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1 **Metabolite Damage and Damage-Control in a Minimal Genome**

2

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41

42 **Abstract**

43 Analysis of the genes retained in the minimized Mycoplasma JCVI-Syn3A genome established
44 that systems that repair or preempt metabolite damage are essential to life. Several genes known
45 to have such functions were identified and experimentally validated, including 5-
46 formyltetrahydrofolate cyclo-ligase, CoA disulfide reductase, and certain
47 hydrolases. Furthermore, we discovered that an enigmatic YqeK hydrolase domain fused to
48 NadD has a novel proofreading function in NAD synthesis and could double as a MutT-like
49 sanitizing enzyme for the nucleotide pool. Finally, we combined metabolomics and
50 cheminformatics approaches to extend the core metabolic map of JCVI-Syn3A to include
51 promiscuous enzymatic reactions and spontaneous side reactions. This extension revealed that
52 several key metabolite damage-control systems remain to be identified in JCVI-Syn3A, such as
53 that for methylglyoxal.

54

55 **Importance**

56 Metabolite damage and repair mechanisms are being increasingly recognized. We present here
57 compelling genetic and biochemical evidence for the universal importance of these mechanisms
58 by demonstrating that stripping a genome down to its barest essentials leaves metabolite damage-
59 control systems in place. Furthermore, our metabolomic and cheminformatic results point to the
60 existence of a network of metabolite damage and damage-control reactions that extends far
61 beyond the corners of it that have been characterized so far. In sum, there can be little room left
62 to doubt that metabolite damage and the systems that counter it are mainstream metabolic
63 processes that cannot be separated from life itself.

64 **Introduction**

65 A foundational goal of synthetic biology was to create a minimal living organism by a
66 bottom-up approach (1). This goal was reached in 2016 with the creation of JCVI-Syn3.0 (2).
67 This organism was built from the ruminant pathogen *Mycoplasma mycoides capri* serovar LC
68 GM12 by DNA synthesis, recombination, and genome transplantation techniques, and included
69 only genes required for survival or to support a reasonable growth rate (428 protein-coding genes
70 and 34 RNA genes) (2). The initial JCVI-Syn3.0 strain was extremely fragile; a derivative with
71 18 more genes, JCVI-Syn3A was more stable and was the basis for a metabolic model (3).
72 Surprisingly, when the JCVI-Syn3.0 was published in 2016, ~30 % of its genes could not be
73 assigned a specific function. The initial annotation has since been improved by manual curation
74 (4), metabolic modeling (3), and further in silico analyses (5) but ~85 proteins with unknown or
75 vaguely defined functions remain (Supplemental data A1). These unknowns cannot all be
76 missing parts of synthesis/breakdown pathways as the metabolic reconstruction identified only
77 four metabolic and eight transport reactions as missing (3).

78 A crucial area of metabolism usually left out of metabolic models is metabolite damage
79 and repair. Enzymes make mistakes and metabolites undergo spontaneous chemical reactions (6,
80 7). These damage reactions are ever-present and, when the resulting products are toxic, can
81 reduce fitness (6, 8). It has been shown recently that many enzymes of formerly unknown
82 function repair or pre-empt metabolite damage (9–11), that mutations in metabolite repair
83 enzymes cause human diseases (12–14), and that pathway engineering can fail unless appropriate
84 repair enzymes are installed (15). The emerging recognition of the nature and extent of
85 metabolite damage and repair raised the question of the importance of metabolite repair for a
86 minimal genome like JCVI-Syn3/3A. By combining expert manual curation, comparative
87 genomics, metabolomics, metabolic modeling, cheminformatics, and experimental validation, we
88 identified a set of chemical damage reactions likely to occur in JCVI-Syn3 and some of the
89 damage repair and preemption activities that this minimal genome encodes.

90

91 **Results and Discussion**

92 **Identification and validation of homologs of known metabolite repair enzymes**

93 We first manually screened the predicted proteome of JCVI-Syn3A for homologs of
94 known metabolite repair enzymes (6, 15, 16) (see Supplemental data S1 and Appendix). Several
95 were found, as follows.

96 1. 5-FCL. 5-Formyltetrahydrofolate (5-CHO-THF) is a by-product of serine
97 hydroxymethyltransferase (SHMT) (17)(Fig. 1A) that inhibits folate-dependent enzymes and
98 must therefore be recycled or destroyed (18). Of various enzymes known to recycle 5-CHO-THF
99 (19), the most widespread is 5-formyltetrahydrofolate cyclo-ligase (5-FCL) (encoded by *fau/ygfA*
100 (16) in *E. coli*). The JCVI-syn3A genome encodes a 5-FCL homolog (JCVISYN3A_0443); this
101 gene was confirmed to encode an active 5-FCL by a complementation assay. Specifically, an *E.*
102 *coli* K12 $\Delta ygfA$ strain does not grow on M9 minimal medium with 0.2% glucose as carbon
103 source and 20 mM glycine as sole nitrogen source (19) (Fig. 1B). Expression of
104 JCVISYN3A_0443 from a plasmid complemented this growth phenotype (Fig. 1B). Note that
105 the essentiality of JCVISYN3A_0443 might be due both to its repair function and to a role as a
106 source of 5,10-methenyltetrahydrofolate-polyglutamate (3).

107 2. Thiol reductases. Like all aerobes, JCVI-syn3A encounters oxidative stress that can damage
108 macromolecules. Maintaining protein and small-molecule thiol groups in their reduced state is
109 critical for cellular redox homeostasis (20). Thioredoxin/thioredoxin reductase is the dominant
110 protein thiol oxidoreductase system in many organisms, using reducing equivalents ultimately
111 derived from NADPH (21, 22). The JCVI-Syn3A genome encodes homologs of the thioredoxin
112 system proteins (TrxB/JCVISYN3A_0819 and TrxA/JCVISYN3A_0065) that are most likely
113 involved in reducing protein disulfide bonds and have been partially characterized in other
114 *Mycoplasma* species (Fig. 2A)(23, 24). Both genes are essential (Supplemental data A1),
115 supporting key roles for TrxA and TrxB in disulfide bond reduction. Note, however, that
116 thioredoxin is also the electron donor for ribonucleotide reductase, so that JCVISYN3A_0819
117 and JCVISYN3A_0065 may be essential for this reason (23, 25).

118 JCVI3_0887 is a homolog of CoA disulfide reductase (CoADR), which may have a
119 major redox role in certain bacteria (26). Because CoA is required for several reactions in the
120 JCVI-syn3A metabolic model and is predicted to be imported from the medium, CoADR could
121 maintain the CoA pool in the reduced state. Testing the CoADR activity of the
122 JCVISYN3A_0887 showed that it is an active CoAD reductase that operates well at
123 physiological pH (pH 7.5) (27) and has reasonable K_M (0.17 mM) and k_{cat} (2.8 s^{-1}) values (Fig.

124 2B). It lacks detectable activity against oxidized glutathione or pantethine (Fig. 2C). While we
125 cannot exclude the possibility that reduced glutathione is imported from the medium and
126 oxidized glutathione is exported, a CoA-based system is a more parsimonious solution to the
127 redox balance problem.

128

129 **Functional analysis of HAD proteins identifies a nucleotide phosphatase with possible dual** 130 **roles**

131 Our second strategy to identify metabolite repair enzymes was based on the
132 demonstration that hydrolases of previously uncertain or unknown function were subsequently
133 shown to participate in metabolite repair (9). Five genes encoding stand-alone members of the
134 HAD (haloacid dehalogenase) hydrolase family (28) were identified in the JCVI-Syn3A genome
135 (Supplemental data S1) and are conserved in closely related *Mesoplasma florum* L1 genome
136 (29) (Table 1). Such HAD hydrolases often participate in metabolite repair or homeostasis, as
137 many damaged or toxic intermediates are phosphorylated (e.g. phosphosugars), and their
138 recycling or removal requires a phosphatase (9, 30).

139 Comparative genomic analysis of the stand-alone HADs did not point to clear functional
140 hypotheses, except for JCVISYN3A_0728, whose location in a predicted operon with triose-
141 phosphate isomerase and phosphoglycerate mutase suggested a role in sugar phosphate
142 metabolism (Table 1). Possible functions for the HAD proteins included: 1) repair of substrates
143 to be identified; 2) missing phosphatases involved in primary metabolism identified by the
144 metabolic model such as sedoheptulose 1,7-bisphosphate phosphatase or phosphatidate
145 phosphatase; 3) nucleotide phosphatases involved in dNTP pool maintenance. To discriminate
146 among these hypotheses, we combined biochemistry, genetics, and metabolomics.

