The type III-B CRISPR-Cas System Affects Energy Metabolism and Adaptation in the Archaeon *Saccharolobus solfataricus*

Authors:

Erika Wimmer[1], Isabelle A. Zink[2], Logan H. Hodgskiss[1], Melina Kerou[1], Christa Schleper[1]

Affiliations:

[1] Department of Functional and Evolutionary Ecology, Faculty of Life Sciences, University of Vienna

[2] Laboratory of Microbiology, Wageningen University and Research, Stippeneng 4, 6708 WE Wageningen, The Netherlands.

ABSTRACT

Type III CRISPR-Cas immune systems that recognize and cleave extrachromosomal RNA when active, are particularly widespread in archaea. Mechanistically, these systems have the potential to regulate gene expression of host genes on a post-transcriptional level, but very little is known about any potential accessory roles of type III-B systems beyond immunity. We have created knockout mutants of a type III-B CRISPR-Cas complex in the thermoacidophilic archaeon *Saccharolobus solfataricus* to investigate potential secondary functions of the type III-B system. Deletion mutants exhibited an accelerate growth but were less quickly adaptable to changes in carbon sources in their growth media. In line with this phenotype, upregulated genes were significantly enriched in functional categories of energy production and conversion, as well as with carbohydrate or amino acid transport and metabolism in RNAseq studies. Generally, a significant accumulation of genes encoding transmembrane proteins in the upregulated proportion of the transcriptome suggests interconnections between the type III-B CRISPR-Cas system and various membrane-associated processes. Notably, the deletion mutants did not lose their general virus- or plasmid defense activities indicating that this particular system might have been partially adopted for cellular regulatory roles.

INTRODUCTION

CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats - CRISPR associated proteins) immune systems are present in roughly a third of fully sequenced bacterial and the majority of archaeal genomes[1]. Despite the impressive diversity of known CRISPR-Cas systems, the principle first steps of the immune reaction remain: A small crRNA (CRISPR RNA) is transcribed from a so-called spacer in a CRISPR array in the host genome. Spacers are DNA snippets of previously encountered viruses and plasmids that have been stored in the host genome during the process of CRISPR adaptation[2,3]. A CRISPR-Cas protein effector forms a complex with one small crRNA to identify complementary nucleic acid targets (so-called protospacers) on invading genetic elements, such as viruses or plasmids[4]. Dependent on the CRISPR-Cas system, the bound nucleic acid target is then either directly cleaved by the Cas enzyme or abortive infection pathways are activated to clear the invader from the population[5].

While most CRISPR-Cas effectors target double stranded DNA, type III and type VI CRISPR-Cas systems exhibit dedicated RNA-targeting mechanisms[6]. Type III CRISPR-Cas systems are frequently found in various archaeal phyla and are particularly abundant among Thermoproteota (formerly Crenarchaeota)[5]. These systems are especially intriguing because they are multi-subunit complexes that execute their immune reaction in three different ways: Upon recognition and binding of a specific RNA target (i.e. nascent mRNA transcribed from a plasmid), the Cas7 backbone of the type III complex cleaves the RNA protospacer. Furthermore, binding to the protospacer activates the Cas10 subunit of the complex which cleaves single stranded DNA in transcription bubbles promiscuously via its HD domain[7–10]. Concomitantly, the cyclase (palm) domain of Cas10 synthesizes cyclic molecules from ATP (cOAs)[11–14] which in turn activate ancillary CARF-domain containing proteins which can be either RNases, DNases or proteases that indiscriminately degrade their substrates in the cell[15,16]. This leads to cell death or cell dormancy which curtails the spread of the invader in the population[17–23].

Interstingly, the nucleolytic and abortive infection pathways of the type III CIRSPR-Cas complex can be inactivated, thereby only allowing specific cleavage of the RNA target. Complementarity of a sequence stretch at the 3’ end of the protospacer (the so called antitag) to the 5’ part of the crRNA or mismatched bases in certain parts of the crRNA:target duplex allosterically inhibit the catalytic activities of Cas10[8,9,11–14,24–26]. Therefore, type III CRISPR-Cas

effector complexes can specifically cleave mRNA targets, which raises the question as to whether they also contribute to the regulation of host gene expression. Indeed, there are some indications for additional roles of type III CRISPR-Cas complexes apart from immunity. For instance, type III loci often contain high numbers of accessory genes potentially constituting a functional connection to cellular processes other than defense[27,28]. Also, in many cases type III CRISPR-*cas* operons are not genomically linked to CRISPR arrays or modules involved in the acquisition of new spacers, thus they are reliant on co-occurring CRISPR-Cas systems for a canonical immune response[5,29]. Furthermore, we find type III CRISPR-Cas systems to be among the most highly expressed genes in *Saccharolobus solfataricus*, also when no plasmid or virus invader is present.

Sa. solfataricus P1 belongs to the archaeal phylum Thermoproteota and contains two type III CRISPR-Cas complexes, a type III-D (Csm) and a type III-B (Cmr-ß), as well as three DNA-targeting type I-A complexes[5,30]. In vitro studies on the Cmr-ß complex from a close relative, *Sa. islandicus* (SisCmr-ß), revealed that catalytic activities of the complex are modulated by the additional subunit Cmr7[31,32] as its presence coincided with starkly reduced cOA production by the Cas10 subunit[33]. In this study, the endogenous type I CRISPR-Cas system was harnessed to generate a *cmr-ß* knockout strain of *Sa. solfataricus* in order to discern its putative links to other processes and alternative roles besides immunity. A wide range of effects of the genomic deletion on energy metabolism and transport mechanisms indicate a general impact on the host's physiology as revealed by RNAseq. In addition, knockout strains were more sensitive to switches from complex into single sugars media, indicating a role of the Cmr-ß complex in metabolic adaptability. Furthermore, we did not observe any effect on plasmid immunity upon type III CRISPR-Cas deletion, indicating that the Cmr-ß might not be a main contributor to type III CRISPR-Cas immune defense in *Sa. solfataricus*.

RESULTS

Type IA-CRISPR-Cas-mediated knockout of the type III-B CRISPR-*cas* gene module in *Sa. solfataricus* P1

The endogenous DNA-targeting type I-A CRISPR-Cas system was harnessed for deletion of the genes encoding the type III-B CRISPR-Cas complex Cmr-ß (SSOP1_RS10085 - SSOP1_RS10115) (Fig. 1A). Two different 37-bp protospacers with 3' protospacer adjacent motif (PAM) 5'-NGG[34] were selected within each of the three targeted genes (*cmr7, cmr6,*

105 *cmr3/cas5*) on the *Sa. solfataricus* chromosome (Fig. 1A). In total, six different mini CRISPR
106 arrays (miniCR) were assembled as described previously[35] containing one spacer or a
107 combination of two to three spacers targeting the respective genes on either coding or
108 template strand (cs or ts, respectively). The miniCRs and two approximately 800-bp donor
109 sequences for DNA repair corresponding to upstream and downstream flanking regions of
110 the *cmr-ß* locus were cloned into the plasmid-based vector pIZ-GW[36] (Fig. 1B). *Sa.*
111 *solfataricus* M18 cells transformed with the genome-editing plasmids were enriched under
112 selection in liquid medium. Although all constructs yielded the intended deletion as detected
113 by culture PCR, one knockout culture was chosen for plating to isolate single colonies (Suppl.
114 Fig. S1). The genomes of two purified knockout mutant cultures (KO1 and KO2) grown from
115 two single colonies were sequenced showing that in comparison to the deposited reference
116 (NZ_LT549890.1) the *cmr-ß* genes and their upstream basal promoter elements had been
117 scarlessly excised from the genome in both mutants (Fig. 2). Interestingly, KO2 had
118 additionally acquired a larger deletion (over 37 kb) between the genes SSOP1_RS14925 and
119 SSOP1_RS15080, both of which are encoding IS5 family transposases (Suppl. tab. 1, Fig. 3).
120

121 **No difference in plasmid immunity between *cmr-ß* knockout mutants and wildtype strains**

122 To investigate the contribution of Cmr-ß to the immune defense against foreign genetic
123 elements in *Sa. solfataricus* P1, both wildtype and *cmr-ß* knockout mutant were challenged
124 with two plasmids generating a transcript of a protospacer (i.e. target) complementary to a
125 crRNA encoded in CRISPR array D (Fig. 4A and B). We have previously demonstrated that
126 cells transfected with a viral vector carrying the same protospacer flanked by a PAM could
127 not survive, even under selective conditions[24], indicating an active type I CRISPR-Cas
128 immune response. To solely study the immune response of Cmr-ß and/or Csm by
129 circumventing type I CRISPR-mediated interference, the PAM of construct D2.109 was now
130 disrupted by nucleotide insertion at the 3' end of the protospacer. Upon recognition of this
131 protospacer transcript, the available type III effector(s) should elicit a multi-layered immune
132 response causing detrimental effects to the cells due to abortive infection. As mentioned
133 above, abortive infection can be abolished by the presence of a sequence stretch at the 3'
134 end of the protospacer (antitag) complementary to the 5' tag of the crRNA while the bound
135 target is still degraded[7–9,11–14,37,38]. Therefore, we equipped the same protospacer with a 3'
136 flanking antitag in construct D2.109-a (Fig. 4A), which in our previous study yielded a type III-
137 CRISPR-Cas-mediated reduction in protospacer transcript levels while DNA interference was
138 not observed[24]. In addition, two different controls were used, namely cells transformed with
139 plasmid A53 lacking a matching protospacer[24,39] and untransformed cells not harboring any

