A random mutagenesis screen enriched for missense mutations in bacterial effector proteins.

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Abstract

To remodel their hosts and escape immune defenses, many pathogens rely on large arsenals of proteins (effectors) that are delivered to the host cell using dedicated translocation machinery. Effectors hold significant insight into the biology of both the pathogens that encode for them and the host pathways that they manipulate. One of the most powerful systems biology tools for studying effectors is the model organism, *Saccharomyces cerevisiae*. For many pathogens, the heterologous expression of effectors in yeast is growth inhibitory at a frequency much higher than housekeeping genes, an observation ascribed to targeting conserved eukaryotic proteins. Abrogation of yeast growth inhibition has been used to identify bacterial suppressors of effector activity, host targets, and functional residues and domains within effector proteins. We present here a yeast-based method for enriching for informative, in-frame, missense mutations in a pool of random effector mutants. We benchmark this approach against three effectors from *Legionella pneumophila*, an intracellular bacterial pathogen that injects a staggering >330 effectors into the host cell. For each protein, we show how *in silico* protein modeling (AlphaFold2) and missense-directed mutagenesis can be combined to reveal important structural features within effectors. We identify known active site residues within the metalloprotease RavK, highly conserved residues in SdbB, and previously unidentified functional motifs within the C-terminal domain of SdbA. We show that this domain has structural similarity with glycosyltransferases and exhibits *in vitro* activity consistent with this predicted function.

Introduction

For many bacterial pathogens, host manipulation derives from the collective activity of large numbers of translocated proteins ("effectors") that are injected into the host cell using dedicated

46 secretion machinery. A striking example is the gram-negative bacterium, *Legionella*
47 *pneumophila*, the causative agent of Legionnaires' disease, an often-fatal severe pneumonia that
48 naturally replicates in freshwater protozoa. *L. pneumophila* encodes for the largest effector
49 arsenal described to date (>330 effectors per isolate, or roughly 10% of the proteome) (Burstein
50 *et al.* 2009; Huang *et al.* 2011; Zhu *et al.* 2011), which is injected into the host cell using the
51 Dot/Icm type IVB secretion system (Segal *et al.* 1998; Vogel *et al.* 1998). *L. pneumophila*
52 effectors modulate host vesicle trafficking, post-translational modification, protein translation,
53 autophagy, vacuolar function, and the cytoskeleton to avoid lysosomal fusion and to establish a
54 replicative, neutral pH vacuole (Isberg *et al.* 2009; Escoll *et al.* 2013; Sherwood and Roy 2016;
55 Qiu and Luo 2017). While over 50 effectors have been studied, the majority of these remain
56 uncharacterized (Finsel and Hilbi 2015).
57 Determining the function of the >330 effectors and the role they play in establishing the
58 *Legionella*-containing vacuole is complicated by extensive genetic redundancy within the
59 effector arsenal (O'Connor *et al.* 2011) and the lack of predicted conserved domains or functions
60 for many substrates (Gomez-Valero *et al.* 2011, 2014; Burstein *et al.* 2016). In a study
61 comparing genomes from 38 *Legionella* species, only half of the predicted effectors contained
62 conserved domains and many of these are of uncharacterized function (Burstein *et al.* 2016).
63 Although the amino acid sequence of many effectors may not yield obvious clues to their
64 function, some effectors have structural homology to characterized proteins or domains, along
65 with conserved active site motifs or other signature motifs (Toulabi *et al.* 2013; Morar *et al.*
66 2015; Wong *et al.* 2015; Urbanus *et al.* 2016; Pinotsis and Waksman 2017; Kozlov *et al.* 2018).
67 Looking beyond *L. pneumophila*, over 18,000 effector genes have been predicted across the

68 entire *Legionella* genus (Burstein *et al.* 2016; Gomez-Valero *et al.* 2019). This number suggests
69 both a wealth of novel effector activities and host biology waiting to be discovered.
70 We set out to develop a method to efficiently identify important motifs or amino acid
71 residues in uncharacterized *Legionella* effectors by random mutagenesis and selection for loss-
72 of-function mutations to facilitate the prediction of mechanism and function. Approximately
73 10% of the effectors severely inhibit yeast growth when overexpressed (Campodonico *et al.*
74 2005; Felipe *et al.* 2008; Heidtman *et al.* 2009; Shen *et al.* 2009; Guo *et al.* 2014; Urbanus *et al.*
75 2016), such that loss-of-function by random mutagenesis can be selected for as an alleviation of
76 the yeast growth defect. However, a random mutant pool contains a multitude of mutations that
77 can potentially cause a loss-of-function phenotype, such as frameshift, nonsense and missense
78 mutations in the effector or regulatory elements such as the promoter region. Most of these
79 mutations are uninformative as only missense loss-of-function mutations in the effector are
80 relevant. To enrich for full-length missense clones, we used a C-terminal in-frame His3 fusion
81 and selected for histidine prototrophic yeast requiring the presence of a full-length protein. A
82 similar strategy (C-terminal His3 fusions) was previously shown to enrich for in-frame human
83 open reading frames amongst a randomly primed pool of cDNAs cloned into a yeast expression
84 vector (Holz *et al.* 2001). Here, we benchmark this in-frame mutagenesis approach against three
85 *L. pneumophila* effectors previously shown to inhibit yeast growth: SdbA and SdbB, whose
86 functions remain uncharacterized and RavK, a previously described metalloprotease. We show
87 our approach identifies active site residues within RavK (Liu *et al.* 2017) highly conserved
88 residues in SdbB, and previously unidentified functional motifs in the C-terminal domain of
89 SdbA. These motifs are part of the donor and acceptor binding regions of glycosyltransferases,

90 which we show the C-terminal domain of SdbA shares homology with. Finally, we show that a

91 C-terminal fragment exhibits *in vitro* activity consistent with this predicted function.

92 **Results**

93 **A random mutagenesis screen to identify regions important for bacterial effector function**

94 To efficiently screen for randomly generated mutations in *Legionella* effectors that cause a loss-
95 of-function phenotype and represent full-length protein rather than frameshift or nonsense
96 mutations, we applied the method of Holz and colleagues to select for expression of full-length
97 proteins in yeast (Holz *et al.* 2001). We cloned the yeast *HIS3* gene in frame behind yeast-
98 cytotoxic *Legionella* effectors on a high-copy galactose-inducible yeast expression plasmid.
99 After confirming that the His3 fusion does not interfere with the yeast-cytotoxic phenotype, and
100 therefore likely does not interfere with effector function, we generated a pool of random mutants
101 using the *E. coli* mutator strain XL1-Red. We then transformed this mutant plasmid pool to yeast
102 and monitored growth on different media types to assess the number of transformable plasmids (-
103 ura/glucose) and the efficiency of the random mutagenesis step (-ura/galactose) selecting all
104 mutations that caused a loss of function (Fig 1). To specifically select for full-length missense
105 loss-of-function mutants, we grew the transformed pool on medium with galactose and lacking
106 uracil and histidine (-ura-his/galactose), which requires the production of full-length effector-
107 His3 fusion protein and mutations in the effector that disrupt its activity.

108

109 **Missense loss-of-function screen identifies conserved SdbB amino acid residues**

110 As a proof of principle, we looked at SdbB which severely inhibits yeast growth (Fig 2A)
111 (Heidtman *et al.* 2009) and is part of the SidB paralog family, whose members are predicted to
112 be lipases from the α/β hydrolase enzyme family (Luo and Isberg 2004). After verifying that the
113 SdbB-His3 fusion was still capable of inhibiting yeast growth (Fig 2A) we created a random
114 mutagenesis pool of SdbB-His3 and quantified the number of colony-forming units (CFUs) on

115 the different selection media. While 1.89% of the transformable plasmids carried a mutation that
116 allowed for growth on SD-ura/galactose medium indicating some type of loss-of-function
117 mutation (Fig 2B), only 0.03% of the transformable plasmids carried a mutation allowing growth
118 on SD-ura/-his/galactose medium which requires the expression of full-length fusion protein.
119 The efficiency of the histidine selection step was verified by sequencing 20 clones from each
120 condition. The loss-of-function clones selected on –ura/galactose consisted of 16 frameshift
121 mutations, 2 nonsense mutations, 1 combination of a missense and frameshift mutation and 1
122 missense mutation in the *sdbB* gene (Fig 2C). In contrast, the loss-of-function clones selected on
123 –ura/-his/galactose only contained missense mutations (Fig 2D,F). For both conditions a number
124 of mutations were recovered several times suggesting that the mutational screen is reaching
125 saturation. To confirm that the identified mutations indeed rescued SdbB toxicity in yeast, we
126 compared the fitness of mutants to an empty vector control in a liquid growth curve assay (see
127 Experimental methods). The wild-type and His3-fused SdbB almost completely inhibited yeast
128 growth, while the SdbB mutants showed a fitness of 60-90% compared to an empty vector
129 control (Fig 2E and 2G).