147 The four HAD proteins that we were able to express in *E. coli* (JCVISYN3A_0066,
148 JCVISYN3A_0077, JCVISYN3A_0728, JCVISYN3A_0907) were tested for activity against a
149 panel of 94 phosphatase substrates (Table A1) (31). The four proteins had detectable activity
150 against the model phosphatase substrate *p*-nitrophenyl phosphate (*p*NPP) and different
151 physiological substrates (Fig. A1). The JCVISYN3A_0728 enzyme hydrolyzed a wide range of
152 nucleoside and sugar phosphates, the JCVISYN3A_0907 and JCVISYN3A_0077 enzymes
153 hydrolyzed narrower ranges of sugar phosphates, and the JCVISYN3A_0066 enzyme
154 hydrolyzed FMN and CoA. That sugar phosphates are good substrates of JCVISYN3A_0728 is

155 consistent with its genomically-predicted role in sugar phosphate metabolism, but no specific
156 function or substrate could be assigned. Note, however, that the 94-substrate panel did not
157 include damaged sugar phosphates.

158 We attempted to delete HAD-encoding genes in JCVI-syn3A, expecting this to be
159 possible because transposon bombardment of the JCVI-syn3A genome indicated all five HADs
160 were quasi-essential (i.e., required for fast growth but not for viability) [(3) and Supplemental
161 data S1]. Deletants were readily obtained for genes JCVISYN3A_0066, JCVISYN3A_0077,
162 JCVISYN3A_0728, and JCVISYN3A_0907 (Supplemental data S2). Attempts to delete
163 JCVISYN3A_0710 using two different methods were unsuccessful (Supplemental data S2).
164 Deletion of this gene could have resulted in an extremely slow-growing strain that was
165 unrecoverable in the conditions used, Alternatively JCVISYN3A_0710 could be essential, the
166 transposon insertions in the gene being artifacts. That the same gene is also essential in *M.*
167 *florum* (Table 1) favors the latter hypothesis.

168 We observed no major differences in growth rates between JCVI_Syn3A and any HAD
169 mutant (Fig. A2). To conduct a metabolomics analysis, the four mutants and the JCVI-Syn3A
170 parent were grown in SP4-KO medium and harvested at the same point of log-phase growth.
171 (Appendix and Tables A2 and A3). A total of 4152 features were detected in the samples using
172 hydrophilic interaction liquid chromatography (HILIC) and mass spectrometry (Supplemental
173 data S3), of which 522 were annotated as known metabolites.

174 Partial least squares discriminant analysis was used to find the variable importance in
175 projection (VIP) scores of each annotated metabolite. The fifteen metabolites with the highest
176 VIP scores (Fig. 3 and Fig. A3) showed little contamination from media, as determined by
177 analysis of unused media along with mutant samples. Most of these metabolites were below the
178 limit of detection in unused media, and most of the rest were present at a >30-fold lower
179 abundance in media than in samples, suggesting little or no contamination from residual media
180 (Supplemental data S3). Two metabolites (cytidine and thiamine) were found at similar
181 abundance in media and samples, suggesting these media contamination.

182 Among the 15 metabolites with high VIP scores, the JCVISYN3A_0728 knockout showed
183 significantly higher abundance of glycerophosphate, oleoyl lysophosphatidic acid, and
184 palmitoylglycerol than other genotypes (Fig. 3 and Fig. A3). We were not able to determine
185 which form of glycerophosphate was increased, although the 3-phosphate is a priori more likely,

186 being found in the metabolic model as a cardiolipin metabolism intermediate that is synthesized
187 via phosphorylation of imported glycerol by GlpK (JCVISYN3A_0218). To further analyze the
188 knockout metabolomics data further, all four HAD hydrolase knockout phenotypes were
189 separately compared to wild type JCVI-Syn3A (Supplementary data S3). The conclusions are
190 summarized below and further discussed in the Appendix.

191 The metabolomics data suggests that JCVISYN3A_0066 is the major dNMPase with
192 activity against the deoxymononucleotides dAMP, dGMP, dUMP, dCMP, dTMP and dIMP, and
193 also the ribomononucleotide IMP. Furthermore, as further discussed in the Appendix, the data
194 also suggest the residual presence of pyrimidine nucleoside phosphorylase (PyNP) activity in
195 JCVI-Syn3A after the known MMSYN1_0734 has been removed. The lack of observed
196 nucleotidase activity for JCVISYN3A_0066 in the *in vitro* substrate screen could be due to the
197 absence of relevant effectors. In contrast it seems that JCVISYN3A_0077 is also a dUMP-
198 specific specific dNMPase that plays a minor role *in vivo* compared to JCVISYN3A_0066. The
199 metabolomics data also suggests that JCVISYN3A_0728 is a glycerol 3-phosphate phosphatase.
200 The other activities detected *in vitro*, if relevant *in vivo*, might not be apparent in the
201 metabolomics data if these substrates do not accumulate in cells. No functional role could be
202 proposed for JCVISYN3A_0907.

203

204 **Comparative genomics uncovers a possible metabolite repair diphosphatase**

205 The YqeK HD family phosphohydrolase is fused to nicotinic acid mononucleotide
206 adenylyltransferase (NadD) in most mycoplasmas and strongly physically clustered with NadD
207 in many other gram-positive organisms (32) (Fig. 4 and Fig. A4A). These genomic associations
208 led us to propose that YqeK repairs mistakes made by NadD. The canonical activity of NadD is
209 to adenylate nicotinate-ribonucleotide (NaMN) using ATP as a donor of the AMP moiety (Fig.
210 4A). However, use of another NTP or the deoxy-form of ATP would create an erroneous product
211 requiring disposal, most likely by hydrolysis. We therefore expressed JCVISYN3A_0380 and its
212 His230Ala variant in *E. coli* (Fig. A4B). (The His230Ala mutation is predicted to abolish
213 phosphatase activity that would interfere with NadD activity measurement.) *Bacillus subtilis*
214 NadD was used as a benchmark. The JCVISYN3A_0380 His230Ala protein and *B. subtilis*
215 NadD were tested for *in vitro* activity with various nucleoside triphosphates as substrates. The
216 adenylating activity of the JCVISYN3A_0380 His230Ala mutant was quite non-specific and

217 actually greater against dATP, CTP, or UTP than against the physiological substrate, ATP,
218 whereas *B. subtilis* NadD strongly preferred ATP (Fig. 5A). JCVI-syn3 NadD can therefore
219 readily form deoxy-adenosine, -cytidine, or -uridine analogs of the NAD precursor nicotinate
220 adenine dinucleotide (NaAD), which can presumably be converted to inhibitory analogs of NAD
221 and NADP.

222 We then tested the JCVI-Syn3 YqeK domain for diphosphatase activity using the NaAD
223 analogs that could be produced by JCVI-syn3A NadD. The YqeK domain had activity towards
224 the cytosine (NaCD) and uracil (NaUD) analogs of NaAD that was at least as high as that against
225 NaAD itself (Fig. 5B), which agrees with the preference of the NadD domain to form these
226 analogs.

227 We also observed that the YqeK domain had high activity against 8-oxo-GTP, although
228 judging from relative activities with 0.05 mM and 0.5 mM substrate, the K_M is likely higher than
229 for the other substrates tested (Fig. 5B). Consistent with this finding, we showed that the genes
230 encoding the JCVI-syn3A NadD-YqeK fusion can partially complement the *E. coli mutT* high
231 mutation rate phenotype (measured as Rif^R ratios) (Fig. 5C). The partial complementation was
232 also observed when expressing the YqeK domain alone, but not the NadD domain alone.
233 Finally, it was recently shown that YqeKs of gram-positive bacteria belong to a novel
234 diadenosine tetraphosphate (Ap₄A) hydrolase family (33). Taken together, these observations
235 suggest that YqeK is a versatile diphosphatase with several functional roles.

236 Indeed, the available transposon insertion data ((3) and Supplemental data S1) suggested that the
237 NadD domain is essential and the YqeK domain is quasi-essential because a few hits in the
238 YqeK region of the gene were detected in the first Tn round and disappeared after the fourth
239 round. We could not isolate a JCVISYN3A_0380 deletant despite several attempts. We were,
240 however, able to construct a strain carrying the His230Ala mutation that inactivates YqeK
241 diphosphatase activity (Supplemental data S2), and this strain showed no growth defect or
242 obvious metabolite imbalance (Fig. A2).

243

244 **Metabolomics-driven exploration of damage and repair chemistry in JCVI-Syn3**

245 Thus far, all of our damage and repair cases began with analysis of genes in the JCVI-
246 Syn3A genome and uncovered clear instances of metabolite damage and repair. But are these
247 examples isolated exceptions, or the tip of an iceberg of uncharacterized metabolic chemistry?

248 To address this question, we adopted a systematic exploratory approach based on the
249 metabolomics data for JCVI-Syn3A cells (see Supplemental Table S3). Because this approach
250 begins with the observed chemical results of potential metabolite damage and is not limited by
251 our current knowledge of gene function, it will certainly find damage mechanisms that our gene-
252 first approach will miss. Still, this approach will also miss any damage mechanisms that fail to be
253 observed through metabolomics, either due to volatility of end products or extremely effective
254 damage mitigation systems.

