). This analysis revealed interesting patterns: all plasmids,  
239 apart from pAMBL1, which produces the largest fitness benefit, were associated with  
240 an over-expression of genes involved in glutamine synthesis (PA5506-PA5509)  
241 (Ladner et al 2012). Conversely, in PAO1/pAMBL1 there was a group of over-  
242 expressed metabolic genes, which were either non-affected or under-expressed in  
243 the presence of the remaining plasmids. These genes were responsible for valine,  
244 leucine and isoleucine degradation (PA2012-PA2014), tyrosine and phenylalanine  
245 metabolism (PA2007-PA2009), glycine, serine and threonine metabolism (PA2442-  
246 PA2446), benzoate degradation (PA1999-PA2000) and fatty acid metabolism  
247 (PA2001 and PA0744). Interestingly, PA4918, which encodes a putative  
248 nicotinamidase (Okon et al 2017), was the only gene whose differential expression  
249 (over-expression) was specifically associated with the presence of costly plasmids.

## 250 *Analysing potential sources of plasmid cost*

### 251 *1. Impaired chromosomal replication is not a general effect of plasmids in PAO1*

252 Previous experimental studies have shown that plasmids can interfere with  
253 chromosomal replication, which may entail a large fitness cost (Ingmer et al 2001,  
254 San Millan et al 2015b, Shintani et al 2010, Yano et al 2016). One symptom of stalled  
255 chromosomal replication is increased expression of the SOS pathway in response to  
256 single-stranded DNA. To test for plasmid-mediated inhibition of chromosomal  
257 replication, we looked for an increase in the expression of SOS pathway genes. Our  
258 transcriptomic data did not show an overexpression of SOS-mediated genes in any  
259 of the five plasmid-carrying bacteria (Supplementary Table S2). To confirm these

260 results we introduced the plasmids, including pNUK73 (which activates the SOS  
261 response (San Millan et al 2015b)), into a PAO1 reporter strain carrying a luciferase  
262 operon under the control of an SOS inducible promoter (Torres-Barcelo et al 2015).  
263 We could not introduce plasmid pBS228 into the reporter strain, because this plasmid  
264 has very poor electroporation efficiency due to its large size. The production of  
265 luminescence was measured during the exponential phase of the growth curves of  
266 the different strains. We included a positive control for which we added sub-inhibitory  
267 concentrations of the SOS-inducing antibiotic ciprofloxacin to the plasmid-free  
268 reporter strain. Our results revealed significant differences in SOS activation among  
269 strains (Figure 4, analysis of variance:  $P < 0.0001$ ,  $F = 74.73$ ,  $df = 6, 49$ ). As expected,  
270 ciprofloxacin and pNUK73 produced an increase in luminescence production  
271 compared to the plasmid-free PAO1 (Tukey's post-hoc test,  $P < 0.0001$ ). The  
272 remaining plasmids, however, produced no significant changes (Tukey's post-hoc  
273 test,  $P > 0.05$ ). These results suggest that impaired chromosomal replication is not a  
274 general effect of plasmids in PAO1.

## 275 *2. Translation inefficiency as a source of plasmid cost*

276 Codon usage imbalance between horizontally acquired genes and the host  
277 chromosomal genes can lead to inefficient ribosome allocation and ribosome pausing  
278 (Plotkin and Kudla 2011). To investigate if translation inefficiency was responsible for  
279 the fitness costs associated with the plasmids under study, we scanned the  
280 expression profiles of chromosomal genes of plasmid-carrying strains and looked for  
281 signatures of increased translational demand. Only PAO1/Rms149 showed an  
282 overexpression of genes involved in translation and ribosomes compared to plasmid-  
283 free PAO1 (Figure 3, Supplementary Table S5), which could be the consequence of  
284 an increase in translational demand (Harrison et al 2015). Interestingly, Rms149  
285 produced the highest cost out of the 5 plasmids analysed by RNA-Seq in this study.

286 However, the remaining plasmids showed no significant effect on the expression  
287 levels of translation-associated genes.

288 To obtain an estimation of the codon usage compatibility between plasmids and  
289 PAO1 genes, we calculated the distribution of codon adaptation index (CAI) for  
290 plasmid genes (Supplementary Figure S4). The CAI quantifies the similarity between  
291 the synonymous codon usage of a given gene and that of a subset of highly  
292 expressed genes in *P. aeruginosa* PAO1 (see methods). CAI therefore provides a  
293 proxy for the transcriptional burden imposed by the gene; genes with lower CAI carry  
294 rare codons that may stall ribosomes. However, it is important to take into account  
295 the expression levels of plasmid genes to obtain a realistic idea of the translational  
296 burden imposed by plasmids. Therefore, we weighted the CAI value of each plasmid  
297 gene by its expression level. We did not find a positive correlation between the  
298 median corrected CAI values of plasmid genes and the relative fitness of plasmid-  
299 carrying strains (Pearson's test,  $r = -0.150$ ,  $P = 0.809$ ,  $t = -0.262$ ,  $df = 3$ , see  
300 Supplementary Tables S6 and S7 for an analysis of CAI and expression levels of  
301 plasmid genes). Taken together, these results indicate that translational inefficiency  
302 produced by plasmid transcripts is probably not a general cause of fitness cost in *P.*  
303 *aeruginosa* PAO1.