130 The positions of the frameshift and nonsense mutations in SdbB (Fig 2D) indicate that a
131 large part of the protein is required for function, as even a nonsense mutation at S365, 84 amino
132 acid residues from the C-terminus, almost completely rescued activity. The missense loss-of-
133 function mutations (Fig 2D and 2F) target four amino acid residues (G116, G189, D273 and
134 H351) that are invariant in SdbB orthologs from *Legionella pneumophila* and other *Legionella*
135 species (Burstein *et al.* 2016) (Fig S1), suggesting they are essential for function or structure.
136 The G198E mutation is part of the GXS/CXG motif that is invariant across the SdbB orthologs
137 and is predicted to align with the so-called nucleophile elbow of the nucleophile-acid-base triad

138 of the α/β hydrolase active site (Brenner 1988; Ollis *et al.* 1992; Schrag and Cygler 1997;
139 Marchler-Bauer *et al.* 2017) (Fig S2). When the mutations are mapped onto the SdbB
140 AlphaFold2 model (Jumper *et al.* 2021; Varadi *et al.* 2021) the majority localize in the pocket
141 with the putative catalytic cysteine (C187), including the invariant D273 and H351 residues
142 captured in the screen suggesting they are the remaining residues of the catalytic triad (Fig 2H).
143 Thus, the SdbB example demonstrates that functionally important amino acid residues
144 can be identified using the random mutagenesis method in conjunction with the histidine
145 selection for full-length protein. Importantly, this approach significantly reduces the number of
146 sequenced clones required to identify amino acid residues or regions of interest, by
147 approximately 60-fold in the case of SdbB.
148
149 **Missense loss-of-function screen identifies the active site of the characterized effector RavK**
150 To benchmark the missense loss-of-function screen on a characterized effector, we looked at
151 RavK which also severely inhibits yeast growth (Heidtman *et al.* 2009). RavK was recently
152 shown to be a small, soluble metalloprotease that specifically cleaves host actin, and mutation of
153 the active site motif HExxH abolishes all activity and yeast toxicity (Liu *et al.* 2017). The RavK-
154 His3 fusion was still able to inhibit yeast growth (Fig 3B) and subjected to random mutagenesis
155 and selection for loss-of-function mutants on medium lacking histidine. Of the 14 loss-of-
156 function clones we sequenced, one clone contained a large, in-frame deletion from amino acid
157 residue 70 to residue 166, which was unexpected but confirms the strength of the histidine
158 selection for full-length protein. All other loss-of-function clones were caused by single point
159 mutations resulting in missense mutations (Fig 3A). In the fitness assay, wild-type and His3-
160 fused RavK almost completely inhibited yeast growth, while the RavK mutants displayed a

161 fitness of 70-90% compared to the empty vector control. The loss-of-function mutations all map
162 to the first half of RavK, suggesting that the N-terminal half of RavK is essential for RavK
163 function. This is in agreement with Liu and colleagues (Liu *et al.* 2017), who identified the
164 active site motif ($_{95}$HExxH$_{99}$) in the N-terminal half of the protein and found that the 50 C-
165 terminal residues of RavK can be deleted without any effect on its activity on actin.

166 Four of the loss-of-function mutations targeted the active site motif H$_{95}$ExxH$_{99}$, three of
167 which are mutations to E$_{96}$. Our screen also identified several residues outside of this motif that
168 are critical for RavK function. To investigate why these might be functionally important, we
169 performed an HHpred analysis which looks for structural homologs of proteins (Zimmermann *et*
170 *al.* 2018). HHpred identified many hits with homology to the HExxH metalloprotease motif.
171 Among the top five HHpred hits are three small soluble metalloproteases or minigluzincins,
172 anthrax lethal factor and a zinc dependent peptidase from the M48 family (Dalkas *et al.* 2010;
173 López-Pelegrín *et al.* 2013; López-Pelegrín *et al.* 2014) (Fig S3). Notably, some of the other
174 loss-of-function mutations occur in areas that have homology with structural elements in the
175 minigluzincins that contribute to the active site cleft (López-Pelegrín *et al.* 2013) (Fig S3).
176 Indeed, when the missense mutations are mapped on the RavK AlphaFold2 model (Jumper *et al.*
177 2021; Varadi *et al.* 2021) (Fig 3C) they cluster around the active site HExxH including the top
178 rim of the active site cleft.

179

180 **The C-terminal domain of SdbA is a putative glycosyltransferase**

181 SdbA is a member of the SidB paralog family (Luo and Isberg 2004). While the function of
182 SdbA remains undefined, experimental evolution of *Legionella* in mouse macrophages selected
183 for parallel SdbA nonsense and frameshift mutations in three out of four independent lineages

184 (Ensminger *et al.* 2012), suggesting that its normal function partially restricts growth in this
185 accidental host. While the N-terminal domain of SdbA has homology with SidB (Luo and Isberg
186 2004), the additional C-terminal domain does not have significant sequence homology to other
187 known proteins (data not shown). SdbA completely inhibits yeast growth when heterologously
188 expressed (Heidtman *et al.* 2009), making the missense loss-of-function screen an informative
189 tool by which to identify functional residues that might suggest a specific activity inside the
190 eukaryotic cell.

191 The missense loss-of-function screen identified 19 mutations in 24 sequenced clones
192 targeting 17 amino acid residues in SdbA (Fig 4A). In contrast to the smaller proteins SdbB and
193 RavK, the SdbA results included several double mutants. In some cases, it is not clear how much
194 each mutation contributes to the phenotype, considering that the fitness of the double mutants is
195 not dramatically different from the single loss-of-function mutations (Fig 4B). However, all the
196 single mutations that lead to a loss-of-function phenotype fall in the C-terminal domain and
197 mainly concentrate in two regions: $_{541}$GGTGHIS$_{547}$ and $_{957}$GGLSVME$_{963}$. An HHpred homology
198 search (Zimmermann *et al.* 2018) predicted with high confidence that the C-terminal domain is a
199 glycosyltransferase of the GT-B fold. When comparing the SdbA C-terminal domain with the
200 sequence of *E. coli* MurG, a well-studied member of the GT-B fold glycosyltransferase family,
201 the two mutated regions align with the G-loop 1 and a consensus region in GT-B fold
202 superfamily involved in binding the donor molecule (Ha *et al.* 2000; Hu *et al.* 2003; Crouvoisier
203 *et al.* 2007) (Fig 4C). Glycosyltranferases hydrolyze UDP-sugar donor molecules and transfer
204 the sugar to the acceptor molecule – which can be a variety of molecules such as small
205 molecules, lipid or proteins (Lairson *et al.* 2008). In MurG amino acid residues A263, L264,
206 L265, E268, Q287 and Q288 contact the donor molecule UDP-GlcNAc (Hu *et al.* 2003) (Fig4C)

207 while the G-loop 1 is thought to be involved in acceptor molecule binding (Ha *et al.* 2000).
208 Notably, mutations in these motifs abrogate MurG enzymatic activity, including mutation of the
209 residues H18 and E268 (Hu *et al.* 2003; Crouvoisier *et al.* 2007), whose corresponding residues
210 in SdbA (H545 and E963) were found to be mutated in our screen. Fig 4D shows the single
211 missense mutants abrogating SdbA activity mapped onto the AlphaFold2 model (Jumper *et al.*
212 2021; Varadi *et al.* 2021)of a C-terminal fragment (amino acid residues 510-1050) with the
213 residues H545 and E963 highlighted in yellow (43)
214 To test whether the C-terminal domain of SdbA is indeed a glycosyltransferase, we
215 purified the C-terminal fragment 510-1050 and the equivalent of the MurG E268A inactive
216 mutant in SdbA (E963A) and tested several UDP-sugars as substrate using the UDP-Glo assay
217 (Fig 4E). Glycosyltransferases can hydrolyze UDP-sugars in the absence of an acceptor molecule
218 (with water acting as an acceptor in the reaction)(Sheikh *et al.* 2017; Vicente *et al.* 2023). Indeed
219 wild-type SdbA 510-1050 hydrolyzes the UDP-GlcNAc donor while the E963A mutant cannot
220 (Fig 4E), confirming that SdbA is a glycosyltransferase with specificity for UDP-GlcNAc and
221 that a mutation in the E963 residue identified by the missense loss-of-function screen abrogates
222 that activity. Using the same assay, we determined the kinetic parameters of UDP-GlcNAc
223 hydrolysis by SdbA (Fig 4F), which revealed high affinity (low micromolar Km) of this enzyme
224 to UDP-GlcNAc suggesting efficient glycosylation activity *in vivo.*
225 Taken together, the missense loss-of-function screen identified critical residues in the C-
226 terminal domain of SdbA, which together with HHpred analysis predict that SdbA contains a
227 glycosyltransferase domain. The discovery and confirmation of the C-terminal
228 glycosyltransferase domain allows for targeted follow-up experiments to elucidate the action and
229 target of SdbA on eukaryotic host cells.
230

231 **Discussion**

232 Our observations demonstrate that the combination of a random mutagenesis loss-of-function
233 screen with a selection for full-length protein is very effective in specifically selecting loss-of-
234 function missense clones. In fact, all but one of the clones recovered in our assay contained
235 missense mutations, while the remaining one contained an in-frame deletion which included the
236 active site of RavK. The screens correctly identified the previously described active site of
237 RavK, and the predicted active site nucleophile motif and the remaining residues of the catalytic
238 triad of SdbB. Surprisingly, the N-terminal α/β hydrolase domain of SdbA did not accumulate
239 loss-of-function mutations, but the C-terminal domain appears to be crucial for the function of
240 SdbA. The prediction that the C-terminal domain of SdbA is a glycosyltransferase was supported
241 by *in vitro* activity towards UDP-GlcNAc and provides a way forward to study this *Legionella*
242 effector.