255 We focused specifically on a set of 480 metabolites (Supplemental Table S4E) that
256 satisfied two criteria: (1) the mass spectral signal was confidently identified with a defined
257 molecular structure; and (2) the metabolite was at least as abundant in the JCVI-Syn3A cells as
258 in the growth medium. We compared the 480 identified peaks to the 304 metabolites in the
259 JCVI-Syn3A model and the 33,978 compounds in the ModelSEED database (34), resulting in 57
260 matches to the model and 217 (45%) matches to the database (Supplemental Table S4E). The
261 comparison to the JCVI-Syn3A model reveals two types of discrepancy: (1) 247 metabolites in
262 our model do not appear in our metabolomics data, which is to be expected as many metabolites
263 are too low in concentration or too volatile to be detected in metabolomics; and (2) 423
264 metabolites that were observed and do not appear in our model, which is more problematic as
265 this implies that there is significant chemistry taking place in this system that our present model
266 cannot explain. The ModelSEED database lookup reveals further discrepancies: (1) 263 observed
267 metabolites do not appear in biochemistry databases, indicating that these is no known
268 biochemical route to any of these compounds that are observed to arise in a biological system;
269 and (2) 160 observed metabolites (217-57) do have known biochemical biosynthesis mechanisms
270 but these mechanisms do not appear in our current JCVI-Syn3 model (3). To predict potential
271 chemical routes to as many of the observed metabolites as possible without limiting our search to
272 known chemistry or straying too far from known JCVI-Syn3A metabolism, we used the PickAxe
273 tool (35). This tool applies generalized reaction rules based on known spontaneous (8) and
274 enzymatic (36, 37) chemical mechanisms to predict potential novel reactions that a given set of
275 metabolites (here, all JCVI-Syn3A metabolites) could undergo. We started with the 304
276 metabolites present in the JCVI-Syn3A model and applied PickAxe for multiple iterations to
277 allow generation of multistep pathways (see Methods). We used both spontaneous and enzymatic
278 reaction rules in the PickAxe expansion, enabling prediction of pathways with a mixture of both

279 (as occurs in many damage and repair pathways). The initial PickAxe iterations uncovered an
280 increasing number of compounds generated that matched the observed metabolites, but these hits
281 tapered off after six iterations to just one new compound produced that matched an observed
282 metabolite (blue line in Fig. 6). The number of compounds predicted by PickAxe that matched
283 known biochemistry in the ModelSEED database (green line in Fig. 6) followed a similar trend.
284 We halted the PickAxe expansion at this stage, given its diminishing returns. The final chemical
285 network generated by PickAxe included 33,934 compounds, 61,939 reactions, and matched a
286 total of 182 distinct metabolites (including the original 57 matching the JCVI-Syn3 model) and
287 1090 ModelSEED compounds (Supplemental data S4C-D).

288 Next, we used a new flux balance analysis formulation, metabo-FBA, to select a minimal
289 subset of these reactions that connect the functioning JCVI-Syn3A model to as many observed
290 metabolites as possible using mass and energy balanced pathways (see Methods). Because our
291 study is of a minimal genome with relatively few enzymes and specifically focuses on metabolite
292 damage, we favored solutions that involved as many reactions generated by spontaneous reaction
293 rules as possible. This approach produced a predicted flux profile that simultaneously pushed
294 flux through reactions involving compounds that matched 182 observed metabolites (see solution
295 depicted in Fig. 7 and data in Supplemental data S4A and E). This solution included 145 (58%)
296 of the 252 reactions in the JCVI-Syn3 model (purple reactions in Fig. 7), 129 additional
297 ModelSEED reactions (primarily predicted enzymatic reactions; green reactions in Fig. 7), 84
298 novel enzymatic reactions (blue reactions in Fig. 7), and 74 novel spontaneous reactions (red
299 reactions in Fig. 7) (data in Supplemental data S4A). The fixed image of our flux solution
300 depicted in Fig. 7 is of limited value for permitting a detailed exploration of the fluxes, so we are
301 also including all data files and instructions needed to replicate this view in a fully functioning
302 dynamic Escher map (see Supplemental data S5). Also, the fully expanded version of the JCVI-
303 Syn3A model used to generate this flux solution is provided in SBML and JSON format in
304 Supplemental data S5.

305 This flux solution is only one of many possible solutions that can explain the observed
306 metabolomics data. While it is unlikely that this solution is completely correct, the true solution
307 must make use of similar chemistry, start with the same initial high-confidence JCVI-Syn3A
308 compounds, and produce the same observed metabolic intermediates, meaning the true solution
309 cannot differ very substantially from our selected one.

310 The map broadly (Fig. 7), shows clear hotspots of chemical expansion (adenine, cytosine,
311 sugars, pyruvate, amino acids, central carbon trunk reactions, CoA) and regions with little or
312 none (deoxynucleotides, guanine, thymidine, THF, riboflavin, NAD). This is probably explained
313 by the intrinsic reactivities and the concentrations of the associated compounds. Many of the
314 hotspot compounds are high-concentration metabolic starting points (e.g., sugars), end points
315 (e.g., amino acids), or high-flux intermediates (e.g., pyruvate). Their high concentrations make it
316 more likely that these compounds will react chemically and that metabolomics will detect the
317 resulting products.

318 The many ModelSEED reactions and predicted novel enzymatic reactions proposed by
319 this approach represent previously unannotated but potential promiscuous side activities of
320 existing annotated gene products in JCVI-Syn3A. The metabolomic evidence for the presence of
321 the products of these reactions points strongly to the presence of the reactions themselves. The
322 cluster of ModelSEED reactions expanding from the glucose-6-phosphate (g6p) node of the
323 JCVI-Syn3A model is a good example (see Fig. 7). These reactions are phosphorylations and
324 hydrolyses that interconvert many diverse sugars and polysaccharides, all of which are supported
325 by our metabolomics data. As the model only contains reactions for glucose as a representative
326 sugar, it probably understates the extent of such reactions.

327 Also of note, is how many of the pathways predicted in JCVI-Syn3A by our metabo-FBA
328 method involve a mixture of database reactions, predicted spontaneous reactions, and novel
329 enzymatic reactions (30/50 total pathways). Any analysis based on just one or two of these three
330 reaction sources would explain a far smaller number of observed metabolites due to gaps and
331 dead-ends in the predicted pathways. This, using all three reaction data sources provides a much
332 fuller understanding of metabolism.

333 Another notable point is that much of the new predicted chemistry surrounds amino
334 acids. Many of the observed metabolomics peaks correspond to amino acid derivatives such
335 as dipeptides and acetylated amino acids (see Fig. 7). The dipeptides serve primarily as nutrients
336 for JCVI-syn3, which contains the peptidases needed to degrade these compounds (a large
337 number of the ModelSEED reactions added by our metabo-FBA approach relate to dipeptide
338 transport and degradation). The acetylated amino acids are different in that only 7 out of 10 of
339 these compounds were found in biochemistry databases, and the databases lacked spontaneous
340 acetylation reactions to produce these compounds. Yet, metabolomics evidence supported the

341 presence of all 10 in the JCVI-Syn3A strain. The metabo-FBA approach added 10 predicted
342 spontaneous acetylation reactions, using acetyl-phosphate as a donor, based on PickAxe
343 predictions. This demonstrates how readily acetylation occurs in these systems, either by
344 spontaneous action or by promiscuous enzyme activity, and it highlights the particular
345 vulnerability of amino acids to this acetylation.

346 These results also support previous hypotheses about the main metabolic network of
347 JCVI-Syn3A (3) with regard to acetyl phosphate and the enzymes producing/consuming it. The
348 *in vivo* essentiality of phosphate acetyltransferase (JCVISYN3_0229) and acetate kinase
349 (JCVISYN3_0230) was previously puzzling, given that the upstream genes in the pathway, the
350 subunits of pyruvate dehydrogenase (JCVISYN3_0227/8), were found to be non-essential *in*
351 *vivo*. It had been hypothesized that the two former enzymes were essential because buildups of
352 acetyl-CoA or acetyl phosphate needed to be prevented, both being known protein acetylation
353 agents (38). The extensive and diverse acetylation damage we found evidenced in our
354 metabolomics data would seem to further support this hypothesis.

355 Relatedly, our results support a role for acetyl phosphate in the acetylation of proteins as
356 well as free amino acids because some of the identified amino acids had side chain acetylations.
357 The results also support the hypothesized essential role of acetate kinase as a means of
358 preventing acetyl phosphate accumulation.

359 These analyses also expose insights into the relative importance of our various proposed
360 mechanisms for spontaneous chemistry, based on which mechanisms are most likely to give rise
361 to metabolic products found in our metabolomics data (see larger discussion in Appendix and
362 Fig. A5). Of course, not all chemically impactful metabolites are readily observed in
363 metabolomics data due to instability or volatility. Methyl-glyoxal is a good example of an
364 important metabolite that arises from and participates in spontaneous damage reactions but could
365 not be observed (Fig. 7). While methylglyoxal was not among the observed metabolites due to
366 small size and volatility, metabo-FBA added reactions involving this compound because it leads
367 to numerous downstream potential damage and repair reactions. A more detailed discussion of
368 methylglyoxal follows.