### 304 *3. Biosynthetic costs of plasmid-encoded proteins*

305 Most of the bioenergetic cost associated with expressing genes comes from protein  
306 synthesis (Bonomo and Gill 2005, Lynch and Marinov 2015). Therefore, the cost  
307 associated with synthesizing newly acquired proteins may be an important limit to  
308 HGT. To determine the burden associated with synthesizing plasmid-encoded  
309 proteins, we calculated the biosynthetic cost of both plasmid and chromosomal  
310 proteins for each plasmid-carrying strain (correcting for the gene expression levels,  
311 see methods and Supplementary Table S8). Our results showed that the biosynthetic

312 cost of plasmid-encoded proteins ranged from 2.46% to 3.72% of the total costs  
313 associated with protein synthesis of the cells (Supplementary Table S9). However,  
314 the relative fitness of the plasmid-carrying clones did not negatively correlate with the  
315 relative cost of plasmid-encoded proteins (Pearson's test,  $r = 0.225$ ,  $P = 0.716$ ,  $t = 0.40$ ,  
316  $df = 3$ ) nor with the biosynthetic cost associated with plasmid-induced change in  
317 chromosomal gene expression (Pearson's test,  $r = 0.858$ ,  $P = 0.063$ ,  $t = 2.89$ ,  $df = 3$ ).

### 318 *Metabolic analysis of plasmid-carrying PAO1*

319 Transcriptomic data revealed that plasmids preferentially altered the expression of  
320 metabolic genes in PAO1. We applied mass spectrometry to investigate how these  
321 alterations impacted the metabolic profiles of plasmid-carrying clones. We performed  
322 metabolic profiling of parental PAO1 and all six plasmid-carrying PAO1, including  
323 PAO1/pNUK73 (since we did not investigate the metabolic profile of this strain in our  
324 previous work (San Millan et al 2015b)). We detected more than 5000 compounds  
325 across the samples and were able to identify 97 based on matching criteria with  
326 authentic standards. To understand the metabolic effects caused by the plasmids, we  
327 compared the abundance of each compound in the plasmid-carrying strains with its  
328 abundance in the plasmid-free PAO1 strains (Supplementary Table 10). The different  
329 plasmids impacted the concentration of multiple compounds and, interestingly, the  
330 changes in the metabolic profiles produced by the different plasmids showed a high  
331 degree of parallelism, both in the compounds affected and in the direction of the  
332 changes (Supplementary Figure S5 and Figure 5). The different plasmids affected  
333 the abundance of a common subset of compounds. For example, out of the total  
334 compounds detected, 462 showed a significant change in abundance in common in  
335 at least four of the 6 plasmid-carrying clones, when chance alone predicted  
336 approximately 9 (Chi-squared test,  $P < 0.0001$ ,  $\chi^2 = 452.43$ ,  $df = 1$ , Supplementary  
337 Figure S5, Supplementary Table S11). For the identified metabolites, 11 were  
338 significantly altered in at least four plasmid-carrying clones in common, while chance

339 predicted less than one (Fisher's test,  $P= 0.005$ ,  $df= 1$ , Figure 5, Supplementary  
340 Table S11).

341 Although analysis of the metabolomics data is complicated by the low number of  
342 identified metabolites, it is possible to tentatively recognise some general trends.  
343 Firstly, it is notable that many of the identified nucleotides have altered abundance in  
344 the plasmid-carrying clones relative to PAO1. While this may suggest dysregulated  
345 nucleotide biosynthesis and/or metabolism in the clones, it is also likely that  
346 expression of plasmid genes increases the demand for nucleotides, potentially  
347 leading to a re-buffering of nucleotide concentrations. It is interesting that the RNA  
348 nucleotides cytidine monophosphate and uridine monophosphate appear down-  
349 regulated in the plasmid-carrying clones, whereas the equivalent deoxynucleotides  
350 are either up-regulated or unchanged. This observation may imply an increase in  
351 RNA biosynthesis in the plasmid-carrying clones. While plasmid-induced protein  
352 biosynthesis does not seem to cause a significant fitness cost (see above), it is  
353 possible it induces toxic knock-on effects. For example, the PAO1/pAMBL1 clone  
354 may overcome the cost of increased protein biosynthesis by over-expressing genes  
355 involved in amino acid metabolism (as we observed), thereby removing potentially  
356 toxic levels of amino acids released after protein degradation. It should also be noted  
357 that levels of pyrimidine nucleotides may be affected by the common over-expression  
358 of glutamine biosynthesis genes (see above); glutamine is a co-substrate of cytidine  
359 synthetase, which interconverts uridine and cytidine triphosphates.