243 To effectively apply the random mutagenesis missense enrichment selection or to extend
244 this approach to bacteria and mammalian cells, a number of considerations need to be taken into
245 account. First, the protein of interest must have a selectable loss-of-function phenotype such as
246 alleviation of growth inhibition. Growth fitness is a universal phenotype that is easy to measure
247 in bacteria, yeasts and mammalian cell lines. Second, the C-terminal fusion of a selection marker
248 must not interfere with protein function. If the function of protein of interest is inhibited by the
249 C-terminal fusion, it could potentially be overcome by introducing linker regions of varying
250 length and flexibility (Chen *et al.* 2013) or by using a cleavable linker such as the ubiquitin K0
251 mutant that is processed by cytosolic deubiquitinases in eukaryotic cells (Bachran *et al.* 2013).
252 Similarly, the C-terminal selection marker must be able to function as a fusion protein or be
253 liberated by an *in vivo* cleavable linker.

254 To extend the random mutagenesis missense enrichment selection to bacteria, the
255 chloramphenicol acetyltransferase (CAT) gene which confers resistance to chloramphenicol
256 could be a good candidate as a C-terminal fusion partner. CAT has been successfully used in
257 protein fusions where it conferred chloramphenicol resistance during colony selection as a C-
258 terminal fusion partner, with increased selection efficiency when mutant fusion proteins are
259 soluble (Maxwell *et al.* 1999). In mammalian cell lines, a positive selection marker such as
260 blasticidin S deamidase could be used as a C-terminal fusion partner. Blasticidin S deamidase is
261 functional as a C-terminally fused protein (Suarez and McElwain 2009) and confers resistance
262 against blasticidin, which rapidly inhibits mammalian cell growth at a low dose (Kimura *et al.*
263 1994). An alternative, if no positive selection marker is available, is GFP, which has been used
264 extensively as a fused localization marker for various cellular compartments and organisms
265 (Margolin 2000; Roessel and Brand 2002; Huh *et al.* 2003). After a standard number of
266 generation doublings, GFP-positive cells, indicative of the presence of full-length protein, can be
267 selected by flow cytometry and cell sorting or by screening for GFP positive colonies in bacteria
268 and yeast. However, these approaches would reduce the screening throughput and/or require
269 specialized equipment.
270 Our initial results suggest that missense-directed mutagenesis will be a useful tool to help
271 identify potential functions for other bacterial effector proteins, many of which have low
272 sequence homology to characterized proteins (Gomez-Valero *et al.* 2011; Burstein *et al.* 2016).
273 Rather than being replaced by *in silico* protein modeling, we show how the two methodologies
274 complement one another and can be used to identify structural features essential for activity
275 against the eukaryotic cell. In *Legionella pneumophila* alone 10% of the translocated effectors
276 (Campodonico *et al.* 2005; Felipe *et al.* 2008; Heidtman *et al.* 2009; Shen *et al.* 2009; Guo *et al.*

277 2014; Urbanus *et al.* 2016), severely inhibit yeast growth and are possible candidates for the
278 random mutagenesis missense enrichment screen. Identifying functional residues within
279 uncharacterized effectors is a logical first step towards validating *in silico* protein models,
280 predicting effector activity, and designing protein-protein interaction studies.

281 **Experimental methods**

282 **In frame effector-His3 fusion by yeast recombinational cloning**

283 The *S. cerevisiae* BY4742 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*; (Brachmann *et al.* 1998))
284 strains overexpressing *lpg0275*, *lpg0969* and *lpg2482* (*sdbA, ravK,* and *sdbB* respectively) from
285 the high-copy vector pYES2 NT/A (Life Technologies, GAL 1 promoter, N-terminal 6X
286 HIS/Xpress tag and URA3 selectable marker) (Heidtman *et al.* 2009) were used to create the
287 effector-His3 fusion mutants by yeast recombinational cloning. The *S. cerevisiae HIS3* gene was
288 PCR amplified from pAG423GAL-ccdB (Alberti *et al.* 2007) using an effector specific forward
289 primer containing the last 50-60 nucleotides of the effector (minus the stop codon) followed by
290 the first 20-30 nucleotides of the *HIS3* sequence and the pYES-HIS3 reverse primer (Table S1).
291 The resulting PCR products were transformed together with XbaI/PmeI digested pYES2 NT/A
292 lpg0275, lpg0969 or lpg2482 to BY4742 using the high-efficiency LiOAC/PEG method (Gietz
293 and Schiestl 2007) and plated onto SD-uracil with 2% glucose. The resulting transformants were
294 screened by PCR and sequence verified. To confirm that the His3 fusion does not interfere with
295 effector function, the ability of the effector-HIS3 fusion to inhibit yeast growth was tested by
296 comparing the growth BY4742 with empty vector control, the wild-type effector and the
297 effector-HIS3 fusion in a yeast spot dilution assay as described previously (Urbanus *et al.* 2016).
298

299 **Selection of loss-of-function mutations**

300 The effector-HIS3 fusion vectors were mutagenized in XL1-red (Agilent) as per manufacturer's
301 instructions. XL1-red transformants were washed off the transformation plate, grown overnight
302 in 50 ml LB with ampicillin and the resulting mutant plasmid pool was purified by midiprep
303 (Promega). The mutant plasmid pool was transformed to BY4742 using the high-efficiency

304 LiOAC/PEG method (Gietz and Schiestl 2007). Four transformation reactions were performed
305 per screen, each using 1 μg of plasmid pool per reaction. One reaction was split in three parts
306 and plated onto different media types to quantify the transformable plasmids (SD- uracil + 2%
307 glucose), loss-of-function mutants (SD- uracil +2% galactose), and missense loss-of-function
308 mutants (SD-uracil/histidine + 2% galactose) and incubated for 2-4 days at 30°C. The remaining
309 transformation reactions were plated onto 150 mm SD-uracil/histidine + 2% galactose plates and
310 allowed to grow until colonies appeared (3-4 days). Plasmids were rescued from missense loss-
311 of-function mutants and transformed to the *E. coli* Top10 strain before sequencing using primers
312 in the vector and effector, if required (Table S1). Details of the sequenced mutants are shown in
313 Table S2.
314
315 **Analysis of mutant fitness**
316 Liquid growth assays were used to assess the effect of missense loss-of-function mutations on
317 yeast fitness as described (Urbanus *et al.* 2016) with the following modifications. Overnight
318 cultures of freshly transformed BY4742 with empty vector control, pYES2 NT/A effector-HIS3
319 wild-type and mutants were diluted 100-fold into 100 μl of SD-ura/2% galactose and grown with
320 Breathe-Easy adhesive seals (EK scientific) in a CellGrower robot (S&P robotics) at 30ºC with
321 intermittent shaking. Yeast growth was monitored for 30 h by measuring the OD_{620} every 15
322 min. Growth fitness was calculated as the ratio of the area under the curve (AUC) of a effector-
323 expressing strain over an empty vector control after 30 h using the R package GrowthCurver
324 (Sprouffske and Wagner 2016). The average AUC ratio and standard deviation was calculated
325 from three technical replicates.
326

327 **HHpred analysis and sequence alignments**

328 The amino acid sequence of RavK and SdbA (amino acid residues 528-1116) were submitted to
329 the HHpred server (https://toolkit.tuebingen.mpg.de/#/) (Zimmermann *et al.* 2018) analyzed
330 using MSA generation HHblits Uniclust20_2017_07 and Uniprot20_2016_02, respectively and
331 otherwise default parameters. The resulting alignments were visualized using Boxshade
332 (https://embnet.vital-it.ch/software/BOX_form.html). Amino acid sequence alignments of SdbB
333 with its orthologs or with SidB were generated using T-coffee (Tommaso *et al.* 2011) and
334 visualized using Jalview (Waterhouse *et al.* 2009) or Boxshade.

335

336 **Nucleotide sugar donor specificity of the SdbA C-terminal domain**

337 The gene fragment corresponding to SdbA (Lpg0275) residues 510-1050 was PCR amplified
338 from *Legionella pneumophila* str Philadelphia-1 genomic DNA and inserted into the pMCSG53
339 plasmid (Eschenfeldt *et al.* 2013) by ligation independent cloning, providing an N-terminal
340 6xHIS-TEV tag. The point mutant E963A was prepared by site-directed mutagenesis using
341 QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer's
342 protocol. Plasmids were sequenced and transformed to the *E. coli* BL21 DE Gold strain for
343 purification. Recombinant proteins were purified to near homogeneity (>95%) using Ni-chelate
344 affinity chromatography on Ni-NTA Superflow resin (Qiagen) using standard protocols. Cultures
345 were grown in TB and expression was induced at an OD_{595} of 0.8 with 0.4 mM IPTG overnight
346 at 16 °C. Cells were harvested by centrifugation at 9,300× *g*, resuspended in 50 mM HEPES pH
347 7.5, 400 mM NaCl, 5% glycerol, 5 mM imidazole, and lysed by sonication. Lysates were
348 clarified by centrifugation at 21,000× *g* at 4°C and loaded onto gravity flow Ni-NTA agarose
349 columns (Qiagen), followed by washing with 50 mM HEPES pH 7.5, 400 mM NaCl, 5%

350 glycerol, 30 mM imidazole. Proteins were eluted using 50 mM HEPES pH 7.5, 400 mM NaCl,
351 5% glycerol, 250 mM imidazole and flash-frozen in liquid nitrogen for storage at −80°C. The
352 purity of the protein samples was assessed by SDS-PAGE and visualized by Coomassie Brilliant
353 Blue R.