369

370 **Possible ways for JCVI-Syn3A to cope with methylglyoxal stress**

371 Methylglyoxal is necessarily formed from the triose phosphates in JCVI-Syn3A central
372 metabolism (39) but the classical glyoxalase system comprising the glutathione-dependent GloA
373 and GloB enzymes (40) is absent. Likewise, the JCVI-Syn3A genome does not encode enzymes
374 with minor methylglyoxal-detoxifying activities, such as aldose reductases and keto-aldehyde
375 reductases (41–43). The only candidate enzyme that we identified as potentially able to counter
376 methylglyoxal-induced damage is JCVISYN3A_0400, which encodes a homolog of DJ-1. The
377 DJ-1 superfamily has several functionally distinct clades, of which four are found in *E. coli*
378 (encoded by *hchA*, *yajL*, *yhbO* and *elbB*). Phylogenetic analysis places JCVISYN3A_0400 in
379 the YajL/DJ-1 clade (Fig. A6).

380 The members of the DJ-1 superfamily that have been functionally characterized
381 participate in stress response and detoxification (44). Some are thought to be deglycases (45),
382 glyoxalases (46), or aldehyde-adduct hydrolases (47). Previous studies showed variability in the
383 phenotypes reported for the *E. coli hchA*, *yajL*, *yhbO* deletion mutants as the sensitivity of the
384 *yajL* reported by the Richarme group (48) was not reproduced in independent studies (46). We
385 also failed to reproduce the reported glyoxal or methylglyoxal sensitivities of the single deletion
386 *yajL* mutant, but did observe a defect both in its growth rate and yield of the the $\Delta yajL/\Delta hchA$
387 *E. coli* K-12 BW25113 strain (Fig. 8A and Fig. S7A). Expression of the *E. coli yajL* or
388 JCVISYN3A_0400 genes *in trans* complemented this growth phenotype (Fig. 8A and Fig. A7A)
389 suggesting JCVISYN3A_0400 is indeed in the same DJ-1 subgroup as YajL.

390 To test the hypothesis that JCVISYN3A_0400 participates in methylglyoxal
391 detoxification, we measured the glyoxalase activity of the recombinant protein.
392 JCVISYN3A_0400 possess a low but measurable methylglyoxalase activity ($k_{cat} = 0.025 \pm 0.002$
393 sec^{-1} , $K_M = 1.23 \pm 0.30$ mM), lower than obtained for the positive control protein human DJ-1
394 ($k_{cat} = 0.126 \pm 0.004 \text{ sec}^{-1}$, $K_M = 0.34 \pm 0.04$ mM) but higher than *E. coli* YajL ($k_{cat} = 0.004 \pm$
395 0.0001 sec^{-1} , $K_M = 0.095 \pm 0.018$ mM) (Fig. 8B). The low k_{cat} for YajL is consistent with a prior
396 report that did not detect glyoxalase activity using methylglyoxal as a substrate (46). The $\sim 20 \text{ M}^{-1}$
397 $\text{sec}^{-1} k_{cat}/K_M$ value for JCVISYN3A_0400 is five to six orders of magnitude lower than that of
398 glyoxalase I, the canonical glutathione-dependent glyoxalase (49). Even compared to other DJ-1
399 superfamily glyoxalases, JCVISYN3A_0400 is a poor enzyme. The lactate oxidase-coupled
400 assay used here is specific to L-lactate, which should detect all the lactate produced by
401 JCVISYN3A_0400, as a prior study indicated that DJ-1 clade enzymes produce only the L

402 enantiomer (50), although we did not test the enantiopurity of the lactate produced by
403 JCVISYN3A_0400 in this study.

404 Because DJ-1 superfamily members have been reported to be generalist deglycases (51),
405 we tested the deglycase activity of JCVISYN3A_0400 against the methylglyoxal-CoA
406 hemithioacetal (Fig. A7B). CoA was used as the thiol because the absence of glutathione
407 biosynthetic enzymes in JCVI-Syn3A suggests that CoA is its main small molecule thiol (see
408 above). JCVISYN3A_0400 had no detectable deglycase activity against methylglyoxal-CoA
409 hemithioacetal (Fig. A7B), while human DJ-1 had a low activity ($k_{\text{cat}} = 0.0068 \pm 0.0007 \text{ sec}^{-1}$, K_M
410 $= 0.144 \pm 0.064 \text{ mM}$) against the same substrate (52). JCVISYN3A_0400 therefore seems
411 unlikely to efficiently detoxify methylglyoxal via either glyoxalase or deglycase pathways. It is
412 possible that JCVISYN3A_0400 and other DJ-1-type glutathione-independent
413 methylglyoxalases have some unidentified positive effector *in vivo* that enhances their activity,
414 and the glyoxalase activity of human DJ-1 is highly sensitive to buffer conditions (53). In
415 summary, while results suggest that JCVISYN3A_0400 and YajL are iso-functional, they do not
416 appear to make a large contribution to methylglyoxal detoxification.

417 The recent observation that human DJ-1, *E. coli* YajL, and *S. pombe* DJ-1 can reduce the
418 levels of modifications derived from 1,3 bisphosphoglycerate suggests an alternative hypothesis
419 for the function of JCVISYN3A_0400 and other close DJ-1 homologs (54). It is possible that
420 these proteins share an evolutionarily conserved function in detoxifying an electrophilic cyclic
421 1,3 phosphoglycerate intermediate that is spontaneously formed by intramolecular cyclization of
422 1,3 bisphosphoglycerate (54). This metabolite should be formed in all organisms that use
423 glycolysis and thus provides a possible explanation for why the minimal Mycoplasma JCVI-
424 Syn3A would need to preserve this pathway.

425

426 **Conclusion**

427 Metabolite damage arising from side-reactions of enzymes and spontaneous chemistry has often
428 been ignored or seen as a minor metabolic inconvenience – even a trivial sideshow – that does
429 not warrant investment in enzymes to prevent or repair it (6). Biochemical, genetic, and
430 engineering evidence accumulating over the past decade have started to change this view (6, 8,
431 13, 15, 55, 56). The biochemical and genetic results presented here constitute persuasive
432 additional evidence by demonstrating that stripping a genome down to its barest essentials leaves

433 metabolite damage-control systems in place. Furthermore, our metabolomic and cheminformatic
434 results point to the existence of a network of metabolite damage and damage-control reactions
435 that extends far beyond the corners of it characterized so far. In sum, there can be little room left
436 to doubt that damage itself and the systems that counter it are mainstream metabolic processes.

437

438 **Methods**

439 **Bioinformatics**

440 The BLAST tools (57) and CDD resources at NCBI (<http://www.ncbi.nlm.nih.gov/>) (58) were
441 routinely used. Sequences were aligned using Clustal Omega (59) or Multialin (60).

442 Phylogenetic distribution was analyzed in the SEED database (61). Results are available in the
443 “YqeK” subsystem on the PubSEED server

444 (<http://pubseed.theseed.org//SubsysEditor.cgi?page=ShowSpreadsheet&subsystem=NadD->

445 [YqeK_fusion_display](http://pubseed.theseed.org//SubsysEditor.cgi?page=ShowSpreadsheet&subsystem=NadD-YqeK_fusion_display)). Physical clustering was analyzed with the SEED subsystem coloring tool

446 or the SeedViewer Compare Regions tool (61) and the clustering figure was generated with

447 GeneGraphics (62). Phylogenetic trees were constructed with Mega 6 (63). Student’s t-test

448 calculations were performed using the VassarStats web-tools (<http://vassarstats.net>).

449

450 **Prediction of novel potential chemistry using PickAxe**

451 Expanded chemistry was generated using the PickAxe app in KBase, as shown in this narrative:

452 <https://narrative.kbase.us/narrative/29280>. This app uses the open source RDKit package to

453 apply sets of SMARTS-based chemical reaction rules, derived from previously published

454 chemical damage (8) and enzyme promiscuity (35) studies, to an input set of compounds to

455 produce all possible reactions and products that might arise from that chemistry. This analysis

456 can be run iteratively through repeated application of the reaction rules to all new products that

457 arise from previous generations. We applied the PickAxe approach for six iterations, retaining all

458 compounds that matched the JCVI-Syn3A model, the ModelSEED database (34), or an observed

459 metabolite.

460

461 **Metabo-flux balance analysis to predict minimal reactions to reach observed metabolites**

462 In metabo-flux balance analysis (metabo-FBA), constraints are added to the standard FBA

463 formulation to force flux through one or more reactions involving an observed metabolite. In this

464 formulation, a variable is added for each observed peak (p_i) and a variable is added for each
465 metabolite that has been mapped to the peak (because peaks lack stereochemistry, they may be
466 mapped to multiple possible stereoisomers). Next, a constraint is added stating that a peak cannot
467 be active unless one or more of its associated metabolites is active (where $\lambda_{i,j}$ is a mapping
468 variable equal to 1 if metabolite j is mapped to peak i and zero otherwise):

$$p_i \leq \sum_j^{Compounds} \lambda_{i,j} m_j$$

469 A constraint is also added stating that no metabolite can be active unless at least one
470 reaction in which the metabolite is involved is carrying flux (where $\gamma_{j,k}$ is a mapping variable
471 equal to 1 if metabolite j is involved in reaction k and zero otherwise):

$$m_j \leq \sum_k^{Reactions} 100\gamma_{j,k} v_k$$

472 To maximize active metabolites, the objective of the problem is then set to maximize the
473 sum of all p_i . While p_i and m_j can be specified as binary variables, it works equally well and is
474 less computationally expensive to use continuous variables bounded between 0 and 0.1. To avoid
475 the trivial solution of activating metabolites by pushing flux through both directions of reversible
476 reactions or around mass balanced flux loops, it is essential to also employ thermodynamics
477 constraints in some form in this formulation (64).