## 360 **Discussion**

361 In this work we applied, for the first time, an integrative approach combining  
362 phenotypic characterization with transcriptomic and metabolomic analyses to  
363 decipher the origin of the fitness effects produced by plasmids. Our results suggest  
364 that the fitness effects produced by plasmids are multifactorial, as none of the

365 different causes analysed could explain the fitness effects individually. However,  
366 several lines of evidence indicate that, as expected, undomesticated plasmids  
367 produce a larger cost in *P. aeruginosa* PAO1. For example, using GC content as a  
368 simple proxy for phylogenetic proximity, we found a clear correlation between  
369 plasmid-chromosome differences in GC content and the cost imposed by the plasmid  
370 (Supplementary Figure S6, Pearson's test,  $r= 0.969$ ,  $P= 0.001$ ,  $t= 7.87$ ,  $df= 4$ ).  
371 Moreover, the plasmid producing the least fitness alteration in PAO1, pAKD1, was  
372 also the plasmid producing the least alteration at molecular and physiological levels  
373 (most "domesticated"): namely low expression levels of plasmid genes, low protein  
374 biosynthetic cost, small alteration of transcription and metabolic profiles of the host,  
375 and a GC and codon usage similar to those in PAO1. These results are in line with  
376 previous research showing that high-level expression or codon usage incompatibility  
377 hampers HGT (Medrano-Soto et al 2004, Park and Zhang 2012, Sorek et al 2007,  
378 Tuller et al 2011), while mechanisms controlling expression of plasmid genes, such  
379 as H-NS proteins, facilitate HGT (Ali et al 2014, Doyle et al 2007, Takahashi et al  
380 2015). Broad host range IncP-1 plasmids, such as pAKD1, possess control circuits  
381 that minimize the expression of genes coding for propagation functions after plasmid  
382 establishment (Thomas 2000), and they usually produce low fitness costs across  
383 different bacterial hosts (De Gelder et al 2007).

384 The most novel finding of this study is that different plasmids alter the expression of a  
385 common set of metabolic genes in PAO1, and lead to convergent changes in host  
386 cell metabolism (Figure 3 and 5 and Supplementary Figure 5). Transcriptomic results  
387 revealed interesting differences between the expression profiles of metabolic genes  
388 of plasmid-carrying strains. All plasmids, except pAMBL1 (which produces the  
389 greatest fitness advantage), were associated with an over-expression of genes  
390 involved in glutamine synthesis. Notably, glutamine is thought to play a key role in  
391 the nitrogen control of *P. aeruginosa* (Janssen et al 1981). On the other hand, the



392 beneficial pAMBL1 showed a unique transcription profile, with over-expression of  
393 genes involved amino acid and fatty acid metabolism. A clear correlation is also  
394 observed between fitness cost and expression of an as yet uncharacterised  
395 nicotinamidase, implying its enzyme activity (i.e. conversion of nicotinamide to niacin  
396 and ammonia) could induce a fitness cost. It is tempting to speculate on how these  
397 changes in expression of chromosomal genes may be responsible for the fitness  
398 effects of plasmids, however we prefer to remain cautious in our interpretations,  
399 because although these changes could be the cause of altered fitness, they could  
400 also be the consequence of those fitness effects. Future work will be needed to  
401 investigate these possibilities.

402 Our transcriptomic data are supported by metabolomic analyses on the PAO1 clones,  
403 which reveal striking changes in metabolite concentrations, particularly in the levels  
404 of nucleotides. While we are cautious not to over-interpret our results, they  
405 tantalizingly suggest that there may be a common metabolic response to plasmids in  
406 *P. aeruginosa* PAO1. It is important to highlight that this metabolic response cannot  
407 explain variation in fitness across the plasmid-carrying PAO1, because it is produced  
408 by both costly and beneficial plasmids. Therefore, the link between these common  
409 metabolic effects and fitness is not completely clear. One possible explanation is that  
410 bacteria respond to the presence of plasmids by altering metabolism to compensate  
411 for the new physiological requirements. In some cases, such as pAKD1 and pAMBL1,  
412 these alterations could be sufficient to eliminate the burden of plasmid carriage, and  
413 there is no net cost associated with plasmid acquisition. In other cases such as  
414 pBS228 and Rms149, these metabolic changes could be insufficient to compensate  
415 for the cost of plasmid carriage, and plasmid carriage reduces fitness. It is not  
416 obvious which genes are responsible for regulating this response, as either plasmid  
417 genes or chromosomal genes could theoretically control it. However, given the

418 diversity of plasmids employed in this study, the most parsimonious explanation  
419 would be that chromosomal genes regulate this response.

420 One limitation of this work is the fact that although the plasmid collection used here  
421 covers a wide range of plasmid families and sizes, we did not include a megaplasmid  
422 (>100 kb), which are relatively common in *P. aeruginosa*. Another limitation is that in  
423 this work we used a quorum sensing (QS) deficient PAO1 strain as model system  
424 (see methods). However, we argue that since the plasmids in our collection do not  
425 disturb QS (see control experiment in Supplementary Figure 7), this fact should not  
426 affect the interpretation of our results.

427 In conclusion, our results reveal new insights on the effects of plasmids in bacteria  
428 and on the routes towards plasmid domestication. Crucially, this work paves the way  
429 for new research on the molecular and evolutionary consequences of plasmid  
430 carriage, which may help to discover new targets in the fight against the  
431 dissemination of plasmid-mediated antibiotic resistance.