140 plasmid (negative control, NC) (Fig. 4A, 4B). After introduction of the different plasmids into
141 both wildtype and deletion mutant, it became evident that growth of cells harboring D2.109
142 was negatively affected to a similar extent in both wildtype and *cmr-ß* knockout mutants (Fig.
143 4D, Suppl. Fig. S2 and S3). In both strains, cells transformed with A53 and D2.109-a were
144 still growing in a 10fold higher dilution on plates compared to cells carrying plasmid D2.109.
145 The same overall trend could also be observed when following their growth in liquid culture
146 (Fig. 4C). Consequently, the absence of the Cmr-ß complex had no obvious effect on the
147 extent of the observed type III CRISPR-Cas-mediated immune response in our experimental
148 setting.
149
150 **Knockout mutants exhibit faster growth and significant changes in their transcriptional**
151 **landscape compared to the wildtype**
152 To test whether growth of *cmr-ß* knockout mutants was affected compared to the wildtype
153 in complex medium, four replicate cultures for each strain were inoculated to the same initial
154 OD_{600} (0.027 ± 5.88E-04). In comparison to the wildtype, similarly accelerated growth was
155 observed for both knockout strains KO1 and KO2, the latter being the strain that attained the
156 additional genomic deletion (Fig. 5A). Samples were taken at the start of exponential growth
157 at an OD_{600} = 0.2 and RNA was extracted to perform RNA-seq. Principal component analysis
158 (PCA) revealed a distinct shift in the transcriptomic landscape upon deletion of Cmr-ß (Fig
159 5B). All four replicates of each strain grouped together, thereby indicating high reproducibility.
160 Additionally, the two deletion strains were overlapping whereas the wildtype was clearly
161 clustering separately. One principal component (PC1) could explain ~39% of the variance
162 between knockout strains and wildtype.
163
164 Changes in gene expression with high statistical significance (cutoff for adjusted *P* value
165 (padj) < 0.001) were taken into consideration in the downstream analysis even in case of
166 smaller log2 fold changes (FC) between deletion mutant and control. This approach is in
167 accordance with previous transcriptomic studies in other archaea that have reported lower
168 log2 fold changes of statistical significance for genes of physiological relevance[40–42]. Of the
169 3002 coding genes in *Sa. solfataricus* P1, roughly 30 % were differentially expressed in both
170 KO1 and KO2 when compared to the wildtype (Fig. 5E and F, respectively). The majority of
171 significantly up- and downregulated genes was shared between the two knockout mutants
172 KO1 and KO2 (Fig. 5C and D, respectively). The top 25 upregulated genes for KO1 and KO2
173 are listed in Supplementary Tables S2 and S3, respectively. Observed differences were
174 seemingly not caused by a polar effect on the up- and downstream genes surrounding the

175 site where the targeted deletion had occurred, as significant changes in transcriptional levels
176 of genes were dispersed throughout the genome (Fig. 5G and H). A functional enrichment
177 analysis based on archaeal clusters of orthologous genes (arCOGs) was performed to
178 determine which general biological functions were affected by the deletion (Fig. 6A and B).
179 Genes involved in energy production and conversion (category C), amino acid transport and
180 metabolism (category E), and nucleotide transport and metabolism (category F) were
181 significantly enriched among the significantly upregulated genes in both KO1 and KO2. In
182 KO1, genes associated with carbohydrate transport and metabolism were additionally found
183 to be overrepresented among the significantly upregulated group of genes (Fig. 5A). In
184 contrast, there were no arCOG categories specifically enriched among the downregulated
185 group of genes.
186
187 **Absence of Cmr-ß results in significant changes in core metabolic modules**
188 In accordance with the functional enrichment analysis and the accelerated growth observed
189 in *cmr-ß* knockout strains, several key components of the energy metabolism were
190 upregulated in both knockout strains (all pathways discussed in this section are summarized
191 in Fig. 7, Supplementary Notes). Regarding the electron transport chain (ETC), most of the
192 genes encoding subunits of complex I (NADH dehydrogenase) were upregulated apart from
193 NADH dehydrogenase subunit B (NuoB), which is encoded elsewhere in the genome. In
194 Sulfolobales, c-type cytochromes are absent and the functions of complex III and IV of the
195 ETC are combined within quinone oxidoreductase supercomplexes[43]. These unusual terminal
196 oxidase complexes accept electrons directly from quinones, then in turn reduce molecular
197 oxygen and pump protons to generate a proton motive force. Transcription levels of two out
198 of three terminal oxidases in *Sa. solfataricus* were increased in *cmr-ß* knockout strains. While
199 all genes encoding for terminal oxidase SoxEFGHIM were upregulated, only half of the genes
200 encoding for terminal oxidase SoxABCDL showed significantly higher expression. The
201 hydrogenase-related subunits of a putative formate hydrogenlyase complex (FHL) present in
202 the genome were significantly upregulated in knockout strains, while the expression levels of
203 the putative formate dehydrogenase subunit (FdhA, a molybdenum-dependent enzyme) were
204 unchanged, or very slightly downregulated. In accordance with that, the periplasmic
205 component of the ABC molybdate transport system located downstream of the FdhA was
206 significantly downregulated.
207
208 Another process that was seemingly impacted on a transcriptional level by the *cmr-ß*
209 knockout was the transport of nutrients, with several different types of transporters being

210 significantly upregulated. Regarding organic carbon compounds, the arabinose ABC
211 transporter (3.A.1.1.14-AraS) and several putative peptide ABC transporters (3.A.1.5-PepT)
212 were clearly upregulated in KO1 and KO2, while the glucose ABC transporter (3.A.1.1.13-
213 GlcS) exhibited mildly increased transcript levels[44,45]. The gene encoding transcriptional
214 regulator XylR (SSOP1_RS15310), which in *S. acidocaldarius* was shown to positively
215 regulate the expression of pentose transporters as well as metabolizing genes[46], was
216 significantly upregulated. Accordingly, all but one gene with the corresponding predicted
217 binding site for XylR[47] were upregulated, among them *araS* and *glcS* encoding the substrate-
218 binding proteins of the respective ABC-transporters (Fig. 7). Interestingly, some genes of the
219 XylR regulon were recently reported to be part of an independently modulated set of genes
220 that also included the *soxEFGHIM* gene cluster, indicating that their expression might be
221 governed by a shared unknown regulator[48]. Transporters putatively involved in the uptake of
222 amino acids (2.A.1.15-The Aromatic Acid:H+ Symporter Family, 2.A.3.11-The
223 Aspartate/Glutamate Transporter Family) were also highly upregulated in both mutants.
224
225 A predicted thiamine transporter[49] (ThiT) was highly upregulated in *cmr-ß* knockout strains,
226 as well as at least one homolog of ThiC (HMP phosphate (HMP-P) synthase) catalyzing the
227 first step in the synthesis of the pyrimidine moiety of thiamine (HMP-PP), and Thi4 (thiamine
228 thiazole synthase) catalyzing the formation of the thiazole ring (HET-P)[50,51]. Additionally,
229 transcript levels of at least one out of two TenA homologs (aminopyrimidine aminohydrolase)
230 implicated in the salvage of thiamine precursors was slightly higher in both mutant strains
231 compared to wildtype. These genes are part of a proposed thiamine regulon in *Sa.*
232 *solfataricus*, which includes genes involved in the biosynthesis pathway and transport of
233 thiamine and/or precursors[49].
234
235 *Sa. solfataricus* employs a reversed monophosphate pathway for the synthesis of ribose-5-
236 phosphate, which serves as pentose moiety in nucleotide biosynthesis[52]. The genes encoding
237 the enzymes partaking in purine de novo biosynthesis until the formation of the intermediate
238 5′-phosphoribosyl-5-aminoimidazole (aminoimidazole ribotide, AIR) were upregulated in KO1
239 and KO2. Interestingly, this intermediate compound is used as a precursor during the
240 generation of HMP-PP, establishing a link to the observations regarding thiamine
241 biosynthesis[49]. In addition, slightly increased expression levels could also be observed for
242 enzymes participating in purine salvage. In contrast, the genes encoding enzymes that
243 catalyze the reaction from cytosine to uracil in the process of pyrimidine salvage were
244 significantly downregulated, likely due to the supplementation of uracil in the medium.

Genes encoding TatC (SSOP1_RS15345) and SecY (SSOP1_RS03615), components of two different protein translocation pathways[53–56], were also significantly upregulated in *cmr-ß* knockout strains. Interestingly, insertion of TatC itself into the membrane was shown to require the Sec pathway together with translocase YidC in E. coli[57], which we also found significantly upregulated in our dataset (SSOP1_RS17510). Overall, a significant enrichment of genes encoding transmembrane domain proteins could be observed among the upregulated fraction of the transcriptomes of both KO1 and KO2 (Fig. 8A and B, respectively).