478

479 **Media, strains, and genetic manipulations**

480 All strains, plasmids and oligonucleotides used in this study are listed in Table A4 and Table A5.
481 Bacterial growth media were solidified with 15 g/l agar (BD Diagnostics Systems) for the
482 preparation of plates. *E. coli* were routinely grown on LB medium (BD Diagnostics Systems) at
483 37 °C unless otherwise stated. Transformations were performed following standard procedures
484 (62). IPTG (100 μ M), Ampicillin (Amp, 100 μ g/ml), Kanamycin (Km, 50 μ g/ml), l-Arabinose
485 (Ara, 0.02–0.2%), Chloramphenicol (Cm, 25 μ g/ml) and Rifampicin (Rif, 25 μ g/ml) were used
486 when appropriate. Bacterial M9 minimal medium (65), 0.4% (w/v) glucose was used either with
487 NH_4Cl (20 mM) or glycine (50 mM) as the nitrogen source. P1 transduction was performed
488 following the classical methods (66). The Kan^R marker was eliminated from the BW2113
489 $\Delta yajL::\text{Kan}^R$ strain by the procedure described by Cherepanov and Wackernagel (67).

490 Transductants from BW2113 $\Delta hchA::Kan^R$ to BW2113 $\Delta yajL$ were checked by PCR for
491 transduction of the $\Delta hchA::Kan^R$ allele into the recipient strains using primer pairs [DH492/493
492 (ext); DH494/495 (int) and DH480/481 (ext); DH482/483 (int)] respectively. Plasmid
493 constructions for expression JCVI-syn3A genes in *E. coli* are described in the supplemental
494 methods.

495 JCVI-syn3A is a near minimal bacterial cell first reported by Breuer *et al.* (3) that
496 contains a subset of the genes in *Mycoplasma mycoides* subspecies *capri* strain GM12.
497 Mycoplasmas were grown in SP4 broth (68) that contains 17% KnockOut Serum Replacement™
498 instead of 17% fetal bovine serum and is referred to as SP4-KO as described in the supplemental
499 Methods. Construction of gene knockout mutants in JCVI-Syn3A was a multistep process, and
500 two different protocols were used. These protocols are described in detail in the **Supplemental**
501 **data S2** file.

502

503 **Mutation frequency assays for *E. coli* derivatives**

504 Overnight cultures in LB with added antibiotics and arabinose (0.02%) were diluted 100-fold in
505 the same conditions and grown for another 24 h before dilutions were plated on LB and LB
506 rifampicin (25 µg/ml) to calculate a mutation ratio (Number of colonies on Rif x dilution factor) /
507 (Number of colonies on LB x dilution factor).

508

509 **Protein expression and purification and enzyme assays**

510 All characterized JCVI-syn3A encoded proteins were expressed as His-tagged variants in *E. coli*
511 and purified using Ni²⁺-NTA columns as described in Supplemental Methods. In vitro activity
512 assays for CoA disulfide reductase, for phosphatase with a range of substrates, NadD,
513 glyoxalase, and deglycase are described in detail in Supplemental Methods.

514 The appendix and supplemental data have been deposited in the Figshare data depository with
515 the DOI: 10.6084/m9.figshare.20020574.

516

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520

521 **Table 1. Members of the HAD family of unknown function encoded by JCVI-Syn3**

Gene	Family	Essential	Best 3 substrates Activity <i>in vitro</i> *	Physical clustering	<i>M. florum</i> ortholog locus tag and essentiality**
JCVISYN3A_0066	Cof subfamily of IIB subfamily of HAD superfamily	no	pNPP, FMN, CoA	Between 5S rRNA gene and thioredoxin	Mfl169 (NE)
JCVISYN3A_0077	Cof-like hydrolase, HAD superfamily	no	Fru-1P, Ery-4P	Between <i>tsaD</i> and <i>aspS</i>	Mfl614 (E)
JCVISYN3A_0710	Cof subfamily of IIB subfamily of HAD superfamily	yes	Could not clone	Between tRNA genes and predicted phosphonate transporter genes	Mfl513 (E)
JCVISYN3A_0728	HAD superfamily hydrolase subfamily IIB, protein	no	GMP XMP 2-deoxy-glucose- 6P	Between glycolysis genes	Mfl503 (E)
JCVISYN3A_0907	Cof-like hydrolase, HAD superfamily	no	N-acetyl-D- glucosamine-6P Fructose-1P N-acetyl-D- glucosamine-1P	Between YidC and choline kinase-like	Mfl680 (NE)

522 *Abbreviations in Table S1; ** (E), essential; (NE)=non-essential in *M. florum*

523

524

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- 707

708 **Figure legends**

709 **Figure 1. 5-FCL activity is encoded by JCVI_0400.** (A) Enzymatic source and repair of 5-
710 CHO-THF. (B) Growth phenotype of a WT *E. coli* BW25113, $\Delta ygfA$ mutant and, $\Delta ygfA$
711 mutant expressing JCVI_0443 gene on M9 minimal medium (0.4% glucose) with (1) 20 mM
712 NH_4Cl or (2) 50 mM glycine as sole nitrogen source. Plates were incubated for 3 days at 37°C.

713

714 **Figure 2. Predicted and validated redox buffering systems in JCVI-Syn3.** (A) Candidates for
715 H_2O_2 detoxification systems of JCVI3, experimentally validated are in solid arrows, only the
716 number of the locus tags are given, P is for protein, R is for small molecule. The predicted source
717 of reductant is NADPH (B) CoADR Michaelis-Menten saturation curve for the determination of
718 the K_m and k_{cat} for CoAD consumption. (C) CoADR is specific towards oxidized CoA with no
719 activity towards other tested disulfides

720

721 **Figure 3. Heatmap including 15 metabolites from JCVI-syn3A mutant metabolomic**
722 **analysis with highest VIP scores.** Samples and genotypes are represented in columns. High
723 intensity measurements as compared to average intensity are red/yellow, and low intensity
724 measurements are represented by green/blue

725

726 **Figure 4. Predicted Hydrolase of unknown function is clustered or fused to NadD in many**
727 **Firmicutes** (A) Predicted NADP^+ synthesis pathway in JCVI-Syn3. (B) Physical clustering and
728 fusions of *nadD* and *yqeK* homologs in several gram-positive Bacteria. The RefSeq identifiers
729 for the *yqeK* genes used in descending order are: NP_975428.1, NP_390441.1, NP_372117.1,
730 NP_816490.1, YP_140036.1. (C) Docked model of 2-deoxy-NaAD bound to the C.

731 *acetobutylicum* YqeK (pdb code: 3CCG). The protein is shown in ribbon format (grey) with side
732 chains as lines, two iron atoms are shown as spheres bound to the diphosphate of
733 dNaAD. Tyrosine 82 (green) is modeled as two conformations in the crystal structure and forms
734 a close interaction with the 2' carbon of dNaAD.

735

736 **Figure 5. Biochemical analysis of the NadD and YqeK activities** (A) Relative reaction rates of
737 *Bacillus subtilis* and JCVI syn3.0 NadD enzymes with NaMN and various nucleotides,
738 calculated as percentage of the canonical reaction with ATP for each NadD enzyme. Enzymes

739 were incubated with 2 mM NTP, 0.5 mM NaMN, 4 mM MgCl₂ and 5 u/ml yeast inorganic
740 pyrophosphatase for 5 min at 37° C. H230A has the conserved H in the active site of the YqeK
741 domain mutated to ablate the HD activity and cleavage of nucleotides. **(B)** Activity of the
742 expressed JCVI syn3.0 YqeK domain with different substrates. YqeK (0.2 µg) was incubated
743 with 0.5 or 0.05 mM substrates, 1 mg/ml BSA and 2.0 mM MgCl₂ for 20 min at at 37° C. Black
744 bars are data for 0.5 mM substrates, white bars are data for 0.05 mM substrates. **(C)** Mutation
745 ratio on LB rifampicin for strain $\Delta mutT$ with empty vector (pBAD24), $\Delta mutT$ with E. coli *mutT*
746 in trans, $\Delta mutT$ with either the *nadD-yqeK* fusion gene JCVI_0380, or the *nadD* or *yqeK*
747 domains alone. *** indicates a P-value <0.001 with experiments performed with four biological
748 replicates and four technical replicates.