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#### 451 **Conflict of interest**

452 There are no competing financial interests in relation to the work described.

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650

651 **Figure legends**

652 **Figure 1.** Effects of different plasmids on the fitness of *P. aeruginosa* PAO1.

653 Plasmids produce different fitness effects in PAO1, and these effects tend to remain  
654 constant across nutrient environments. (A) Relative fitnesses of plasmid-bearing  
655 PAO1 compared to plasmid-free parental strain in LB. Error bars represent the  
656 standard error of the mean (SEM, n= 5). (B) Comparison of the growth rates of  
657 plasmid-free and plasmid-carrying PAO1 in different carbon sources provided by  
658 Biolog EcoPlates and in LB broth. The results are the average of six biological  
659 replicates of PAO1 and three biological replicates of each of the six plasmid-carrying  
660 PAO1 clones. Each panel shows the results for a different plasmid. The colour points  
661 indicate growth rates comparisons in the different carbon sources (see legend). The  
662 points above the black diagonal line indicate environments where the plasmid  
663 produces a benefit, while the points below the line indicate environments where  
664 plasmids produce a cost. The dashed line represents the linear regression model of  
665 the growth rates of the plasmid-bearing clone against the growth rates of PAO1 over  
666 all the environments.

667 **Figure 2.** Plasmid genes are highly expressed.

668 Plasmid-encoded genes showed a higher level of expression than chromosome-  
669 encoded genes. Violin plot representing the distribution of transcript abundances (in  
670  $\log_2$  of transcripts per million, TPM) of chromosome (red) and plasmid (blue) genes in  
671 each plasmid-carrying PAO1. The grey dot represents the median values of the  
672 distributions. For clarity, we removed non-expressed chromosomal and plasmid  
673 genes in this figure (see main text for statistical analyses including and excluding  
674 non-expressed genes and correcting for plasmid copy numbers). Plasmid-encoded  
675 antibiotic resistance genes are depicted in the figure according to their level of  
676 expression. Note that antibiotic resistance genes are, in general, among the plasmid  
677 genes with highest level of expression.



678 **Figure 3.** Differentially expressed genes in the chromosome of plasmid-carrying  
679 PAO1.

680 Differential expression of chromosomal genes in PAO1 carrying different plasmids,  
681 compared to plasmid-free PAO1. In this heatmap we present the genes that are  
682 significantly differentially expressed (DE) in at least one of the plasmid-carrying  
683 clones compared to plasmid-free PAO1. The genes significantly DE are indicated by  
684 the green bars to the right of the figure. Red bars in the heatmap indicate those  
685 genes that are over-expressed and blue bars those under-expressed. The intensity of  
686 the colour is proportional to the level of differential expression, as indicated in the  
687 colour legend ( $\log_2$  fold-change). Genes up or downregulated by more than four fold  
688 are coloured at the same (maximum) intensity. To the left of the figure we indicated  
689 functions significantly enriched in the different clusters of genes formed in the  
690 heatmap. Note that “metabolism” is the function most commonly enriched, indicating  
691 that plasmids tend to alter the expression of metabolic genes in PAO1.

692 **Figure 4.** Plasmids do not mediate activation of SOS response.

693 Not all plasmids induce the SOS response in PAO1. The figure represents the area  
694 under the curve of luminescence production over  $OD_{600}$  [AUC (lux/ $OD_{600}$ )] during the  
695 exponential phase of the growth curves of PAO1 WTp/*ex:luc* reporter strain, which  
696 encodes the luciferase operon under the control of an SOS inducible promoter  
697 (PAO1 in the figure). We also present the AUC (lux/ $OD_{600}$ ) of the different plasmid-  
698 carrying WTp/*ex:luc*, and a control with the plasmid-free WTp/*ex:luc* growing in the  
699 presence of a sub-inhibitory concentration of the SOS-inducing antibiotic  
700 ciprofloxacin (45  $\mu$ g/L). The bars indicate the average of 8 biological replicates and  
701 the error bars indicate the standard error. Asterisks indicate significant differences in  
702 SOS activation (Tukey's post-hoc test,  $P < 0.05$ ). As expected, plasmid pNUK73 and  
703 the presence of ciprofloxacin induced the SOS response. However, none of the  
704 remaining plasmids produced the activation of the stress response.

705 **Figure 5.** Change in abundance of identified metabolites.

706 Different plasmids produce similar changes in the abundance of the identified  
707 metabolites in the host bacterium *P. aeruginosa* PAO1. Heatmap representing those  
708 identified metabolites with significant differences in abundance in at least one of the  
709 plasmid-carrying PAO1 compared to plasmid-free PAO1 (indicated by the green  
710 squares to the right of the figure). Metabolites with higher abundance are  
711 represented in red, and metabolites with lower abundance are represented in blue.  
712 The intensity of the colour is proportional to the differences in metabolite  
713 concentration, as indicated in the colour legend (log<sub>2</sub> fold-change). We performed 5  
714 replicates per strain for the metabolomic analysis.