354 The nucleotide sugar donor specificity of SdbA 510-1050 was assayed using the UDP-
355 Glo Glycosyltransferase Assay (Promega) according to manufacturer's protocol. Briefly, 0.09
356 µM of purified wild-type and E963A mutant SbdA 510-1050 protein was incubated with 100 µM
357 UDP-glucose, UDP-glcNAc, UDP-glucuron, UDP-galactose or UDP-galNAc for 1h at 30°C in
358 50 mM HEPES, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 1 mM $MnCl_2$. The hydrolase activity of the
359 UDP-substrate was detected as the release of UDP by the UDP-Glo assay (Promega) after 20
360 min of incubation with UDP-Glo detection reagent. Luminescence was measured using a
361 SpectraMax M2 plate reader. Three technical repeats were performed per reaction.

362 The V_{max}, K_m and k_{cat} for wild-type SdbA 510-1050 with UDP-glcNAc was determined
363 by incubating 0.16 µM SdbA 510-1050 with UDP-glcNAc concentration range of 0.0039 – 2
364 mM for 1h at 30°C in 50 mM HEPES, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 1 mM $MnCl_2$. Three
365 technical repeats were performed per reaction. Kinetic parameters were determined by non-linear
366 curve fitting from the Michaelis Menten plot using GraphPad Prism (version 5.00 for Windows,
367 GraphPad Software).

368

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373 162256).

374 **Figure legends**

375 **Figure 1: A method to enrich full-length missense mutants in a random mutagenesis screen**.
376 *Legionella* effectors with a severe growth phenotype in yeast are fused in frame with the yeast
377 *HIS3* gene in a high-copy yeast expression vector with galactose-inducible expression and an
378 uracil selection marker. The plasmid is randomly mutated in a *E. coli* mutator strain, such as
379 XL1-red. The resulting mutant pool contains a variety of mutants; sense, nonsense, missense and
380 frameshifts, of which the latter three can cause loss-of-function phenotypes. The plasmid pool is
381 transformed to *S. cerevisiae* strain BY4742 and grown under conditions that select all
382 transformable plasmids (–ura/glucose), all loss-of-function mutations (-ura with galactose
383 induction) and only full-length proteins containing missense loss-of-function mutations (-ura-his
384 with galactose induction).

385

386 **Figure 2: The full-length ORF enriched random mutagenesis screen identifies missense**
387 **mutations in the putative active site pocket. A** SdbB (lpg2482) and SdbB in-frame fusion with
388 His3 show a severe growth defect in a yeast spot dilution assay. **B** The percentage of loss-of-
389 function mutation in the pool of transformable plasmids selected on medium lacking uracil (-ura,
390 grey) and lacking uracil and histidine (-ura-his, black) with galactose induction. The average and
391 standard deviation of three independent replicates are shown. **C** Occurrence of frameshift,
392 nonsense and missense mutations in 20 sequenced clones selected on -ura and -ura-his with
393 galactose induction. **D** A schematic representation of loss-of-function clones selected on –ura
394 with galactose induction. Mutations recovered from the same clone are shown in black,
395 connected by a line while single mutations are shown in red. Mutation type is indicated as a
396 closed triangle (frameshift), open hexagon (nonsense) or closed circle (missense), and the

397 number of symbols reflects the occurrence of the mutation in the dataset. **E** The fitness of wild-
398 type SdbB, His3 fusion and loss-of-function mutations (selected on –ura/galactose) compared to
399 empty vector controls in liquid growth assays confirms the loss-of-function phenotype for the
400 SdbB random mutagenesis clones. The average and standard deviation of three technical
401 replicates are shown. **F** A schematic representation of SdbB loss-of-function clones selected on -
402 ura-his/galactose. Missense loss-of-function mutations are shown in red with a closed circle, the
403 number of symbols reflects the occurrence of the mutation in the dataset. Amino acid residues
404 shown for presentation purposes are shown in grey. **G** The fitness of wild-type SdbB, His3
405 fusion and loss-of-function mutations (selected on -ura,-his/galactose) compared to empty vector
406 controls in liquid growth assays confirms the loss-of-function phenotype for the SdbB random
407 mutagenesis clones. The average and standard deviation of three technical replicates are shown.
408 **H** The missense mutations identified by the random mutagenesis screen (D,F) are shown in
409 orange on an Alphafold2 model of SdbB (AF-Q5ZSN5-F1-model_v4), and residues from the
410 putative active site 185-GxCxG189 not captured by the screen are shown in magenta. Putative
411 catalytic triad C187-D273-H351 residies are shown with sticks and the box shows the
412 enlargement of the putative catalytic site. The AlphaFold2 model was visualized using Pymol 2.2
413 (Schrödinger).
414
415 **Figure 3: RavK random mutagenesis captures residues lining the active site cleft. A** A
416 schematic representation of the mutations causing a RavK loss-of-function phenotype when
417 expressed in yeast. Mutated residues are shown in red, amino acid residues shown for
418 presentation purposes are in grey and the number of symbols reflects the occurrence of the
419 mutation in the dataset. **B** The growth fitness of wild-type RavK, RavK-His3 and loss-of-

420 function mutants normalized to empty vector controls (see Methods) confirms the loss-of-
421 function phenotype for the RavK random mutagenesis clones. The average and standard
422 deviation of three technical replicates are shown. **C** The Alphafold2 model of RavK (AF-
423 Q5ZWW5-F1-model_v4.pdb) with missense mutations shown in orange. The histidine residues
424 of the active site motive $_{95}$HExxH$_{99}$ (Liu *et al.* 2017)not captured by the screen are shown in red.
425 The residues in the active site cleft are shown as sticks. The AlphaFold2 model was visualized
426 using Pymol 2.2 (Schrödinger).
427
428 **Figure 4: The C-terminal domain of SdbA is a putative glycosyltransferase domain.**
429 **A** A schematic representation of the mutations causing a SdbA loss-of-function phenotype when
430 expressed in yeast. Mutations recovered from the same clone are shown in black, connected by a
431 line and single mutations alleviating a SdbA induced growth defect are shown in red. Amino acid
432 residues shown for presentation purposes are shown in grey. Black and red closed circles
433 indicate the number of times the mutation was identified. **B** The growth fitness of wild-type
434 SdbA and loss-of-function mutants normalized to empty vector controls (see Methods for
435 details). SdbA and SdbA-His3 display a severe growth defect, while the loss-of-function
436 mutations rescue growth up to 50% of the empty vector control. The average and standard
437 deviation of three technical replicates are shown. **C** HHpred alignment of SdbA with 1F0K (Ha
438 *et al.* 2000) (*E. coli* MurG). The G-loop 1 and consensus sequence are shown with identical
439 residues (black) and similar residues (grey) highlighted. SdbA amino acid residues that when
440 mutated abrogate activity are indicated by a red closed circle. Amino acid residues in the MurG
441 consensus sequence contacting UDP-GlcNac are indicated by a black star and a red start if
442 mutations abrogate MurG activity. **D** The Alphafold2 model of SdbA (AF-Q5ZYT6-F1-

443 model_v4.pdb) with missense mutations shown in orange. Residues H545 and E963,
444 corresponding to residues H18 and E268 in MurG, are shown in yellow (43). The AlphaFold2
445 model was visualized using Pymol 2.2 (Schrödinger). **E** The SdbA glycosyltransferase domain
446 uses GlcNac as a donor substrate. Donor substrate specificity was tested by incubating 0.09 µM
447 of purified fragment (residues 510-1050) of wild-type SdbA or an inactive mutant (E963A) with
448 100 µM UDP-glucose, UDP-glcNAc, UDP-glucuron, UDP-galactose or UDP-galNAc for 1h at
449 30°C. The hydrolase activity of the UDP-substrate was detected as the release of UDP by the
450 UDP-Glo assay (Promega) after 20 min of incubation with UDP-Glo detection reagent.
451 Luminescence was measured using a SpectraMax M2 platereader. Three technical repeats were
452 performed per reaction. **F** Determination of the kinetic parameters for GlcNac hydrolysis by
453 SdbA. Reactions with 0.16 µg purified wild-type C-terminal domain of SdbA (residues 510-
454 1050) and a range of 0.0039 – 2 mM GlcNac and were incubated for 1 h at 30°C. GlcNac
455 hydrolysis was measured using the UDP-Glo glycosyltransferase assay as described above; three
456 technical replicates were performed per reaction. Kinetic parameters were determined by non-
457 linear curve fitting from the Michaelis-Menten plot.
458
459

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619

Figure 1: A method to enrich full-length missense mutants in a random mutagenesis screen