749
750 **Figure 6. Number of predicted potential metabolites arising from promiscuous enzymatic**
751 **reactions and spontaneous/damage chemistry operating on known compounds in JCVI-**
752 **syn3 metabolism.** Total predicted metabolites are shown, as well as the number of metabolites
753 matching observed peaks (blue line) or ModelSEED compounds (green line). The x-axis
754 indicates the number of reactions steps explored outward from the known JCVI-syn3
755 metabolism, while the y-axis shows the number of new metabolites predicted with each new
756 reaction step.

757
758 **Figure 7. Map of predicted extensions to the JCVI-syn3 model to push flux through as**
759 **many observed peaks as possible.** Reactions and metabolites are color coded as shown in the
760 figure's inset. Model reactions with no flux (black); and with flux (magenta). Predicted and
761 active reactions that are in the database (green); or that are novel and spontaneous (red); or that
762 are novel enzymatic ones (blue). All active predicted spontaneous reactions and nearly all active
763 model reactions are shown on the map; some ModelSEED and predicted enzymatic reactions are
764 excluded. The color code for metabolites is as follows: absent in the mass spec analysis (white);
765 observed metabolites that are also in the model (yellow); in the database (ochre); or novel in
766 themselves or in the way they are produced (brown). Most enzymatic reactions are identified by
767 their EC numbers. Some reactants' names have been omitted since they don't give relevant
768 information. Common abbreviations have been used for the name labels. The map has been
769 divided by panels shown on the figure's background. These panels are labeled according to the

770 major pathway they display. The complete metabolic map in interactive format (Escher map) is
771 given in the supplemental data S5 material.

772

773 **Figure 8. Characterization of JCVI_0400.** (A) Growth of WT, $\Delta yajL$, $\Delta yajL \Delta hchA$, $\Delta yajL$
774 $\Delta hchA$ with *hchA* in trans and $\Delta yajL \Delta hchA$ with JCVI_0400 in trans. pUC19 was used as
775 empty vector. Each strain was tested in 5 replicates Plates were incubated 2 days at 37°C in LB
776 with agitation in a Bioscreen C device. (B) Methylglyoxalase activity of JCVIsyn3A_0400
777 compared to human DJ-1 (DJ1) and *E. coli* YajL. Conversion of methylglyoxal to L-lactate was
778 measured in a coupled assay with L-lactate oxidase and Amplex red. Data were measured in
779 triplicate with error bars shown (sometimes smaller than the symbol) and fitted using the
780 Michaelis-Menten model. JCVIsyn3_0400 is a weak methylglyoxalase.

781

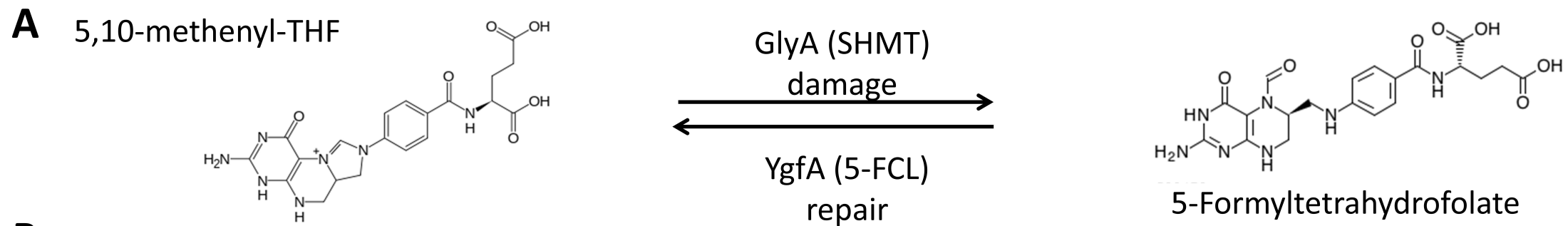
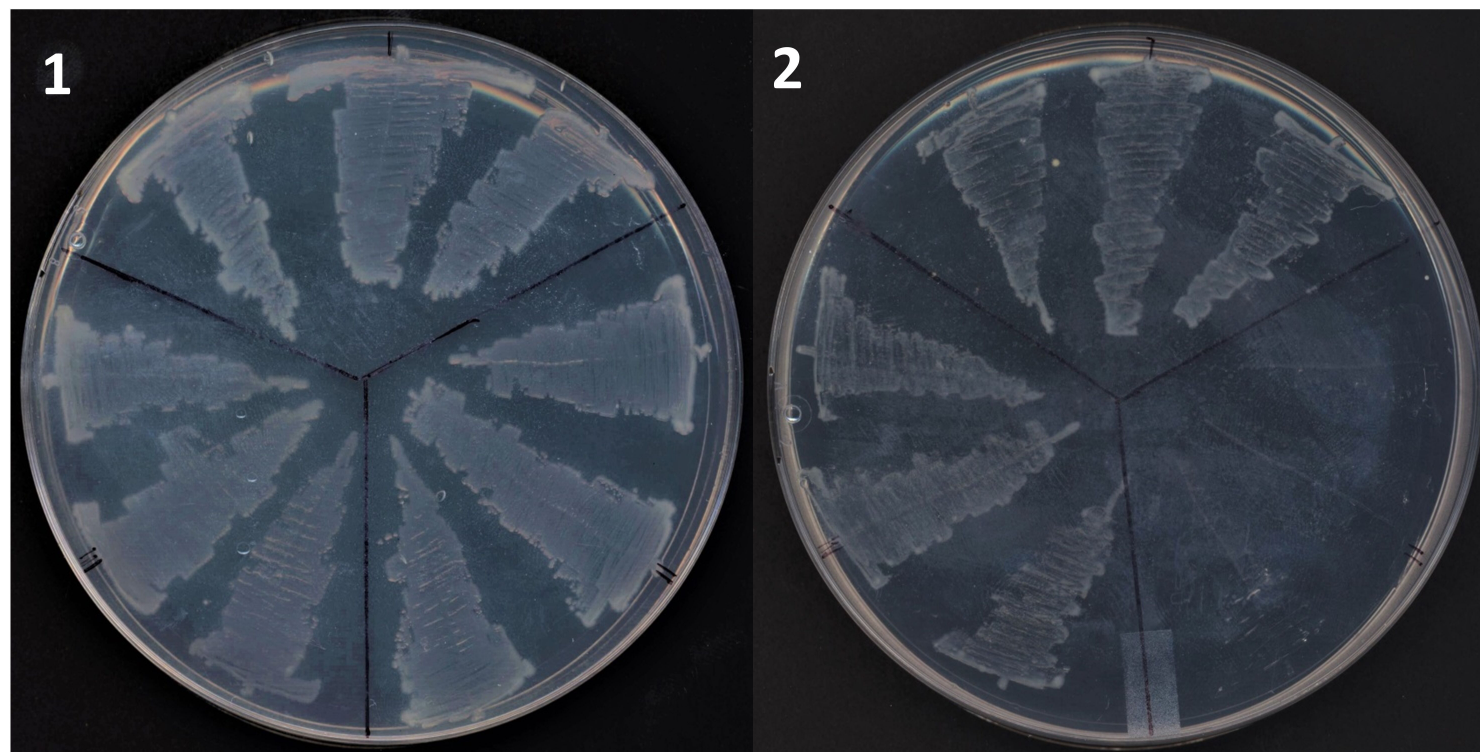
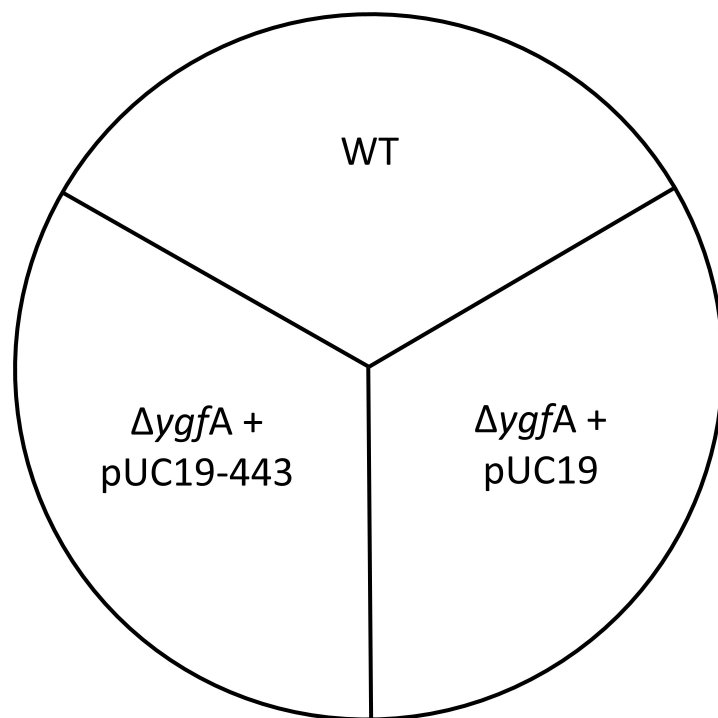
**B**