715

716

717 **Table legends**

718 Table 1. Plasmids used in this study

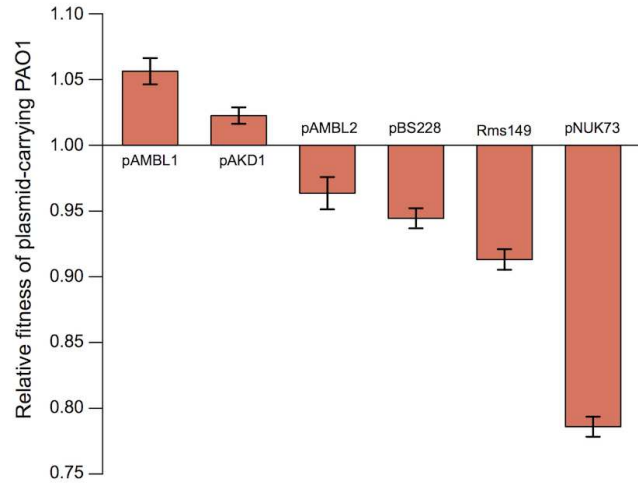
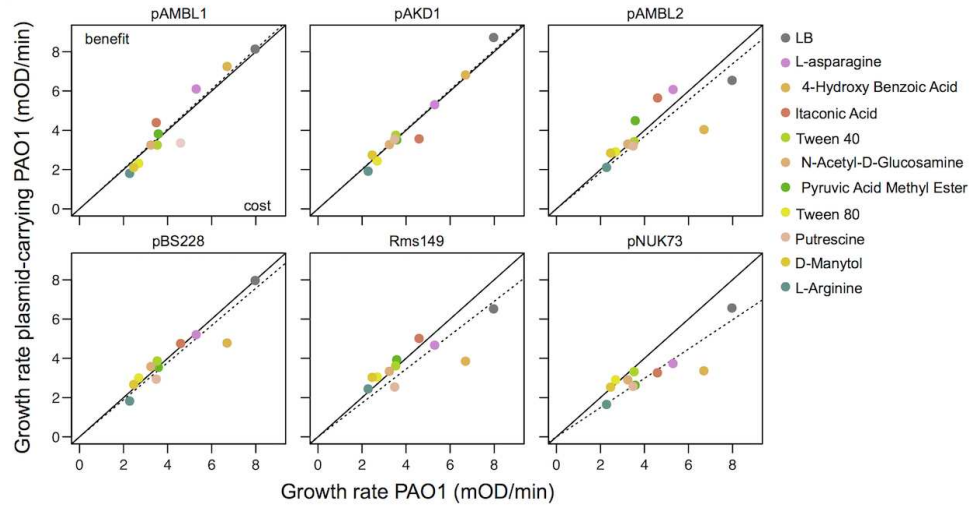
Name	Group	Size (bp)	Transmission <sup>1</sup>	Origin	Year <sup>2</sup>	Reference
pBS228	IncP-1α	89,147	Mobilizable	Waste water	1981	(Haines et al 2007)
Rms149	IncP-6	57,121	Mobilizable	Clinical	1975	(Haines et al 2005)
pAKD1	IncP-1β	58,246	Conjugative	Soil	1998	(Sen et al 2011)
pAMBL1	RepA/C	26,440	Mobilizable	Clinical	2006	(San Millan et al 2015a)
pAMBL2	Rep_3	24,133	Non-transmissible	Clinical	2007	(San Millan et al 2015a)
pNUK73	NA <sup>3</sup>	5,128	Non-transmissible	Soil	2003	(Itoh et al 2003)