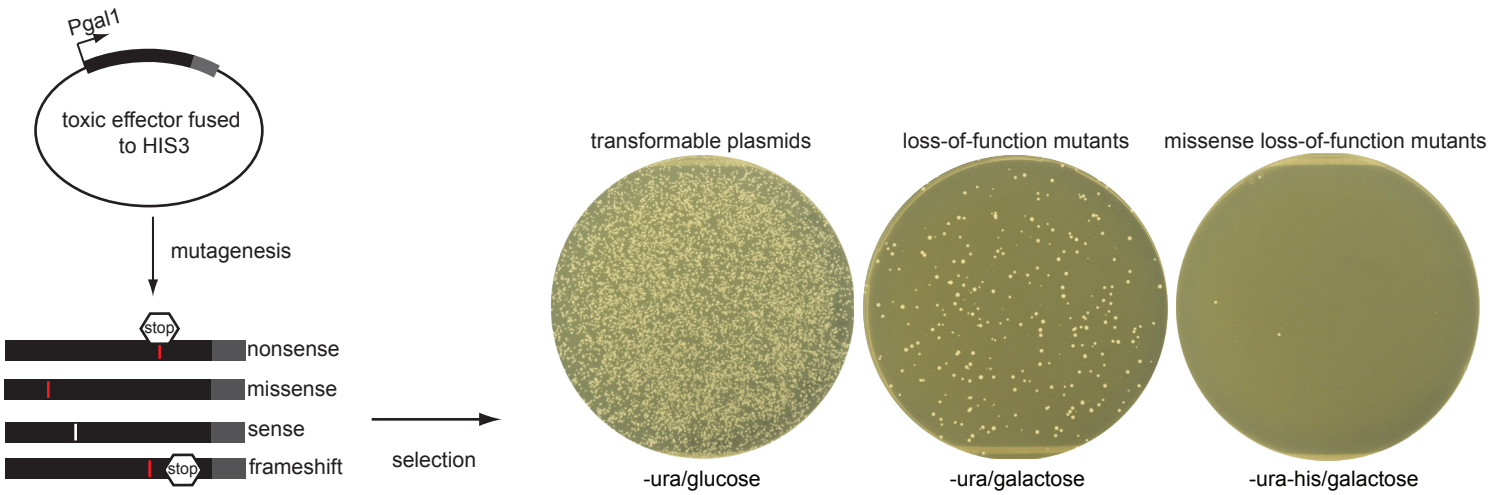


Figure 2: Random mutagenesis screen identifies missense mutations in amino acid residues of the putative active site pocket

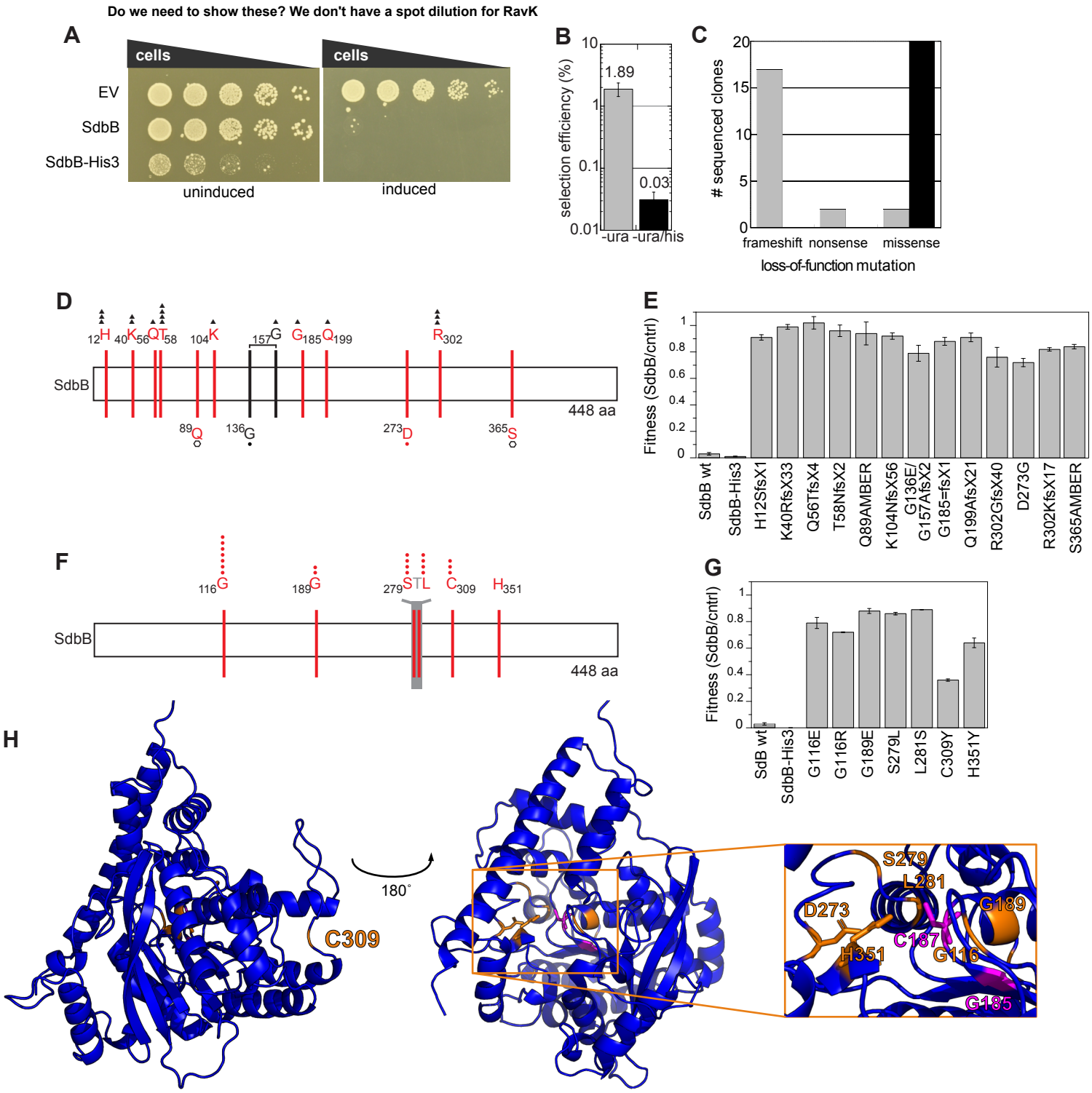


Figure 3 - RavK random mutagenesis captures residues lining the active site cleft