A number of genes involved in the isoprenoid lipid biosynthesis were significantly upregulated in the *cmr-ß* knockout strains. Specifically, the genes responsible for the synthesis of intermediates mevalonate (thiolase - *aact*, hydroxymethylglutaryl-CoA synthase - *hmgs*, hydroxymethylglutaryl-CoA reductase - *hmgr*), glycerol-1-phosphate (glycerol-1-phosphate dehydrogenase) and the ether bond formation step to geranylgeranylglyceryl phosphate (GGGP) synthase), as well as geranylgeranyl reductase (*ggr*) and tetraether synthase (*tes*), leading to the formation of the membrane-spanning glycerol dibiphytanyl glycerol tetraethers (GDGTs). Interestingly, the modifying enzyme calditol synthase (*cds*) responsible for the synthesis of the cyclopentenyl head group calditol[58] was significantly upregulated, while GDGT ring synthases *grsA*, *grsB1* and *grsB2*[59] were downregulated, the latter two significantly.

Lastly, 28-32 putative transcriptional regulators (arCOG functional category K), characterized and uncharacterized, were significantly differentially expressed in the knockout strains, indicating a strong involvement of the Cmr-ß complex to various regulatory circuits. However, the CRISPR-associated Csa3 transcription factors involved in regulation of various CRISPR-Cas system components and coordinating the immune response[60–64] were not differentially expressed according to our cutoffs.

Response of CRISPR-Cas defense systems and transposases to the deletion of Cmr-ß

Next, we wanted to investigate whether the absence of Cmr-ß had influenced the expression of other genes implicated in antiviral defense. Looking at the expression levels of CRISPR-*cas* modules in our wildtype samples, it became evident that the first out of three type I-A *cas* modules (SSOP1_RS07365-SSOP1_RS07390) as well as the *cmr-ß* module were most highly expressed. The second and third type I-A module were considerably lower and basically not expressed, respectively. In comparison, the expression level of the *csm* module

280 was more than ten times lower than the one of the *cmr-ß* module under standard growth
281 conditions. Upon *cmr-ß* knockout, we could detect an upregulation of the majority of genes
282 of the first type I-A *cas* module as well as increased expression levels for the *csm* module
283 (Fig. 7).
284
285 Several transposase genes were significantly downregulated in absence of Cmr-ß. The genus
286 *Saccharolobus* generally carries a remarkable number of insertion sequence (IS) elements[65–
287 68]. For *Sa. solfataricus* P2 specifically it is estimated that IS elements make up approximately
288 10% of its total genome sequence (in bp)[69]. In the P1 reference genome, almost 170
289 functional transposases are currently annotated (based on eggnog annotations of
290 NZ_LT549890.1). Several transposases of the most prominent IS4 (ISC1225, ISC1359,
291 ISC1439) and IS5 (ISC1058, ISC1212, ISC1234) families were found to be downregulated in
292 both deletion mutants. Transposase genes belonging to the less abundant IS630 (ISC1048,
293 ISC1078), ISC1217 and IS110 (ISC1190, ISC1229) elements were also exhibiting lower
294 transcript levels in absence of the Cmr-ß complex. In contrast, although some transposases
295 of the IS200/IS605 and IS607 families were found to be downregulated, certain
296 representatives were upregulated (especially in KO2).
297
298 **Adaptation to minimal media is affected in *cmr-ß* knockout mutant**
299 Since we observed several genes related to transport being differentially expressed in
300 complex (sucrose-tryptone) media, we were interested in examining to which extent this
301 phenotype would still be observable in defined media. Wildtype and KO2 strain were adapted
302 to grow in media containing either glucose or sucrose as sole carbon source. The switch from
303 complex to defined media caused a lag phase in both strains, however it was more
304 pronounced in the *cmr-ß* knockout strain (Fig. 9A). The switch to sucrose resulted in a lag
305 phase of about 48 hours (hr) for the wildtype strain, after which it assumed a growth rate
306 similar to the one exhibited under complex media and reached stationary after 100 hr, while
307 the knockout endured both a prolonged lag phase of about 72-96 hr as well as an overall
308 reduced growth rate with linear growth for the next 96 hr, reaching stationary phase after 192
309 hr. The switch to glucose media proved more cumbersome for both strains and resulted a
310 slightly different profile. The wildtype endured a lag phase of about 72 hours, after which it
311 followed linear growth for the next 138 hours, while the knockout exhibited a very long lag
312 phase of 264 hr, after which it showed exponential growth with a growth rate only slightly
313 lower than in complex media. After adaptation, the growth curves of wildtype and KO2
314 ultimately converged in glucose medium, but not in sucrose, where the wildtype grew slower

315 than the knockout (Fig. 9B and C, respectively). Of the differentially expressed genes in
316 glucose medium, 234 (~8 % of protein-coding genes) were upregulated and 260 (~9 % of
317 protein-coding genes) were downregulated in KO2 (not shown). In sucrose medium, more
318 than twice as many genes were found to be significantly differentially expressed in KO2,
319 among those 621 (~20 %) were upregulated and 560 (~19 %) were downregulated (not
320 shown). The complex media condition served as an internal control in this second
321 experiment, and we found 287 (~10 %) and 254 (~9 %) genes to be up- and downregulated
322 in KO2, respectively (not shown). Here, the vast majority of upregulated genes was consistent
323 with our previous dataset (~73 %) (Fig 10A and Supplementary Information). Interestingly, 20
324 genes were similarly upregulated in all three conditions according to DESeq analysis (Suppl.
325 Tab. S4). Together with genes exhibiting a strong correlation with all *cmr-ß* knockout samples
326 in combined PCA analyses (Fig. 10B, Suppl. Tab. S5), the overarching theme again points
327 towards perturbations in energy metabolism, transport and lipid biosynthesis following the
328 *cmr-ß* knockout. The higher expression of these genes, regardless of medium composition,
329 strongly implicate a role of the Cmr-ß in the regulation of metabolism either directly or
330 indirectly.
331
332
333 **DISCUSSION**
334
335 **Cmr-ß might not be a major contributor to CRISPR-Cas-mediated plasmid defense in *Sa.***
336 ***solfataricus***
337 The co-existence of two different type III CRISPR-Cas complexes in *Sa. solfataricus* and other
338 Sulfolobales[70] might seem redundant at first, however is most likely indicative of different
339 mechanistic specialization which enables complementation in response to varying load and
340 types of MGEs[71,72]. In this study, we set out to investigate the specific roles of Cmr-ß in the
341 immune response of *Sa. solfataricus* P1. Interestingly, while *cmr-ß* knockout mutants were
342 readily obtained, similar attempts at deleting the *csm* locus caused large genomic deletions
343 (Wimmer unpublished) and isolated mutants could therefore not be tested. Our plasmid
344 challenge assay indicated that the transcription-dependent targeting effect of a protospacer-
345 carrying plasmid in *cmr-ß* knockout mutant was comparable to the wildtype. Hence, *Sa.*
346 *solfataricus* P1 Csm-mediated immunity leads to a similar outcome irrespective of the
347 presence of the Cmr-ß system (see Fig. 4). In analogy to SisCmr-ß[33], one could assume that
348 Cmr-ß of *Sa. solfataricus* P1 does not synthesize significant amounts of cOA molecules and

349 is possibly not a major contributor to the canonical multi-faceted type III CRISPR - mediated
350 defense.
351
352
353 **Transcriptional response to the *cmr-ß* knockout supports accelerated growth phenotype**
354 **and interaction with regulatory networks**
355 The findings of our transcriptomic analysis show that the deletion of Cmr-ß had significant
356 effects on the transcriptional landscape and growth behavior. In accordance with accelerated
357 growth following the deletion, we could observe upregulation of genes involved in distinct
358 functional categories of energy production and conversion, as well as metabolism and
359 transport of amino acids, nucleotides, and carbohydrates. The upregulation of complex I
360 (NADH dehydrogenase) and two out of three complex IVs is interesting regarding their
361 importance in maintaining pH homeostasis and generating a proton motif force in
362 thermoacidophilic organisms[43]. This observation is even more compelling considering that
363 complex I is capable of pumping protons as opposed to complex II (succinate
364 dehydrogenase)[73] and the bb3-type terminal oxidase has a higher electron to proton pumping
365 ratio than the other terminal oxidases (aa3-type SoxABCDL and DoxBCE) due to the
366 presence of two proton pumping sites[74,75]. Moreover, its subunit SoxE was suggested to act
367 as a pH sensor and regulate electron flow[74,76]. The activity of characterized FHL complexes
368 has also been connected to counteracting cytoplasm acidification through removal of
369 formate and H2 evolution[77,78]. Moreover, being membrane-bound complexes with similarities
370 to complex I, it has long been hypothesized that they might be capable of proton pumping
371 and therefore contribute to proton motive force generation, a capacity strongly supported by
372 the formate-driven growth in *Thermococcus onnurineus*[79,80]. Both these activities might
373 explain the upregulation profile of the hydrogenase component of the FHL in the knockout
374 strains.
375
376 Upregulation of ABC-type and other transporters for the uptake of substrates such as sugars,
377 peptides/amino acids and phosphate further supports the accelerated growth phenotype of
378 the knockout strains. The additional upregulation of thiamine transport and biosynthesis as
379 well as nucleotide biosynthesis seemingly underlines the need for higher ATP synthesis and
380 energy generation. The increased incorporation of membrane-anchored proteins could be
381 supported by the upregulation of pivotal components of protein translocation pathways,
382 namely *tatC, secY* and *yidC*. As central component of the Tat pathway, TatC captures folded
383 proteins destined for translocation such as subunits of terminal oxidases that assemble their