Figure 2

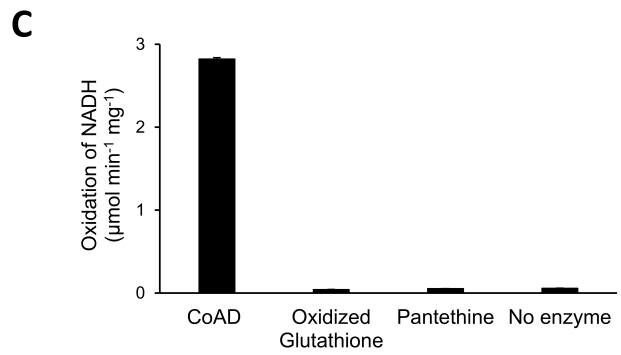
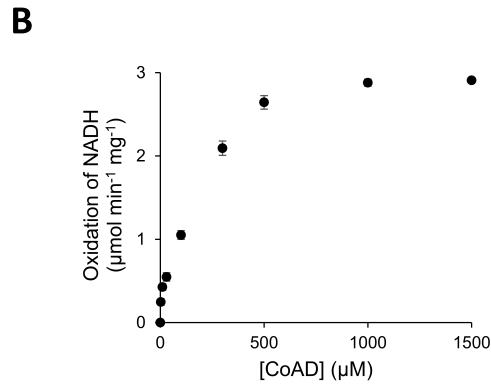
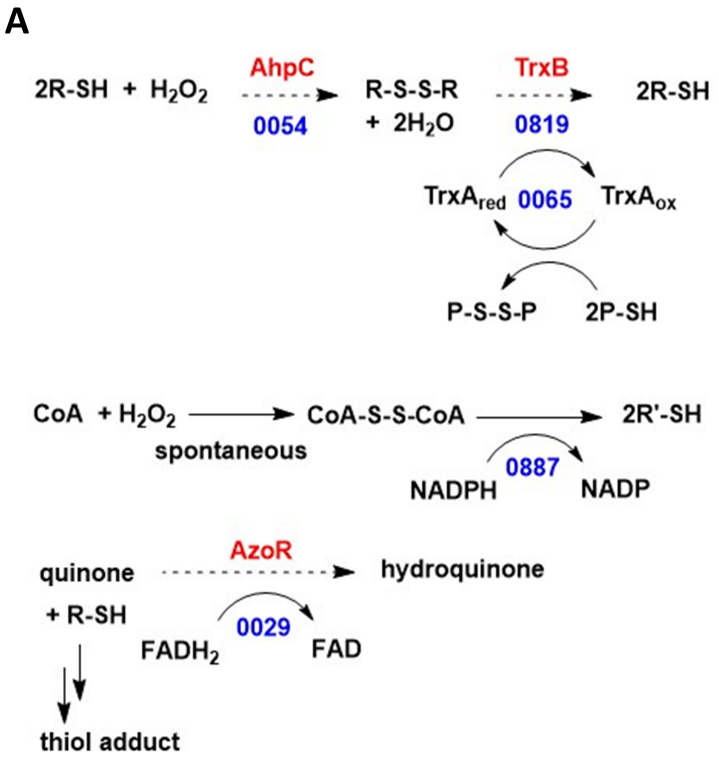


Figure 3

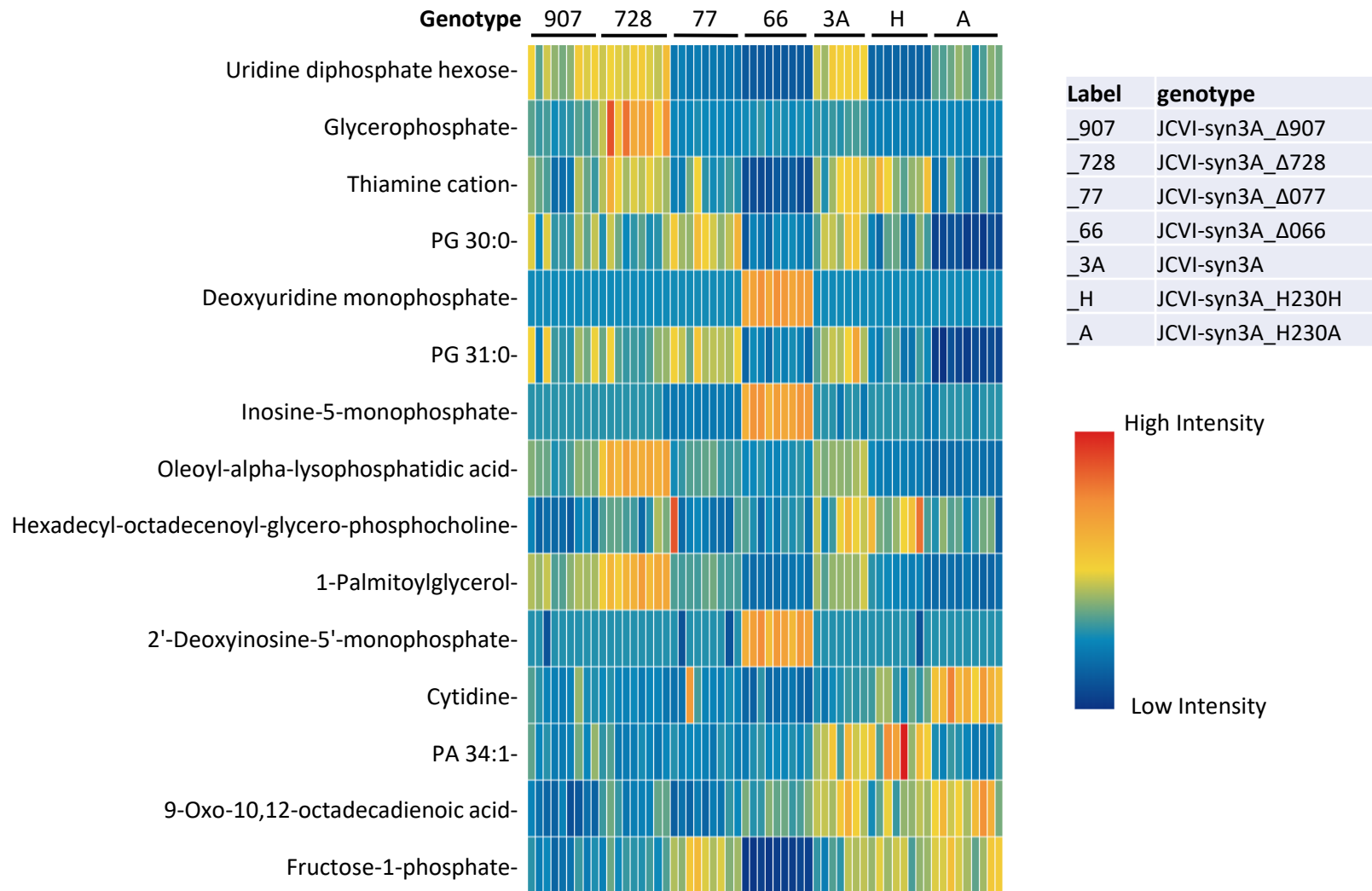
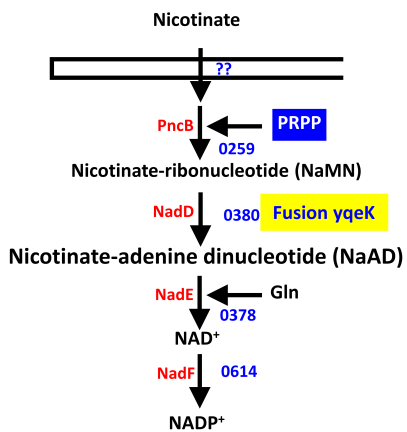