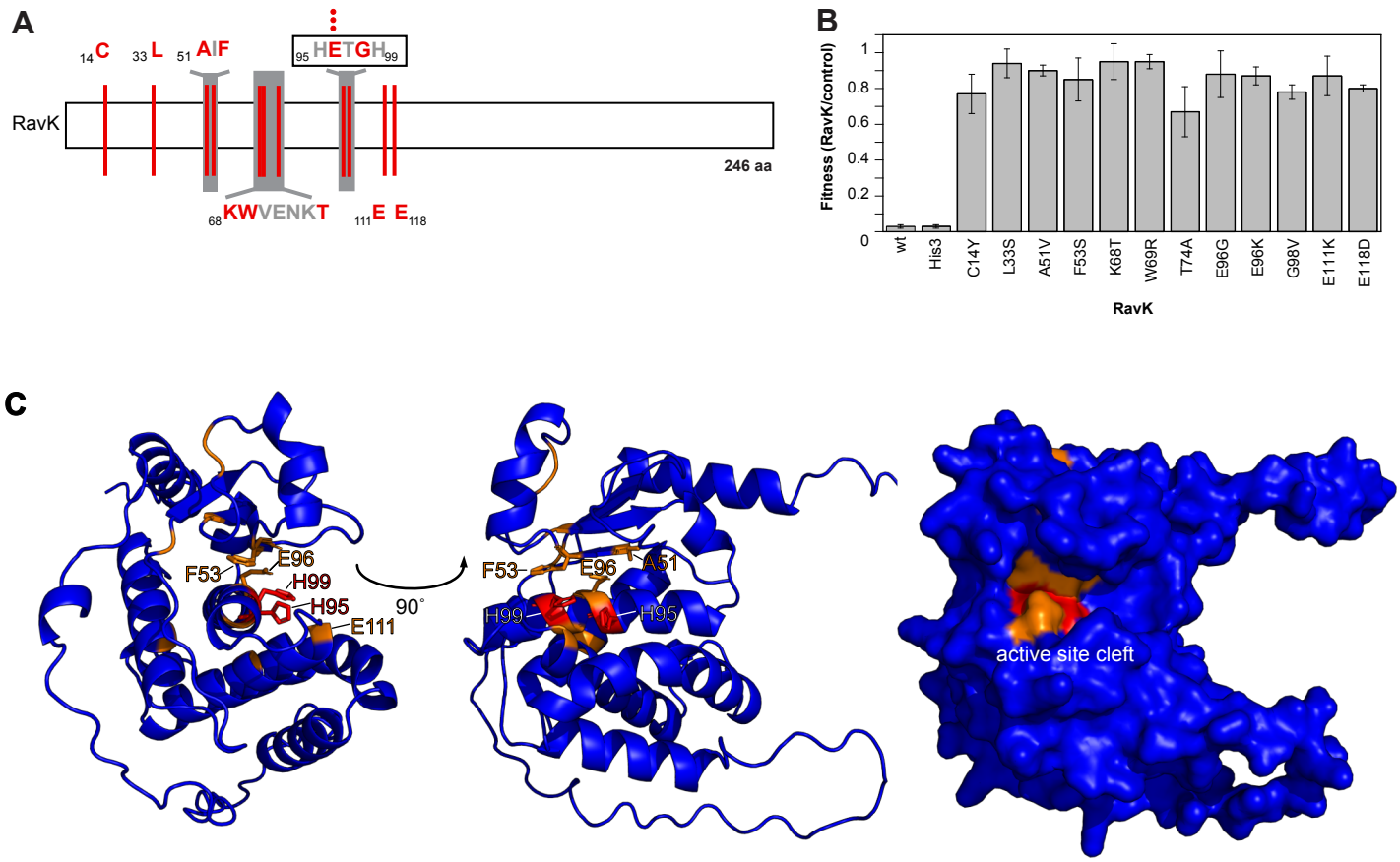
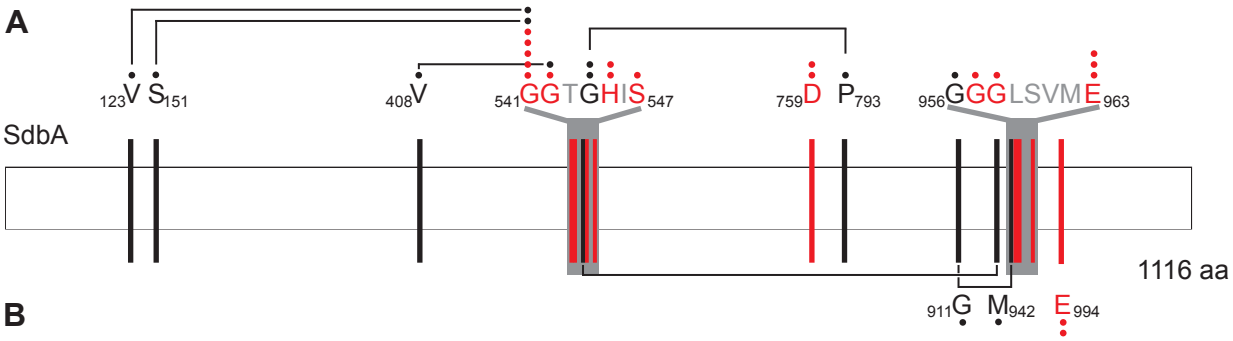
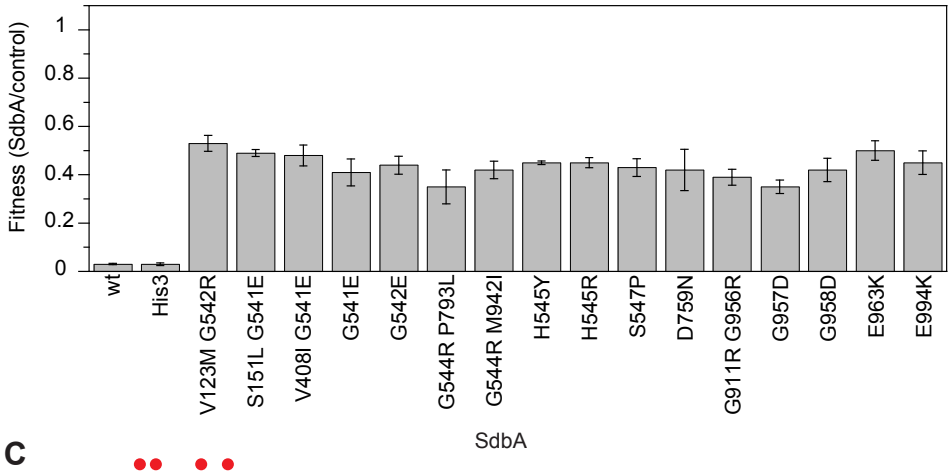
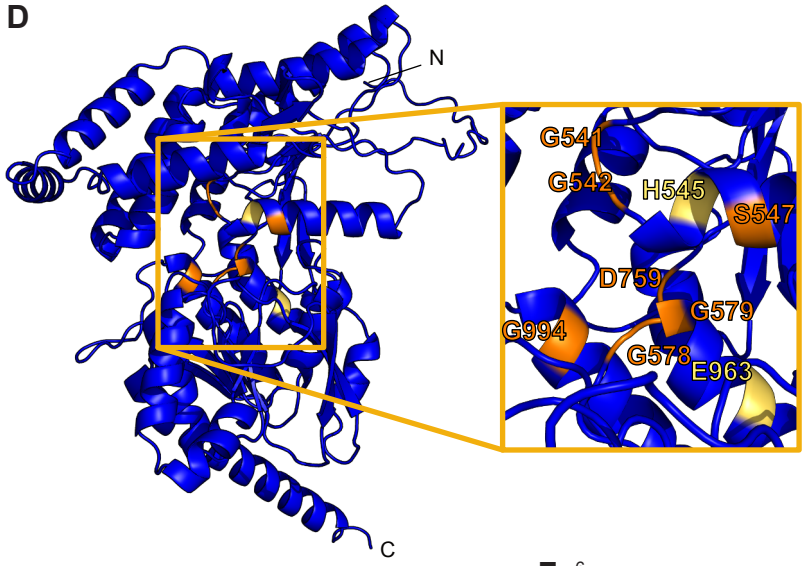
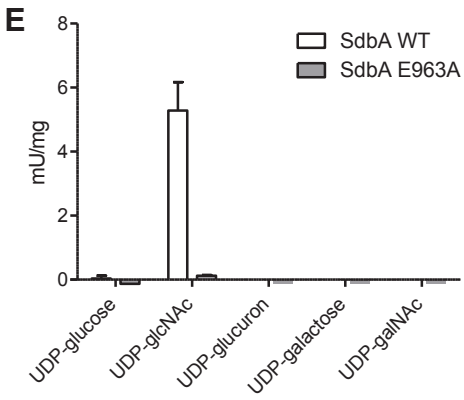


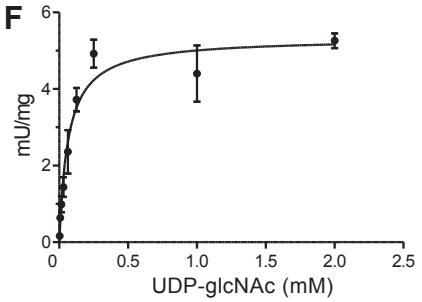
Figure 4: The C-terminal domain of SdbA is a putative glycosyltransferase domain