384 cofactors prior to translocation in the cytoplasm[81,82]. In contrast, ABC transporters of CUT
385 class of *Sa. solfataricus* harbor type IV pilin like signal peptides and are presumed to be
386 secreted via the Sec pathway[44,45,83].
387
388 As summarized above, the upregulation of the ETC complexes and the FHL, as well as the
389 increased organic carbon availability implied by the upregulation of the respective
390 transporters supports the increase in growth rate observed in the deletion mutants under
391 complex media. The observed upregulation in main lipid metabolism genes as well as
392 downregulation of the ring synthases would also agree with this phenotype, as it would be
393 the expected profile under logarithmic growth without energy limitations[84–87]. The observed
394 profile in the *cmr-ß* knockout could also indicate that *Sa. solfataricus* is experiencing (or
395 displaying the transcriptional profile of) a mild case of acid stress, as suggested by the
396 upregulation of calditol synthase[58]. The general transcriptional profile however is not
397 reminiscent of a classic case of acid stress, as documented in *Sa. islandicus*, which displayed
398 decreased energy production, upregulation of the proton-pumping ATPase, upregulation of
399 *grsB*, upregulation of repair and turnover pathways and increased anabolism[88]. As always
400 though, transcriptomic profiles need to be interpreted with caution, as it has been shown that
401 the observed profiles do not always translate into matching proteomic or lipidomic profiles[88].
402 An unclear profile in our case is nevertheless not surprising, as the actual imposed condition
403 (the absence of the *cmr-ß* module) is neither an environmental stressor nor a manipulation of
404 nutrient levels, and therefore the observed phenotypic changes cannot be interpreted in a
405 strict framework. Rather, we are getting a glimpse of the regulatory networks and processes
406 that the Cmr-ß is involved in in the absence of external stimulants, thereby sometimes leading
407 to contradictory profiles.
408
409 **Functional link between CRISPR systems and cell envelope processes**
410 As outlined above, our transcriptomic analysis of type III-B CRISPR-Cas deletion mutants
411 repeatedly pointed towards an involvement with membrane-associated processes.
412 Accordingly, genes encoding for transmembrane proteins were significantly enriched among
413 the upregulated fraction in type III-B deletion mutants under all conditions examined (Fig. 8).
414 In general, a connection between CRISPR-Cas systems and membrane-associated
415 processes seems intuitive as it would allow for quick responses upon entry of infecting
416 viruses.
417

418 First hints at the functional link between the cell envelope and CRISPR-Cas were already
419 gathered relatively early on, when it was shown that the incorporation of misfolded proteins
420 translocated by the Tat system likely caused proton leakage at the membrane prompting the
421 activation of the type I CRISPR-Cas system in *E.coli*[89]. In the human pathogen *Francisella*
422 *novicida*, Cas9 complexed with a small CRISPR-associated RNA (scaRNA) was found to
423 transcriptionally repress Blp, a membrane-exposed lipoprotein usually recognized by the
424 hosts immune cells[90,91]. In the gram-negative bacterium *Serratia*, the Rcs signaling pathway
425 involved in outer membrane stress response[92] downregulates type I and type III CRISPR-Cas
426 defense while simultaneously enhancing innate immunity, e.g through repression of viral cell-
427 surface receptors[93]. Genome analyses have also pointed out the enriched presence of various
428 predicted membrane proteins in the genomic neighborhood of type III CRISPR-Cas systems,
429 indicating a close association with the cell envelope[27,28], although the exact nature of this
430 proposed functional connections largely remains to be investigated *in vivo*.
431
432 **The metabolic burden of maintaining CRISPR-Cas systems**
433 Some of the observed changes in the transcriptional landscape upon deletion of the *cmr-ß*
434 gene cluster could also be explained by the reduced metabolic burden and cost associated
435 with the maintenance of defense systems. In *Streptococcus thermophilus* DGCC7710
436 harboring multiple CRISPR-Cas systems (2 x type II, type I and type III), the disruption of one
437 of the *cas9* genes gives mutants a competitive advantage in competition with wildtype cells,
438 possibly due to a reduced lag phase[94]. In contrast, *Pseudomonas aeruginosa* PA14
439 possesses only one type I-F CRISPR-Cas system, and its deletion caused reduced
440 comparative fitness in competition assays[95]. In general, the studies in *P. aeruginosa* suggest
441 that constitutive fitness costs associated with the presence of functional CRISPR-Cas
442 systems are low, however conditional costs arise due to the elicitation of the immune
443 response upon MGE exposure[95–97]. Similar to what has been observed for *S. thermophilus*[94],
444 the deletion of one out of several co-existing *cas* gene modules also caused an accelerated
445 growth phenotype in *Sa. solfataricus* P1. However, the significant congruence between
446 transcriptomes from two different deletion mutant strains despite the presence of a second
447 large genomic deletion in only one of them suggests that we are, at least in part, witnessing
448 valid functional connections with certain degree of specificity.
449
450 **Are the observed changes the result of direct regulation by Cmr-ß or secondary effects?**
451 The observed profiles of anabolic pathways and membrane composition could be interpreted
452 either as direct regulatory targets or indirectly affected due to the increase in available energy

453 and resulting increased growth rate. The amount of (both characterized and uncharacterized)
454 differentially regulated transcription factors in our dataset also indicates the entanglement of
455 CRISPR-Cas systems with global regulatory networks. On this note, a LysR-type
456 transcriptional regulator involved in control of secondary metabolites and motility was shown
457 to repress type III-A immunity in the gram-negative bacterium *Serratia*[98]. Moreover, the
458 observed differential expression of transposase genes in response to the Cmr-ß deletion
459 could also influence host gene expression and are a known source of various ncRNAs in *Sa.*
460 *solfataricus*[99–101] and other archaea[102–106]. Former studies analyzing the content of isolated
461 Cmr-ß and Csm protein complexes have revealed that both complexes are sequestering a
462 proportion non-canonical guides in addition to canonical crRNAs *in vivo*[32,107], some of which
463 could match potential cellular targets, providing the possibility for direct CRISPR-mediated
464 regulation. However, we also expect that many effects are downstream events of primary
465 direct key regulation targets such as transcription factors, or reaction to the physiological
466 changes invoked by presumed direct effects (e.g. direct upregulation of ETC transcripts
467 would result in a change in the energy budget of the cell). Our current experimental design
468 does not allow for an investigation into the molecular mechanisms by which the Cmr-ß could
469 convey the observed regulatory effects, but we are working on elucidating the direct and
470 indirect effects in the future.
471
472 **CONCLUSION**
473
474 The deletion of the type III system consistently leads to an upregulation of genes encoding
475 for membrane proteins and membrane-associated processes, the most important of which
476 concern energy metabolism, the establishment of a proton motive force and essential nutrient
477 acquisition, accompanied by an accelerated growth phenotype. As these results are in the
478 absence of MGEs, we conclude that the type III system is exerting an influence on the greater
479 metabolic functions, and therefore fitness, of the organism irrespective of its role in cellular
480 defense.
481
482
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485 John van der Oost, Raymond Staals, Sonja V. Albers and Xu Peng for scientific discussions.
486
487

488 **DATA AVAILABILITY**

489 All datasets and supplementary tables will be made available upon publication of the
490 manuscript.

491

492

493

494

495 **MATERIALS & METHODS**

496

497 **Design of genetic constructs and cloning.** The synthetic mini CRISPR arrays (miniCRs)
498 containing one to three targeting spacers were assembled and cloned into pENTRY vectors
499 as described previously[35]. Two 800 to 900 bp sequences homologous to the upstream and
500 downstream regions of the *cmr-ß* locus were introduced into each vector to direct DNA repair
501 mechanisms. Flank 1 corresponding to the upstream region was amplified using primers
502 flank1_fw_SalI and flank1_rv, flank 2 corresponding to the downstream region was amplified
503 using primers flank2_fw and flank2_rv_EagI. The sequences of all primers used for
504 construction of genome editing constructs can be found in Suppl. Tab. S6. The resulting PCR
505 products were checked by gel-electrophoresis and purified (Monarch PCR&DNA Cleanup
506 Kit, NEB) according to manufacturer's instructions. Flanks were phosphorylated using the T4
507 Polynucleotide Kinase (NEB) and cleaved with corresponding restriction enzyme SalI-HF or
508 EagI-HF (NEB). pENTRY-miniCR vectors were double digested with SalI-HF and EagI-HF
509 (NEB). Linearized pENTRY-miniCR vectors and both flanks were fused using the Quick
510 Ligation™ Kit (NEB). Correct sequences of miniCRs and flanks were verified by Sanger
511 sequencing and consequently transferred from pENTRY vectors into destination vector pIZ-
512 GW[36,108] using the Gateway™ LR Clonase™ II Enzyme mix (Invitrogen™).