719

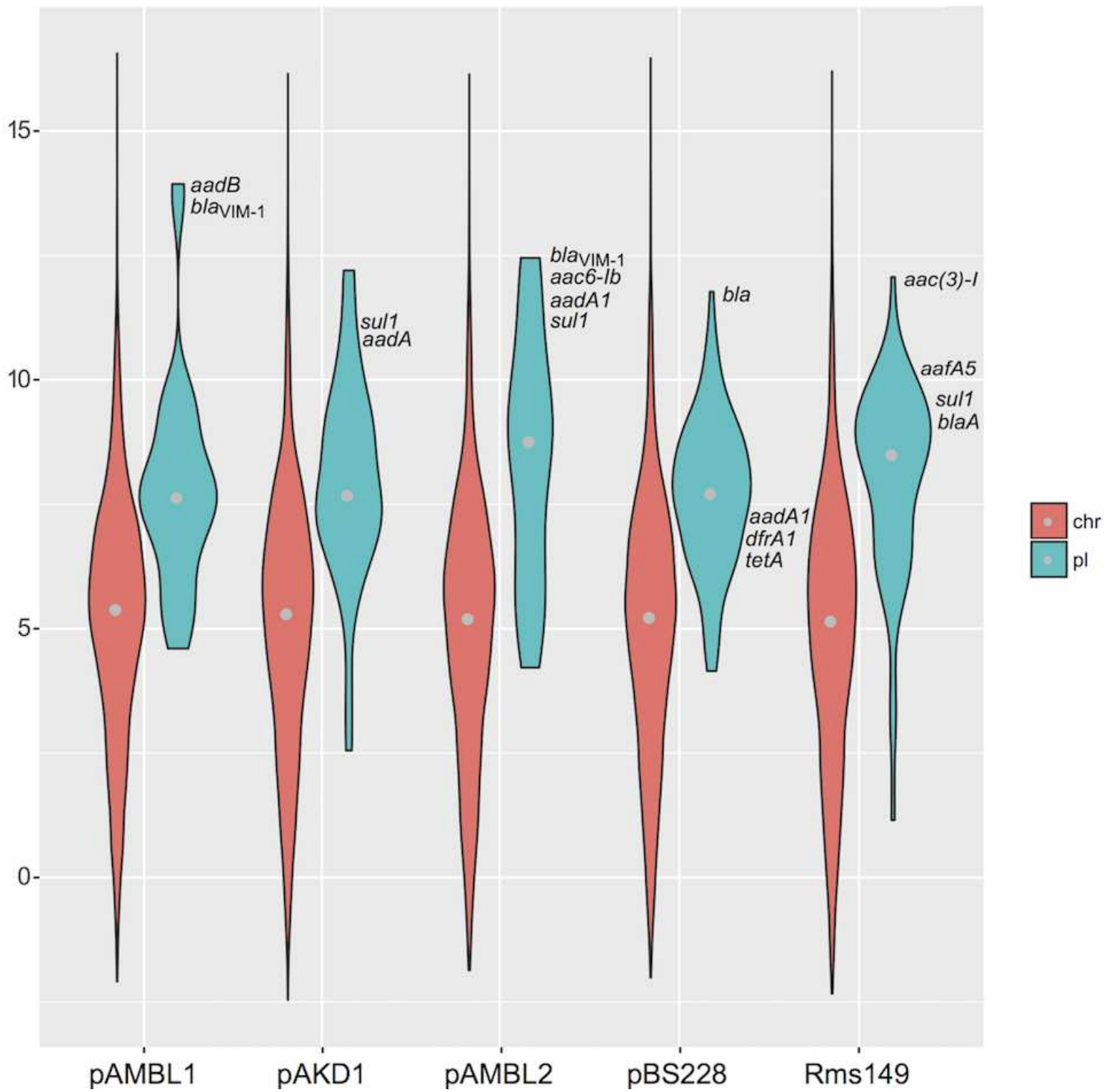
720 <sup>1</sup> Plasmid classification according to conjugative ability: Conjugative: self-  
 721 transmissible by conjugation. Mobilizable: able to conjugate using the conjugative  
 722 machinery of a helper conjugative element. Non-transferable: not able to conjugate  
 723 or to be mobilized.

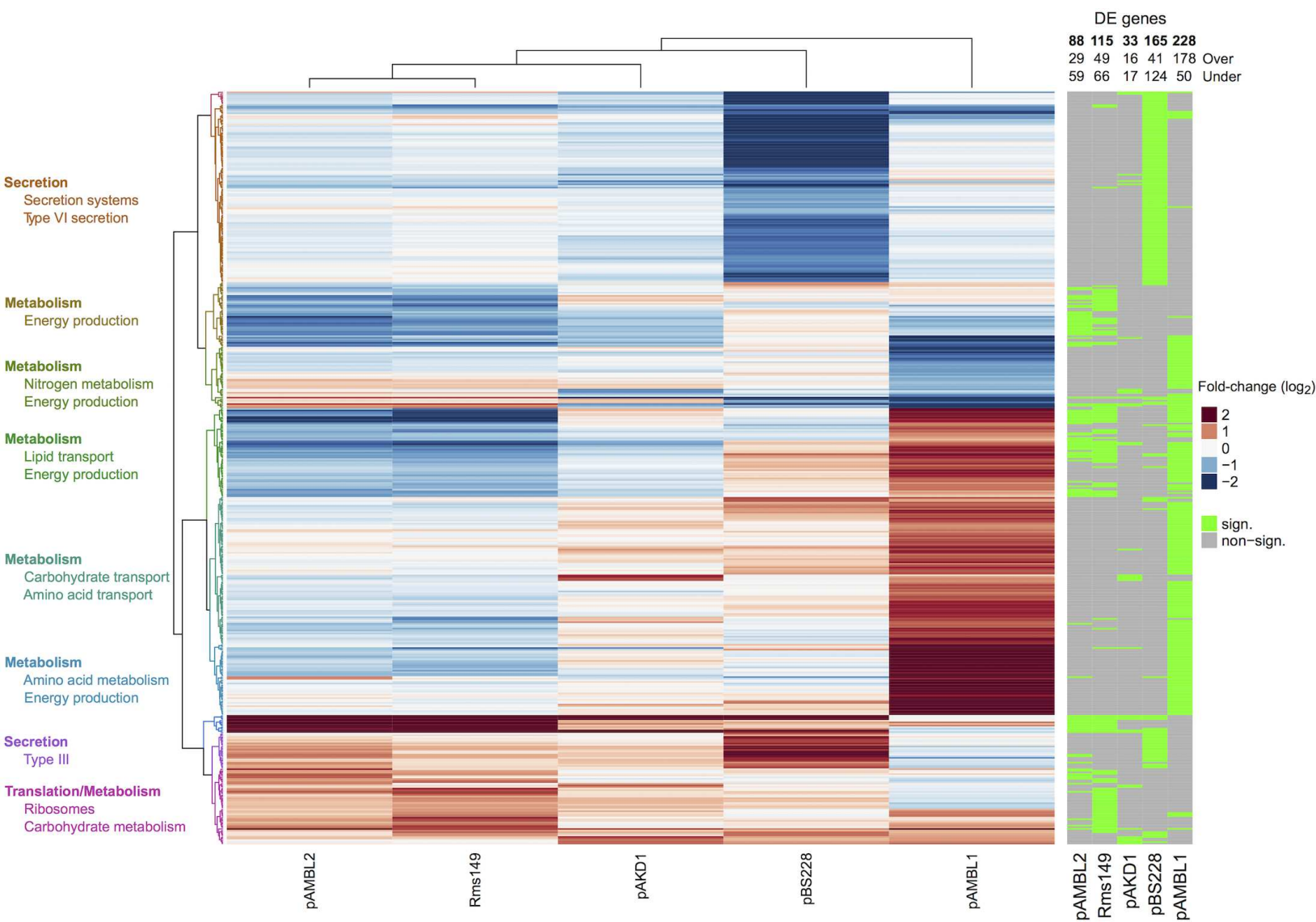
724 <sup>2</sup> Year of description.