V_{max} (mU/mg)	K_m (μM)	K_{cat} (min^{-1})
5.33 ± 0.27	56.3 ± 12.7	0.33 ± 0.02

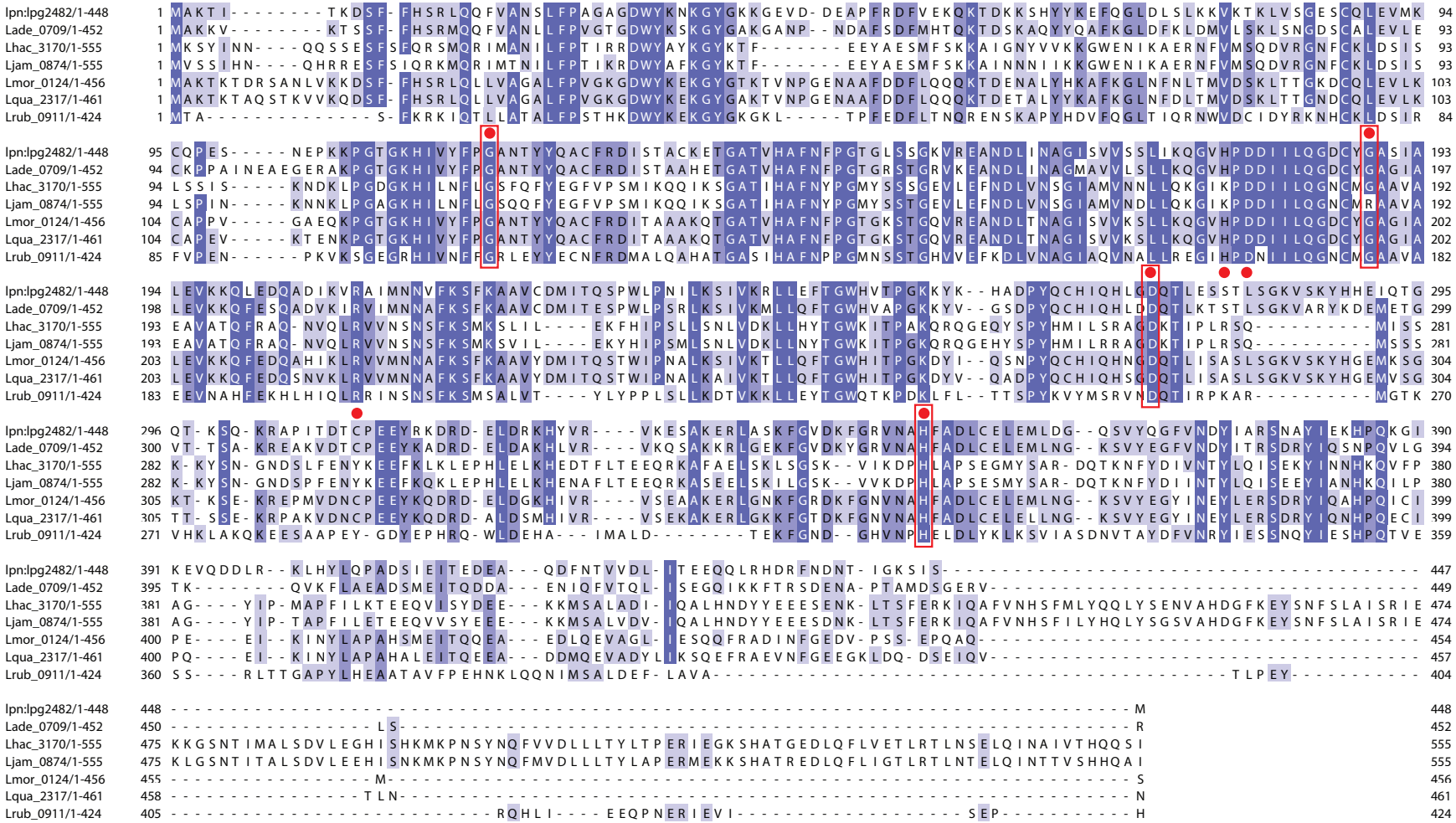


Figure S1: Random mutagenesis targets conserved residues in SdbB orthologs from seven *Legionella* species. The sequences of SdbB orthologs identified by Burstein et al (Nat Genet 48(2): 167-175, 2016) were aligned using T-coffee, visualized with Jalview and coloured by % identity. The missense mutations identified by the random mutagenesis screen are indicated with a red closed circle above the SdbB (lpg2482) sequence. Four of the seven mutations target invariant residues; G116, G189, D273 and H351 shown in red boxes.

```
SidB    1 MAKIYNAPKPKYSGWEWFKFIAIRTVFPPVLLWDLIKIGANKLLGEWVSGLVLPAQNENF
SdbB    1 MAKTITKDSFFHSRLQ--QFVA-NSLFPAG-------------AGDWYKNKGYGKKGE-V

SidB   61 DDLAISDDTVSNYNED------------D--LICEKHDVITHDGAHLDTFEVRHRSQESI
SdbB   44 DDEAPFRDFVEKQKTDKKSHYYKEFQGLDLSLKKVKTKLVSGESCQLEVMKCQPESNEPK

SidB  107 DPKYQKYIINLVGNGMCYEHIIDDIKEDSKALKANVIGFNLRGVGQSTGKAKSSEDLVAD
SdbB  104 KPGTGKHIVYFPGANTYYQACFRDISTACKETGATVHAFNFPGTGLSSGKVREANDLINA

                                GxS/CxG
SidB  167 GIAQVQRLLDQGVSPQNITLKGHSLGAGVASLVAQHFHQLGQ-PINLFNSRSFSTITNFL
SdbB  164 GISVVSSLIKQGVHPDDIILQGDCYGASIALEVKKQLEDQADIKVRAIMNNVFKSFKAAV

SidB  226 VGHMRLERDEIGRAIGHKDSTVGTILGWLAKPFIKFGVALAKWEINAGSAFKSVPEAYKD
SdbB  224 C----------------DMITQSPWLPNILKSIVKRLLEFTGWHVTPGKKYKHA-DPYQC

SidB  286 YIVVRSRKEIRGERIDDAVIPHYASIHKELASERHKKKAEIDEEIANLDDIIRKADPLAK
SdbB  267 HIQHLGDQTLESSTLSGKVSKYHHEIQT--GQTKSQKRAPITDTC--PEEYRKDRDELDR

SidB  346 PGLANARDALVQAREKIKSDRKMETDVQYANGHNSDWNALHNRSGKSA-QTFFREFVQRT
SdbB  323 KHYVRVKE---SAKERLASKFGVD-KFGRVNAHFADLCELEMLDGQSVYQGFVNDYIARS

SidB  405 EA---DHAVKSIPEIN---------------------------------------------
SdbB  379 NAYIEKHPQKGIKEVQDDLRKLHYLQPADSIEITEDEAQDFNTVVDLITEEQQLRHDRFN

SidB      ----------
SdbB  439 DNTIGKSISM
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Figure S2: SidB and SdbB amino acid sequence alignment. The SidB (lpg1642) and SdbB (lpg2482) were aligned with T-coffee and visualized with Boxshade, where identical residues are shown in black and similar residues in grey. The active site motif GxS/CxG predicted by NCBI conserved domain search (Marchler-Bauer A et al.,2017, Nucleic Acids Res.45(D)200-3.) for SidB is indicated, suggesting that SdbB C187 is part of the active site catalytic triad.

```
                                                                                                β2
                                                                                                 ●  ●
ravK       1 MVSLEHIQKLISECRKLGKDGLDNGTNGLIPELEIDVVPPSAFLG-VGNNPAIFVNSKTY
4QHJ_B     1 MKDRKILNEILSNTINE------LNLNDKKANIKIKIKPLKRKIA-SISL----------
4JIU_A     1 ---------------------------------------------LG-YQRPVKVRIRPLK-
4JIX_B     1 ---------------------------------------------LN-INESINIEIKPMK-
2L0R_A     1 ------------------------------------------------------------
3C37_A     1 -VNDPEVQRYVDKVGKRLLSGARAV----EFDYVFKVVKDDSVNAFAIPGGRVYVHTGLL
                rim of active site cleft
                                  ●  ●      ● β3                    ●  ●
ravK      60 KLMRTTHEKWVENKTIVFKSYLL-S-QPAIKIIGAIVHETGHAFN------------V--
4QHJ_B    44 -----------TNKTIYINKNILPY-LSDEEIRFILAHELLHLKY------------GKY
4JIU_A    16 ---MSIARVSFKYGTITLDPAVL-N-LEEEEMFYILIHELAHLKAETSYHSSSFWREV--
4JIX_B    16 ---QKIASFSFKTKTLRLNKYVV-ENFDEELLHYIILHELIHFKI---------------
2L0R_A     1 ------------------------------NDSEGFIHEFGHAVD------------D--
3C37_A    56 KAAD-----------------------NETELAGVLAHEINHAVA---------------

                                      ●  ●
ravK     104 -----AAKIPNT-EANACIFEIEVLMRLFQVKSPLLLGCTELDMQSYFKSRLTDYNKC--
4QHJ_B    80 HINEFEEELLFL-FPNKEAILFNLINKLFQKK----------------------------
4JIU_A    69 -----EKVFPGE-R--AKEIEDRIMTKLQR------------------------------
4JIX_B       ------------------------------------------------------------
2L0R_A    17 -----YAGYLLDKNQSDLVTNSKKFIDIFKEEGSNLTSYGRTNEAEFFAEAFRLMHSTDH
3C37_A       ------------------------------------------------------------