513

514 **Culturing, transformation and isolation of knockout mutants.** Uracil-auxotrophic derivate
515 M18 of *Sa. solfataricus* P1 (DSM 1616, ATCC 35091) was cultivated in Brock salts medium
516 supplemented with D(+)-Sucrose (0.2 %, w/v) (Serva), Tryptone (0.1%, w/v) (Roth) and Uracil
517 (0.0125 mg/mL) (Sigma Aldrich), in short Brock medium S/T/U. Media pH was initially
518 adjusted to 3.5 with H_2SO_4 and cultures were incubated under shaking conditions at 77 °C
519 (Zebec 2016). Growth was assessed by measuring optical density (OD) at a wavelength of
520 600nm in a spectrophotometer. Competent cells for electroporation were prepared as
521 described previously[109]. Per construct, 50 µl of M18 competent cells were mixed with 150 ng
522 of plasmid DNA and electroporated using 1-mm cuvettes in a Gene Pulser Xcell (Biorad) with

523 the following settings: 1240 V, 1000 Ω, 25 µF. Electroporated cells were transferred to equal
524 volumes of recovery solution[110] and incubated for 1 hour at 77 °C. After recovery,
525 electroporated cells were inoculated directly into 50mL selective medium containing D(+)-
526 Sucrose (0.2%, w/v) and NZ-Amine (0.1%, w/v) (Fluka Analytics). For plasmid
527 counterselection, cells harboring pIZ-cmr73ts were transferred to medium containing
528 Sucrose, Tryptone, Uracil and 5-Fluoroorotic acid (0.05 mg/mL) (5-FOA, Sigma Aldrich).
529 Consequently, dilution streaks on gellan gum plates (0.64% w/v) (Gelrite, Roth) with the same
530 media composition were performed. Deletion mutant colonies finally underwent three
531 consecutive rounds of plating (without 5-FOA) to ensure the presence of a single genotype.
532 As a control, a purified isolate of untransformed *Sa. solfataricus* M18 (wildtype) was
533 generated using the same approach. Plates were incubated under high humidity at 77°C.
534
535 **Culture PCR.** The presence of the *cmr-ß* genes was checked by adding 2 µl aliquots from
536 OD samples or colony suspensions as template in PCR reaction with primer pairs cmr-KO-
537 check_fw/rv or cmr-middle_fw/cmr-KO-check_rv. Successful removal of plasmid and
538 persistence of IS-element conferring uracil auxotrophy were verified using primers
539 ORF904_fw/rv and pyrBE_fw/rv, respectively. All primers used for culture PCR are listed in
540 Suppl. Tab S7. For all culture PCRs, full reaction volumes were loaded on 1% agarose gels
541 together with 1 kb Plus DNA ladder (Thermo Scientific). Knockout of *cmr-ß* indicated by
542 smaller bands in gel-electrophoresis of PCR products was verified by sequencing of DNA
543 from gel-purified bands (Monarch DNA extraction Kit, NEB).
544
545 **Plasmid challenge assay.** ORF406 of conjugative plasmid pNOB8 was amplified using
546 primers ORF406_fw/rv. Matching overhangs were introduced into pENTRY vectors
547 downstream of a constitutive promoter (i.e truncated version of leader sequence of CRISPR
548 array D in *Sa. solfataricus* P2) with primers pENTRY-ORF406_fw/rv. The resulting product
549 was used as template in a touchdown PCR reaction (57°C, TΔ-0,2) for fusion with the ORF406
550 amplicon. ORF406 harbors a protospacer with a 5'NGA PAM exhibiting partial
551 complementarity to spacer A53 of *Sa. solfataricus* P2[39] which is absent from P1. Using
552 primers OE-D2.109_fw/rv, this protospacer region was exchanged to match spacer 109 of
553 CRISPR array D2 (D2.109) in P1 as described previously[24]. The insertion of an additional
554 nucleotide directly downstream of the protospacer rendered the original PAM sequence
555 dysfunctional impeding type I-A CRISPR-mediated interference. Additionally, we constructed
556 a protospacer D2.109-a with an antitag (5' CTTTCAAT) matching to the 5' tag of the D2.109
557 crRNA using primer OE-D2.109-a_fw. The sequences of all primers used for construction of

558 ORF406 target constructs are listed in Suppl. Tab. S6. Transformation of wildtype and
559 deletions strain was carried out as described above, with the following alterations: prior to
560 electroporation competent cells from both strains were adjusted to the same optical densities
561 with 20 mM Sucrose, then three replicates of 50 μL cells mixed with 350 ng of plasmid DNA
562 were set up for each construct. Cells without the addition of plasmid DNA were used as
563 control. Following 1 hour recovery, each replicate was inoculated in 50 mL selective medium.
564 After 41 hours (i.e. before cultures had started to grow), 45 μl of serial dilutions from 10^1 to
565 10^{-5} from all cultures were spotted on selective gellan gum plates containing D(+)-Sucrose
566 (0.2%, w/v) and NZ-Amine (0.1%, w/v).
567
568 **Adaptation experiment.** M18 wildtype and KO2 were inoculated from glycerol stocks into
569 prewarmed Brock medium S/T/U, 50 mL, pH 3.5, and incubated under shaking conditions at
570 77°C. As a preadaptation step, aliquots of both strains were then transferred to Brock
571 medium G/T/U supplemented with D(+)-Glucose (Sigma Aldrich) instead of Sucrose at the
572 same final concentration. A second aliquot was again transferred to Brock S/T/U. For the
573 adaptation growth curve, 4 replicate cultures were set up for the following media
574 compositions: Brock medium Sucrose (0.3%, w/v) with Uracil, Brock medium Glucose (0.3%,
575 w/v) with Uracil, and standard complex Brock medium S/T/U. When cultures had reached
576 late exponential growth, one replicate was transferred to 4 replicate flasks with fresh medium
577 of the same composition until growth had stabilized for two consecutive transfers. At that
578 point, 5 mL samples were taken at OD_{600}=0.2 for RNA preparation. Throughout the
579 experiment, cultures were inoculated to the same initial OD_{600}.
580
581 **DNA preparation and genome mutation analysis.** DNA was extracted as described in ref[24].
582 The sequencing was performed by the Next Generation Sequencing Facility at at Vienna
583 BioCenter Core Facilities (VBCF), member of the Vienna BioCenter (VBC), Austria. Samples
584 were sequenced (150 PE) sequencing on an Illumina MiSeq platform with a Westburg DNA
585 preparation kit resulting in 300,000-500,000 reads per sample. Raw reads were downloaded
586 from the VBCF website and checked using md5sum. FastQC[111] version 0.11.8 and MultiQC[112]
587 version 1.8 were used to initially analyze sequences and the samtools package[113] version
588 1.10 (using htslib 1.10[114]) along with bedtools [115] version 2.29.0 was used to convert file types.
589 Trimmomatic[116] version 0.36 trimmed adapter sequences and cropped reads using the
590 options: SLIDINGWINDOW:5:20 LEADING:5 TRAILING:5 MINLEN:50 HEADCROP:12. The
591 stand-alone program PrinSeq Lite[117] version 0.20.4 was used to remove sequences < 38 bp
592 and/or with quality scores < 30. Next, Bowtie2[118] version 2.3.5.1 was used to align the

593 remaining RNA sequences to the *Sa. solfataricus* strain P1 reference genome
594 (NZ_LT549890.1) downloaded from the National Center for Biotechnology Information
595 (NCBI). Mutations, insertions, and deletions were then found using breseq[119,120] version
596 0.32.1 with default settings using the NCBI RefSeq annotation from 02/19/2020.
597
598 **RNA preparation and sequencing analysis.** RNA was extracted from 5 mL of culture
599 sampled at OD_{600}=0.2 as described in ref[24]. Sample quality control, library preparation (150
600 PE) from rRNA-depleted samples (to approx. 50%) and sequencing on an Illumina NovaSeq
601 platform was performed by Novogene (UK) Co., Ltd. Raw reads were downloaded from the
602 sequencing facilities' website and checked using md5sum. Adapter sequences were trimmed
603 using Trimmomatic[116] version 0.39 using the following settings: HEADCROP:12
604 SLIDINGWINDOW:4:15 LEADING:3 TRAILING:3 MINLEN:30. Reads were filtered for a
605 minimum mean quality score of 30 using PrinSeq Lite[117] version 0.20.4. Reads mapping to
606 ribosomal RNA were removed from total reads with the sortmeRNA tool[121] version 4.3.6 by
607 supplying a curated reference file containing *Sa. solfataricus* P1 rRNA sequences. The
608 remaining reads were aligned to the *Sa. solfataricus* P1 reference genome (NCBI:
609 NZ_LT549890.1) using Hisat2[122] version 2.2.1 with parameter --rna-strandness RF. The
610 resulting sequence alignment files were converted to binary format using the samtools
611 package[113] version 1.20. featureCounts from the Subread package [123] version 2.0.6 assigned
612 mapped reads to genomic features (feature and attribute type specified as "gene" and "ID",
613 respectively) using a General Feature Format (GFF) annotation file with RefSeq annotations
614 downloaded from the NCBI website on as a reference. To assess expression of transposase
615 genes, a separate run with featureCounts was performed to inlcude reads mapping at
616 multiple positions within the genome ('-M' option for multimapping reads). The output count
617 matrix was used to perform differential expression analysis with the DESseq2 package[124]
618 version 1.42.1 in R[125] version 4.3.3 with Rstudio[126] version 2023.12.1+402. Default p-value
619 adjustment in DESeq2 (Benjamini-Hochberg method) was used to determine relevant p-
620 values (referred to as p-adj in result tables). Principal component analysis (PCA) was
621 performed as described previoulsy[40].
622
623 **Assignment of transporter families, arCOGs and transmembrane domains, and functional**
624 **enrichment analysis.** The BLAST tool integrated into the curated Transporter Classification
625 Database (TCDB)[127] was used to classify transporters. Archaeal Clusters of Orthologous
626 Groups (arCOGs)[128] were assigned to genes according to database version 2018. Genes
627 without an arCOG assignment were annotated using eggNOG-mapper[129] version 2.1.12.