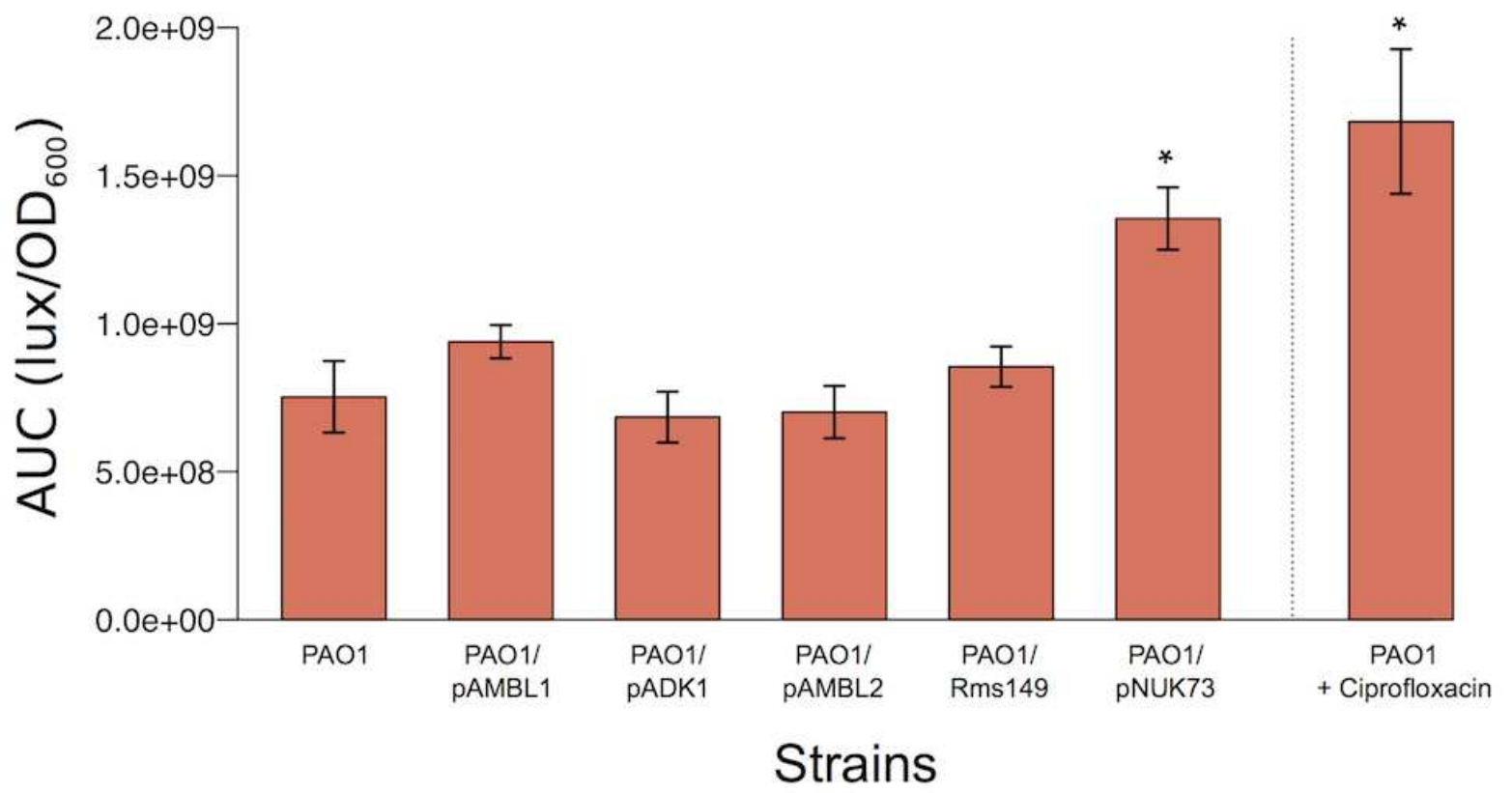
725 <sup>3</sup> Not applicable. The small plasmid pNUK73 does not belong to a specific plasmid  
 726 group.