ravK     156 ------VKDCQCLAEMVEFITHQ
4QHJ_B       -----------------------
4JIU_A       -----------------------
4JIX_B       -----------------------
2L0R_A    72 AERLKVQKNAPKTFQFINDQIKF
3C37_A       -----------------------
```

Supplemental figure 3 - HHpred alignment of RavK with metalloproteases.
Alignment of the HHpred top hits; MJ1213 (4QHJ), proabylysin (4JIU), projannalysin (4JIX) anthrax lethal factor (2L0R) and a zinc-dependent endopeptidase of the M84 family (3C37) with RavK is shown where grey and black background indicate the level of amino acid residue conservation as 50% or more similarity or 50% or more identity, respectively. Structural elements of proabylysin (4JIU) are indicated on top of the alignment and mutations that abolish RavK activity are indicated by a red closed circle.

Table S1: Primers used for yeast recombinational cloning and sequencing. In the yeast recombinational cloning primers the effector sequence is underlined, the HIS3 sequence in bold and the pYES2 vector sequence unchanged.

Primer	Sequence (5'-3')	Use
lpg0275-HIS3 FW	AAGGAAGATTTACATCATAATGAGCTTTCCAGTCAAAGTTTGGTTAGTGTCAGTGCTGGT**atgacagagcagaaagccctagtaaagcgt**	yeast recombinational cloning
lpg0969-HIS3 FW	TGGTTAAAGAGCATTTTCATTCTCCTGAAACAGAGATAAAGCTTGATATA**atgacagagcagaaagccctagtaaagcgt**	yeast recombinational cloning
lpg2482-HIS3 FW	GAACAACAACTAAGGCATGATCGATTCAACGATAATACAATAGGTAAAAGCATCTCTATG**atgacagagcagaaagccctagtaaagcgt**	yeast recombinational cloning
pYES2-HIS3 rv	CGTGAATGTAAGCGTGACATAACTAATTACATGATGCGGCCCTCTAGGATCAGCGGGTTT**ctacataagaacacctttggtggagggaac**	yeast recombinational cloning
scHIS3_seqF	GCTCTGGCCAAGCATTCC	verification of his3 fusion
scHIS3_seqR	TTCAGTGGTGTGATGGTCGT	verification of his3 fusion , sdbB and ravK random mutant sequencing
Gal1 fw	AATATACCTCTATACTTTAACGTC	sdbB and ravK random mutant sequencing
lpg0969seqF	GCTGCCAAAATTCCCAATAC	ravK random mutant sequencing
lpg0969seqR	CCAGTTCCGTACACCCTAGC	ravK random mutant sequencing
lpg0275_467F	AGGGATTGGAGTTCCTGGAC	sdbA random mutant sequencing
lpg0275_637R	CGTCTTCACCTGTAACTTGCAC	sdbA random mutant sequencing
lpg0275_942F	TGAGAAGGGCATGAAAGTCA	sdbA random mutant sequencing
lpg0275_1417F	TCCCGACAAGATTTGGTAGC	sdbA random mutant sequencing
lpg0275_1920F	CAGAACCCGTCCTTATGTGG	sdbA random mutant sequencing
lpg0275_2435F	ACCCACGCAATAACCCTATG	sdbA random mutant sequencing
lpg0275_2825F	TGGCACCAATAATGACCAGA	sdbA random mutant sequencing

Table S2. Random mutagenesis mutants for RavK, SdbA and SdbB. Mutants suppressing the growth defect of RavK,SdbA and SdbB in yeast were selected on SD-gal-ura (induced expression and plasmid selection) or SD-gal-ura-his (induced expression, plasmid selection and full-length fusion protein selection respectively). This table summarizes the identified mutations, the type of mutation and the consequences of the mutation.

Mutant no	Locus no	Gene name	Selection	Nucleotide mutation	Type of mutation	Amino acid mutation(s)	1st mutation	2nd mutation
1	lpg0275	sdbA	+ gal, -ura-his	367G>A, 1624G>A	amino acid substitution	V123M, G542R	123	542
2	lpg0275	sdbA	+ gal, -ura-his	1622G>A, 452C>T	amino acid substitution	S151L, G541E	151	541
3	lpg0275	sdbA	+ gal, -ura-his	1222G>A, 1622G>A	amino acid substitution	V408I, G541E	408	541
4	lpg0275	sdbA	+ gal, -ura-his	1622G>A	amino acid substitution	G541E	541	
5	lpg0275	sdbA	+ gal, -ura-his	1621G>A	amino acid substitution	G541E	541	
6	lpg0275	sdbA	+ gal, -ura-his	1622G>A	amino acid substitution	G541E	541	
7	lpg0275	sdbA	+ gal, -ura-his	1622G>A	amino acid substitution	G541E	541	
8	lpg0275	sdbA	+ gal, -ura-his	1622G>A	amino acid substitution	G541E	541	
9	lpg0275	sdbA	+ gal, -ura-his	1625G>A	amino acid substitution	G542E	542	
10	lpg0275	sdbA	+ gal, -ura-his	1630G>A, 2378C>T	amino acid substitution	G544R, P793L	544	793
11	lpg0275	sdbA	+ gal, -ura-his	1630G>A, 2826G>A	amino acid substitution	G544R, M942I	544	942
12	lpg0275	sdbA	+ gal, -ura-his	1634A>G	amino acid substitution	H545R	545	
13	lpg0275	sdbA	+ gal, -ura-his	1633C>T	amino acid substitution	H545Y	545	
14	lpg0275	sdbA	+ gal, -ura-his	1639T>C	amino acid substitution	S547P	547	
15	lpg0275	sdbA	+ gal, -ura-his	2275G>A	amino acid substitution	D759N	759	
16	lpg0275	sdbA	+ gal, -ura-his	2275G>A	amino acid substitution	D759N	759	
17	lpg0275	sdbA	+ gal, -ura-his	2731C>A, 2866G>A	amino acid substitution	G911R, G956R	911	956
18	lpg0275	sdbA	+ gal, -ura-his	2870G>A	amino acid substitution	G957D	957	
19	lpg0275	sdbA	+ gal, -ura-his	2873G>A	amino acid substitution	G958D	958	
20	lpg0275	sdbA	+ gal, -ura-his	2887G>A	amino acid substitution	E963K	963	
21	lpg0275	sdbA	+ gal, -ura-his	2887G>A	amino acid substitution	E963K	963	
22	lpg0275	sdbA	+ gal, -ura-his	2887G>A	amino acid substitution	E963K	963	
23	lpg0275	sdbA	+ gal, -ura-his	2980G>A	amino acid substitution	E994K	994	
24	lpg0275	sdbA	+ gal, -ura-his	2980G>A	amino acid substitution	E994K	994	
25	lpg0969	ravK	+ gal, -ura-his	41G>A	amino acid substitution	C14Y	14	
26	lpg0969	ravK	+ gal, -ura-his	98T>C	amino acid substitution	L33S	33	
27	lpg0969	ravK	+ gal, -ura-his	152C>T	amino acid substitution	A51V	51	
28	lpg0969	ravK	+ gal, -ura-his	158T>C	amino acid substitution	F53S	53	
29	lpg0969	ravK	+ gal, -ura-his	203A>C	amino acid substitution	K68T	68	
30	lpg0969	ravK	+ gal, -ura-his	205T>A	amino acid substitution	W69R	69	
31	lpg0969	ravK	+ gal, -ura-his	220A>G	amino acid substitution	T74A	74	
32	lpg0969	ravK	+ gal, -ura-his	286G>A	amino acid substitution	E96K	96	
33	lpg0969	ravK	+ gal, -ura-his	286G>A	amino acid substitution	E96K	96	
34	lpg0969	ravK	+ gal, -ura-his	287A>G	amino acid substitution	E96G	96	
35	lpg0969	ravK	+ gal, -ura-his	293G>T	amino acid substitution	G98V	98	
36	lpg0969	ravK	+ gal, -ura-his	331G>A	amino acid substitution	E111K	111	
37	lpg0969	ravK	+ gal, -ura-his	354A>C	amino acid substitution	E118D	118	
38	lpg2482	sdbB	+gal,-ura	33_34insT	frameshift	H12SfsX1	12	
39	lpg2482	sdbB	+gal,-ura	33_34insT	frameshift	H12SfsX1	12	
40	lpg2482	sdbB	+gal,-ura	33_34insT	frameshift	H12SfsX1	12	
41	lpg2482	sdbB	+gal,-ura	119delA	frameshift	K40RfsX33	39	
42	lpg2482	sdbB	+gal,-ura	119delA	frameshift	K40RfsX33	39	

43	lpg2482	sdbB	+gal,-ura	166_167insA	frameshift	Q56TfsX4	56	
44	lpg2482	sdbB	+gal,-ura	172_173insA	frameshift	T58NfsX2	58	
45	lpg2482	sdbB	+gal,-ura	172_173insA	frameshift	T58NfsX2	58	
46	lpg2482	sdbB	+gal,-ura	172_173insA	frameshift	T58NfsX2	58	
47	lpg2482	sdbB	+gal,-ura	172delA	frameshift	T58PfsX15	58	
48	lpg2482	sdbB	+gal,-ura	265C>T	premature stop codon	Q89AMBER	89	
49	lpg2482	sdbB	+gal,-ura	312delA	frameshift	K104NfsX56	104	
50	lpg2482	sdbB	+gal,-ura	407G>A, 469_470insG	amino acid substitution and frameshift	G136E, G157AfsX2	136	157
51	lpg2482	sdbB	+gal,-ura	555_556insG	frameshift	G185=fsX1	185	
52	lpg2482	sdbB	+gal,-ura	594_595insA	frameshift	Q199AfsX21	199	
53	lpg2482	sdbB	+gal,-ura	818A>G	amino acid substitution	D273G	273	
54	lpg2482	sdbB	+gal,-ura	905_906insA	frameshift	R302KfsX17	302	
55	lpg2482	sdbB	+gal,-ura	905_906insA	frameshift	R302KfsX17	302	
56	lpg2482	sdbB	+gal,-ura	905delA	frameshift	R302GfsX40	302	
57	lpg2482	sdbB	+gal,-ura	1094C>A	premature stop codon	S365AMBER	365	
58	lpg2482	sdbB	+ gal, -ura-his	347G>A	amino acid substitution	G116E	116	
59	lpg2482	sdbB	+ gal, -ura-his	347G>A	amino acid substitution	G116E	116	
60	lpg2482	sdbB	+ gal, -ura-his	346G>A	amino acid substitution	G116R	116	
61	lpg2482	sdbB	+ gal, -ura-his	347G>A	amino acid substitution	G116E	116	
62	lpg2482	sdbB	+ gal, -ura-his	347G>A	amino acid substitution	G116E	116	
63	lpg2482	sdbB	+ gal, -ura-his	347G>A	amino acid substitution	G116E	116	
64	lpg2482	sdbB	+ gal, -ura-his	347G>A	amino acid substitution	G116E	116	
65	lpg2482	sdbB	+ gal, -ura-his	347G>A	amino acid substitution	G116E	116	
66	lpg2482	sdbB	+ gal, -ura-his	566G>A	amino acid substitution	G189E	189	
67	lpg2482	sdbB	+ gal, -ura-his	566G>A	amino acid substitution	G189E	189	
68	lpg2482	sdbB	+ gal, -ura-his	836C>T	amino acid substitution	S279L	279	
69	lpg2482	sdbB	+ gal, -ura-his	836C>T	amino acid substitution	S279L	279	
70	lpg2482	sdbB	+ gal, -ura-his	836C>T	amino acid substitution	S279L	279	
71	lpg2482	sdbB	+ gal, -ura-his	842T>C	amino acid substitution	L281S	281	
72	lpg2482	sdbB	+ gal, -ura-his	842T>C	amino acid substitution	L281S	281	
73	lpg2482	sdbB	+ gal, -ura-his	842T>C	amino acid substitution	L281S	281	
74	lpg2482	sdbB	+ gal, -ura-his	926G>A	amino acid substitution	C309Y	309	
75	lpg2482	sdbB	+ gal, -ura-his	926G>A	amino acid substitution	C309Y	309	
76	lpg2482	sdbB	+ gal, -ura-his	926G>A	amino acid substitution	C309Y	309	
77	lpg2482	sdbB	+ gal, -ura-his	1051C>T	amino acid substitution	H351Y	351	