628 Genes encooding proteins with predicted transmembrane domains were identified using
629 Phobius[130]. In both cases, a modified statistical hypergeometric test to determine functional
630 categories significantly enriched for *Sa. solfataricus* (*P*-value < 0.05) among the up- or
631 downregulated genes was conducted using the phyper function in R[40].
632
633
634
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FIGURES

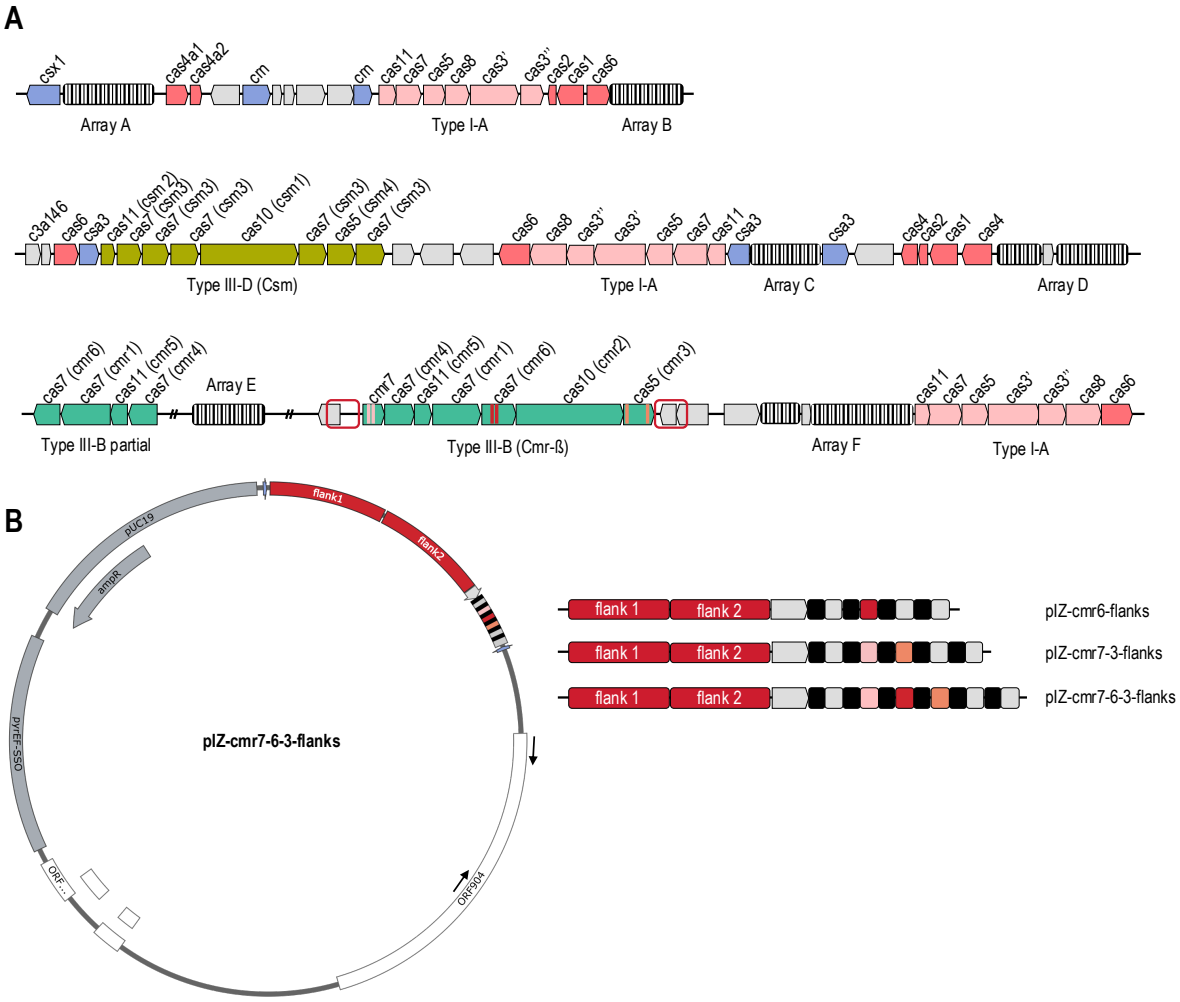


Figure 1. (A) Overview of the CRISPR-Cas systems in *Sa. solfataricus* P1. Three type I-A (pink), one type III-D (lime green), and one type III-B (green) system are present. In addition, a partial type III-B *cas* gene cluster can be detected. The strain harbors six CRISPR arrays (striped boxes), two are split through the insertion of fragments, in the case of array F stemming from a pNOB8-like conjugative plasmid. Six different protospacers that were chosen for the type I-A CRISPR-mediated deletion of the *cmr-ß* gene module are indicated. Two protospacers each are located in genes *cmr7* (pink), *cmr6* (red) and *cmr3* (salmon). Flanking sequences of the *cmr-ß* genes (red boxes) are provided as donor for the DNA repair process (see B). **(B)** Different combinations of artificial spacers (single to triple) all targeting either template or coding strand of the respective genes were cloned into a miniCR array on the plasmid-based vector pIZ, preceded by donor DNA sequences (red). Color code for miniCR: self-targeting spacers – equivalent to (A); non-targeting spacers (used for cloning procedure)– grey; repeats – black.

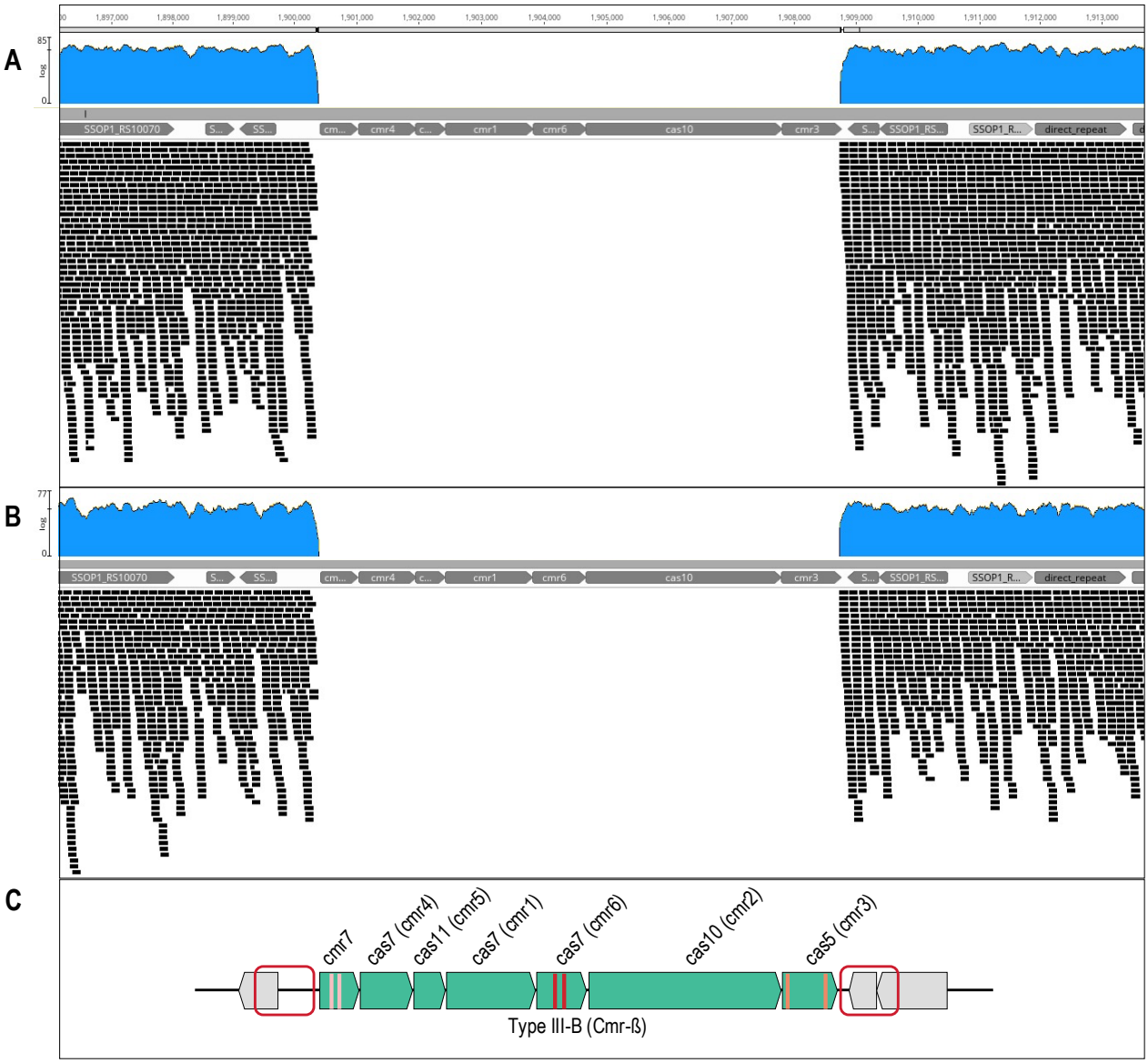


Figure 2. Genome viewer snapshot of *cmr-ß* knockout mutants. Paired-end reads (150 bp) generated from **(A)** KO1 and **(B)** KO2 were mapped to the *Sa. solfataricus* P1 NCBI reference genome (NZ_LT549890.1). Reference genes are displayed as grey arrows. The upper panel shows read coverage (blue) and the lower panel shows aligned reads (black). The excision of the *cmr-ß* gene cluster and its promoter as depicted in **(C)** was confirmed to have happened as intended for both isolated mutant strains.