Figure 4

A



B

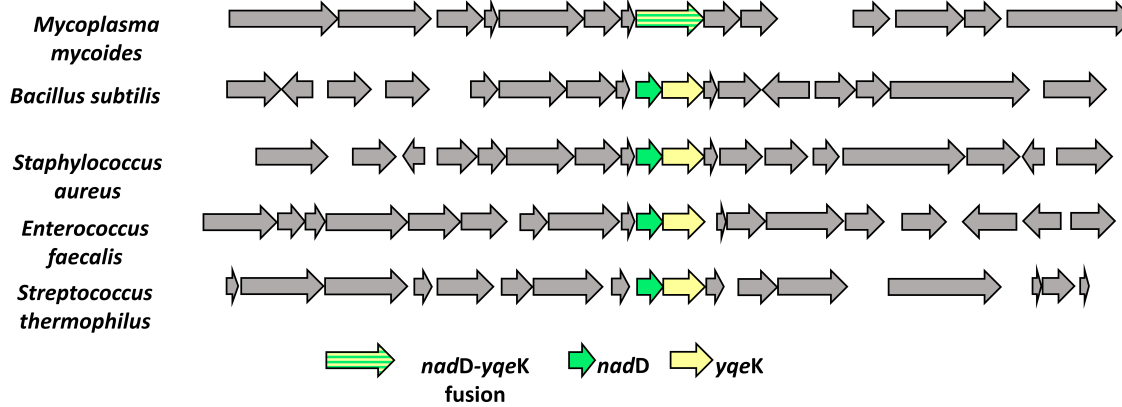
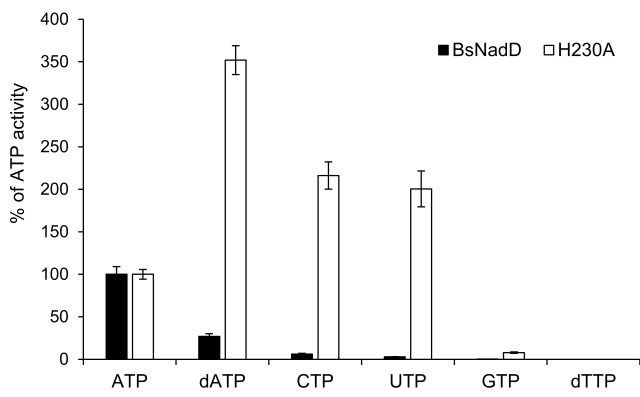
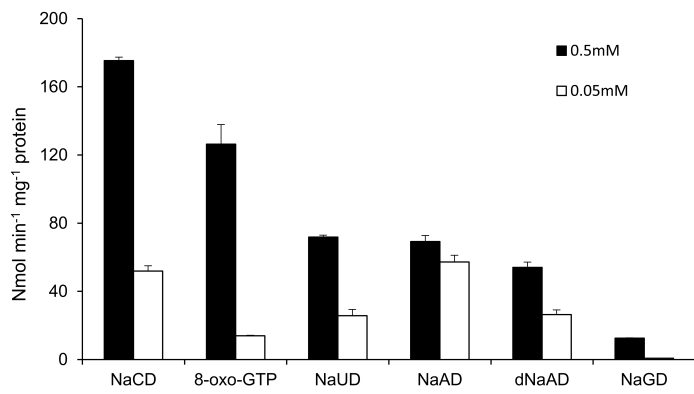


Figure 5

A



B



C

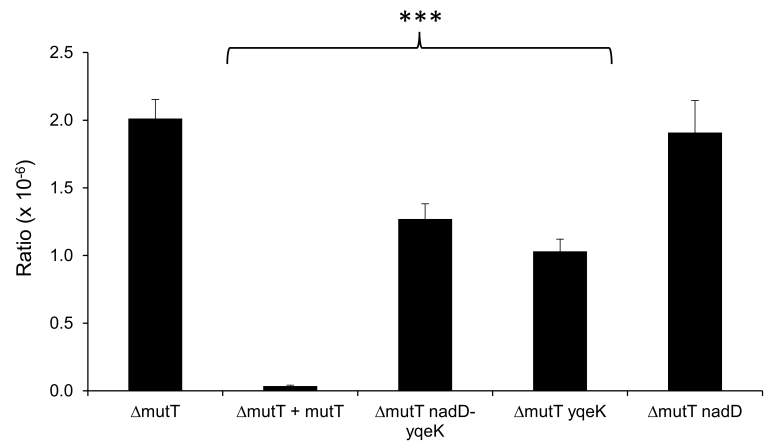
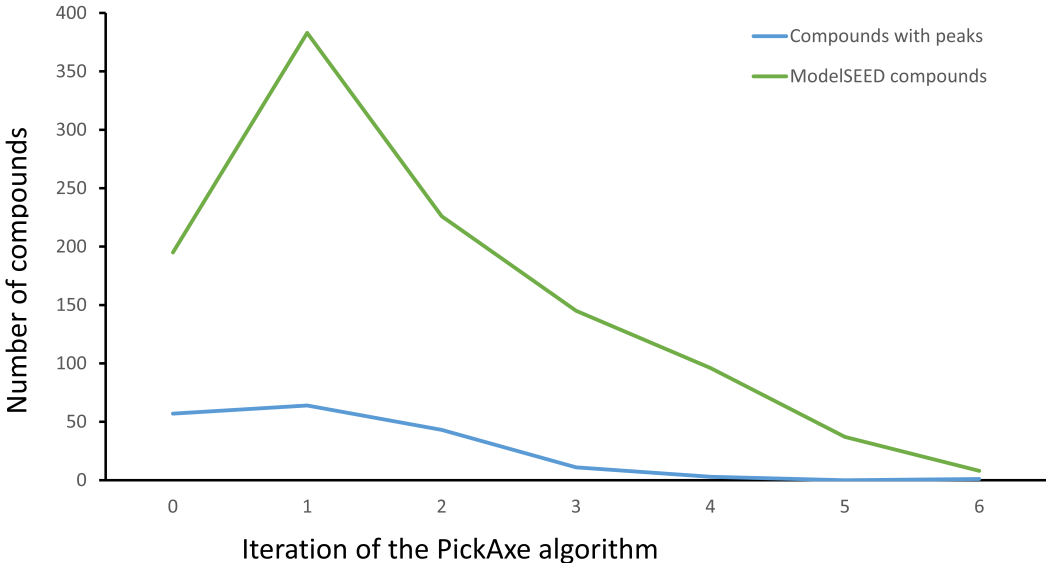


Figure 6



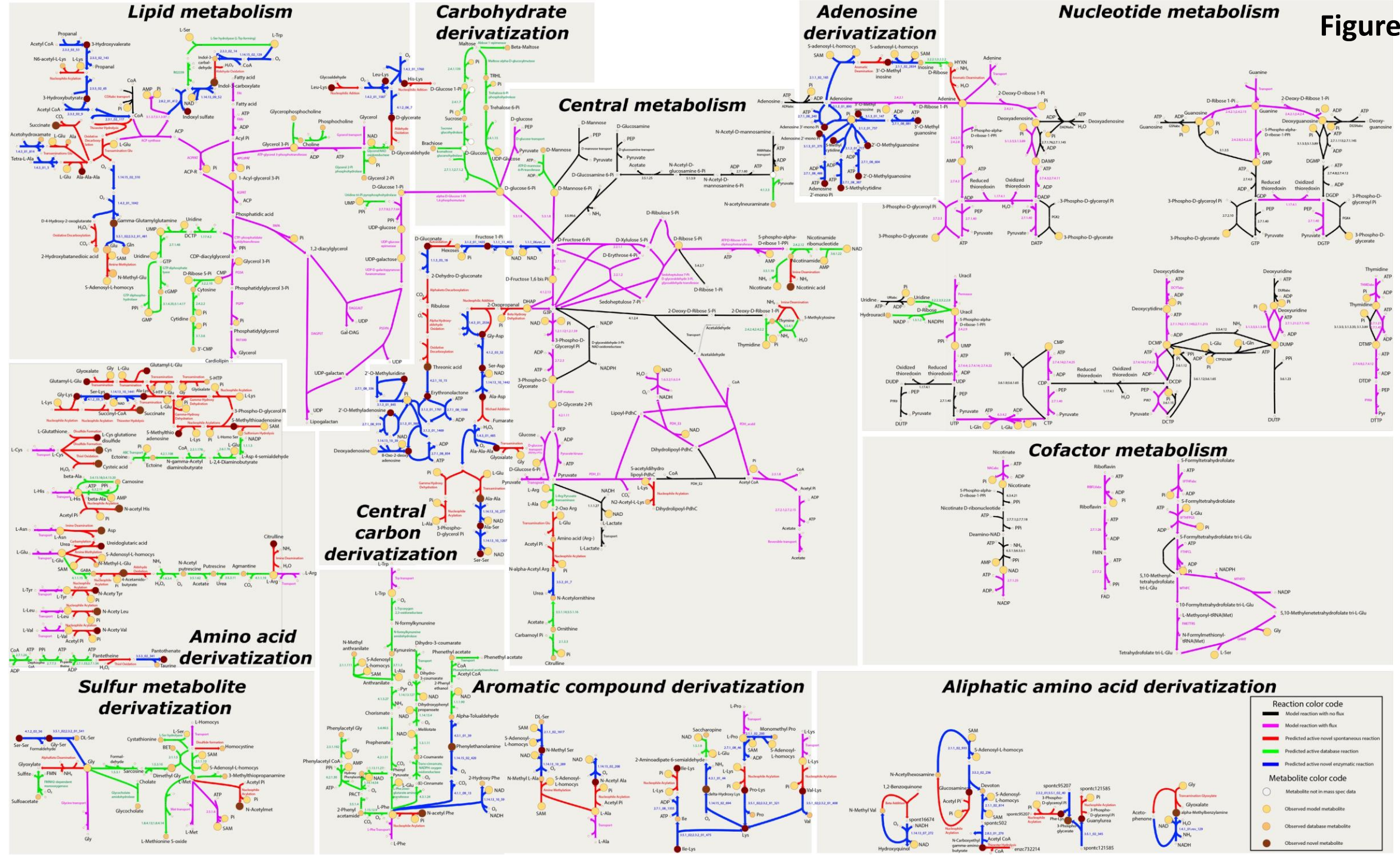


Figure 8