**a****b**

Expression ( $\log_2$  TPM)







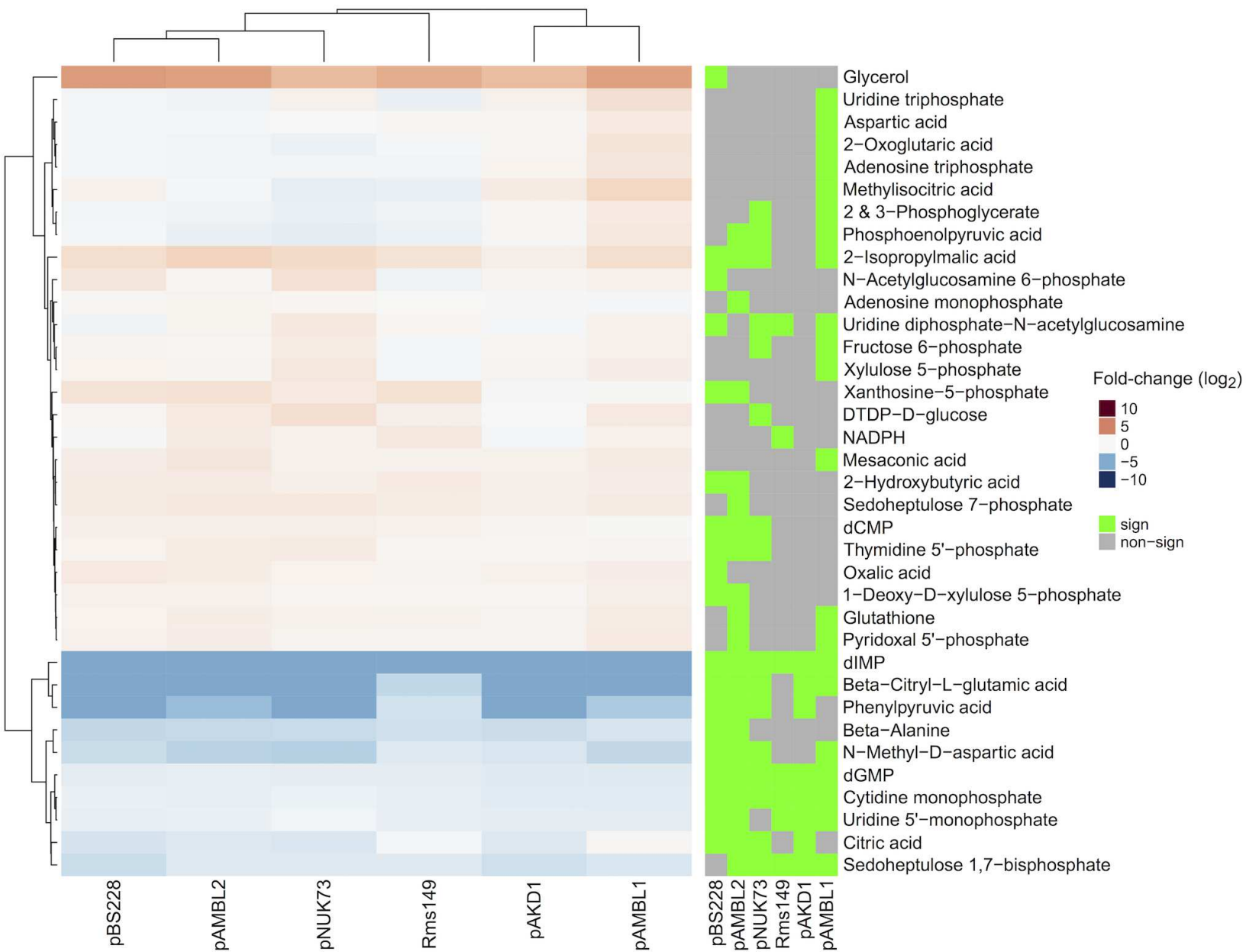




Table 1. Plasmids used in this study

Name	Group	Size (bp)	Transmission <sup>1</sup>	Origin	Year <sup>2</sup>	Reference
pBS228	IncP-1 $\alpha$	89,147	Mobilizable	Waste water	1981	(Haines et al 2007)
Rms149	IncP-6	57,121	Mobilizable	Clinical	1975	(Haines et al 2005)
pAKD1	IncP-1 $\beta$	58,246	Conjugative	Soil	1998	(Sen et al 2011)
pAMBL1	RepA/C	26,440	Mobilizable	Clinical	2006	(San Millan et al 2015a)
pAMBL2	Rep_3	24,133	Non-transmissible	Clinical	2007	(San Millan et al 2015a)
pNUK73	NA <sup>3</sup>	5,128	Non-transmissible	Soil	2003	(Itoh et al 2003)

<sup>1</sup> Plasmid classification according to conjugative ability: Conjugative: self-transmissible by conjugation. Mobilizable: able to conjugate using the conjugative machinery of a helper conjugative element. Non-transferable: not able to conjugate or to be mobilized.

<sup>2</sup> Year of description.

<sup>3</sup> Not applicable. The small plasmid pNUK73 does not belong to a specific plasmid group.