Figure 3. Genome viewer snapshot of second deletion that occurred spontaneously in *cmr-ß* mutant KO2. Paired-end reads (150 bp) were mapped to the *Sa. solfataricus* P1 NCBI reference genome (NZ_LT549890.1). Reference genes are displayed as grey arrows. The upper panel shows read coverage (blue) and the lower panel shows aligned reads (black). The deleted region indicated by a red arrow spans over 37 kb and is borderd by two IS5 family transposase (pseudo-) genes. Read alignments within the indicated deleted region correspond to copies of different IS4 family transposase genes.

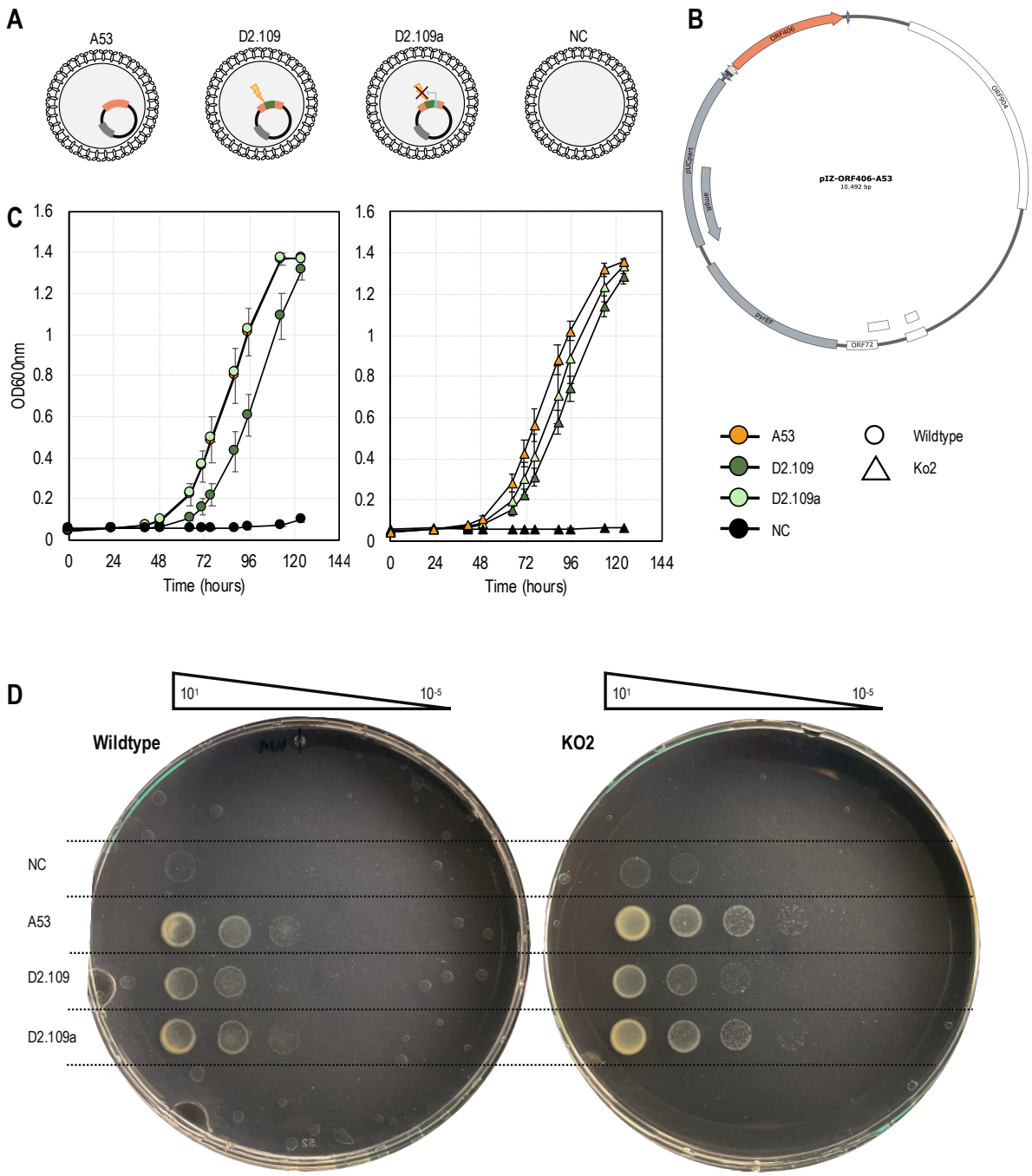
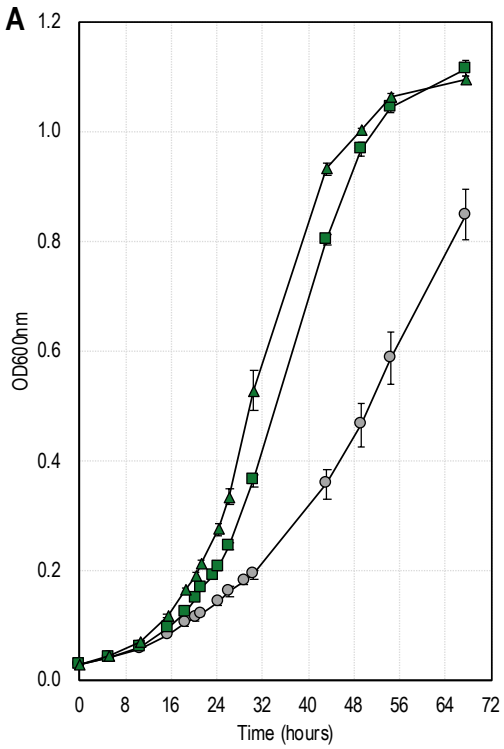
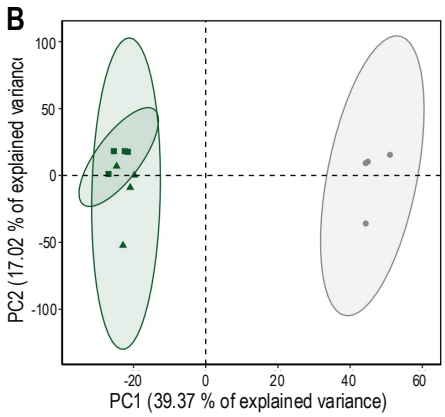
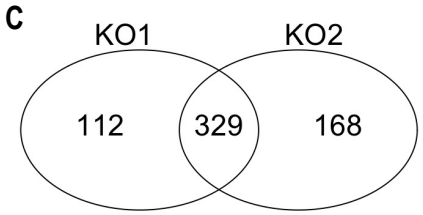
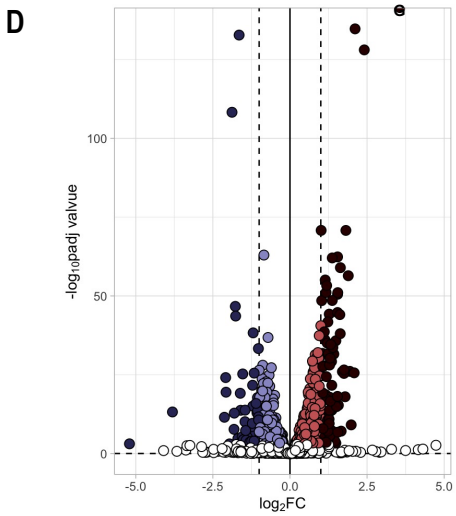


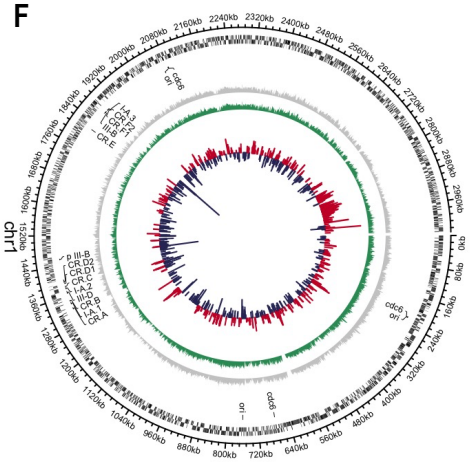
Figure 4. Plasmid challenge experiment. (A) Wildtype and deletion mutant KO2 were transformed with plasmids harboring different protospacers: A53 is not recognized by the CRISPR immune system due the lack of a matching spacer; D2.109 matches an endogenous spacer in CRISPR locus D eliciting type III CRISPR-mediated immunity; D2.109a harbors the same protospacer as in D2.109 and a matching 3' antitag to inhibit abortive infection. Negative control (NC) cells did not receive a plasmid and therefore lack an intact *pyrEF* locus. **(B)** Plasmid map of pIZ-ORF406-A53. **(C)** Growth curves for control (left) and KO2 (right) were generated by measuring optical density at 600nm, samples are color coded as follows: NC - black; A53 - orange; D2.109 - dark green; D2.109a - light green. Error bars: mean ± SD (n = 3, i.e. three separate inoculations after electroporation). **(D)** After transformation, cells were recovered in selective media before performing 10-fold serial dilutions (10^1 to 10^{-5}) and spotted on plates. One representative plate per strain is shown, the remaining plates are provided in the supplementary material (Suppl. Fig. S2 and S3).

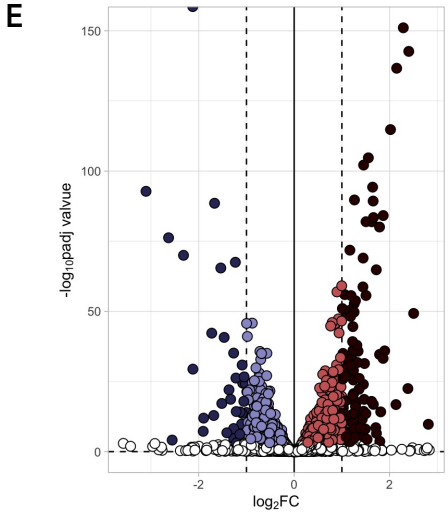

A
OD600nm
Time (hours)


B
PC2 (17.02 % of explained variance)
PC1 (39.37 % of explained variance)


C
KO1
KO2
112
329
168


D
-log10padj valvue
log2FC


F
chr1


E
-log10padj valvue
log2FC

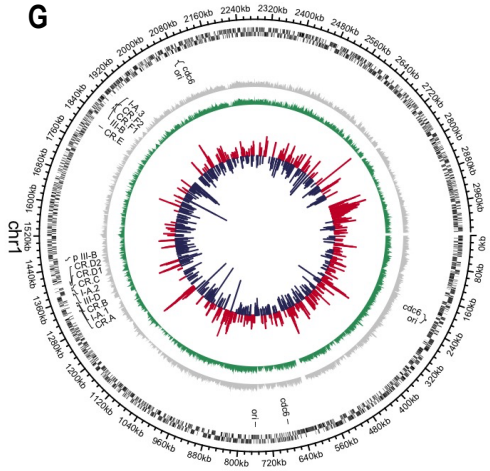

G
chr1

Figure 5. Knockout of the *cmr-ß* genes leads to growth accelaration and significant changes on the transcriptomic level. **(A)** Initial growth curve following isolation of two separate *cmr-ß* knockout mutants in comparison to wildtype undergoing the same procedure for the isolation of a single colony. Growth was followed by measuring optical density at 600nm. Color code: wildtype control – grey; deletion mutant 1 (KO1) – dark green; deletion mutant 2 (KO2) – light green. Error bars, mean ± SD (n = 4, i.e. four separate inoculations). **(B)** Principal component analysis of log transformed transcriptomic data from early exponential growth phase. Ellipses indicate 95% confidence interval. Same color code as in growth curve is used. **(C)** Venn diagram showing overlap between KO1 and KO2 regarding significantly upregulated genes in comparison to wildtype. **(D)** and **(E)** Volcano plots displaying log2-fold changes (x-axis) and associated significance (-log10 adjusted p value, y-axis), comparing KO1 and KO2, respectively. Color code indicates genes that are strongly upregulated (log2FC > 1, padj ≤ 0.001, dark red), upregulated (log2FC < 1, padj ≤ 0.001, red), strongly downregulated (log2FC < -1, padj ≤ 0.001, dark blue), downregulated (log2FC > -1, padj ≤ 0.001, blue). **(F)** and **(G)** Circular genome-wide plot for KO1 and KO2, respectively, showing *Sa. solfataricus* genome with position 0 to the right. Position of origins of replication (ori) and corresponding *cdc6* genes, as well as CRISPR-Cas systems are indicated. From outside inwards, rings indicate annotated coding sequences (black), log2-transformed TPM values for control (grey) and knockout mutant (green). Log2-fold changes in expression levels are displayed for significantly upregulated (padj ≤ 0.001, red) and downregulated (padj ≤ 0.001, blue) genes.

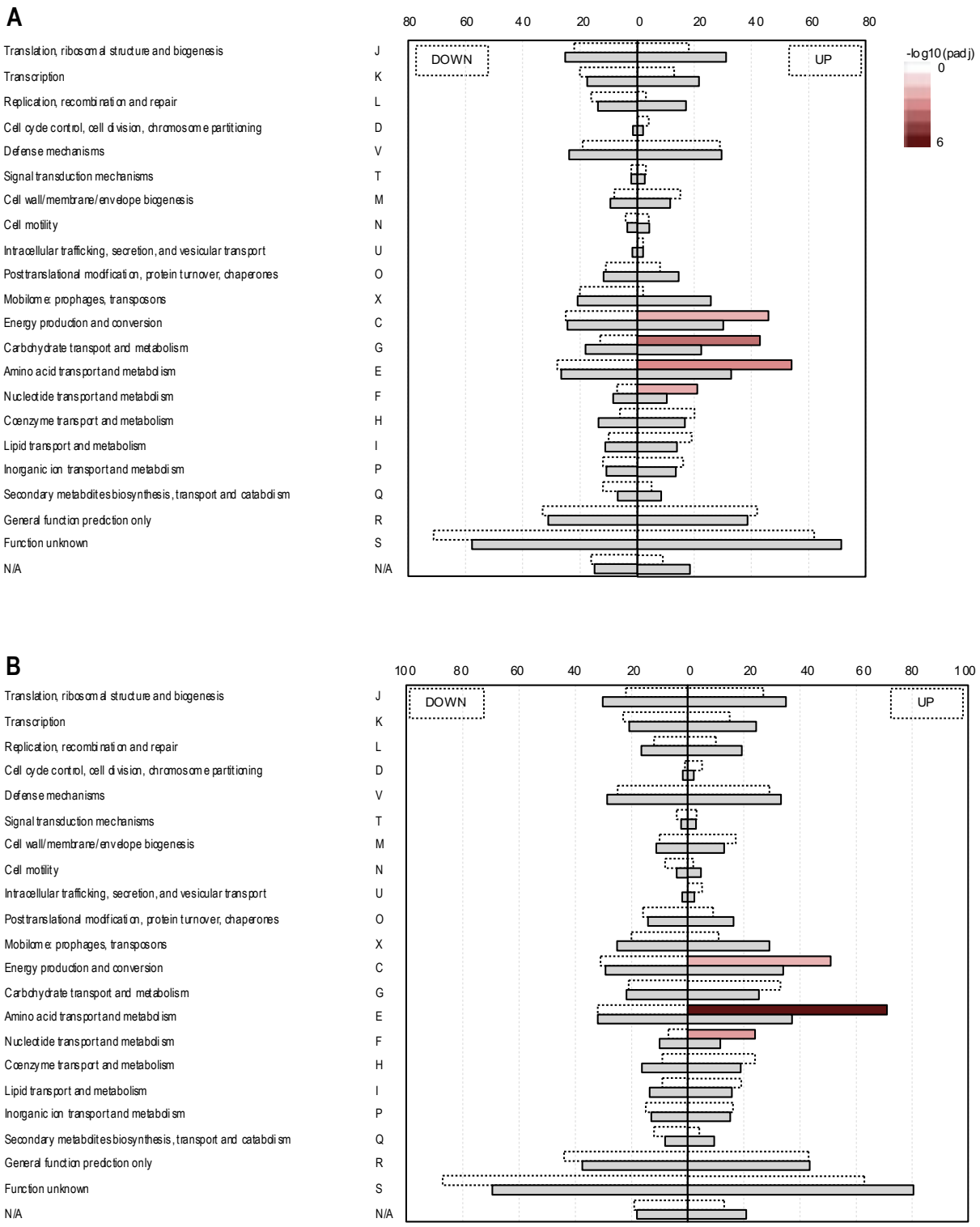


Figure 6. Functional enrichment analysis based archaeal clusters of orthologous genes (arCOGs) for **(A)** KO1 and **(B)** KO2. Left and right side of the plot show results for significantly downregulated and upregulated genes, respectively. Grey bar size represents the number of genes expected for each category based on a hypergeometric test, whereas neighboring bars represent the found number of genes for each category. Categories with padj-value < 0.05 are considered significantly overrepresented and highlighted by solid lines around the bar. Significance levels (-log10 transformed adjusted p-value) are indicated by color scale from white to dark red.

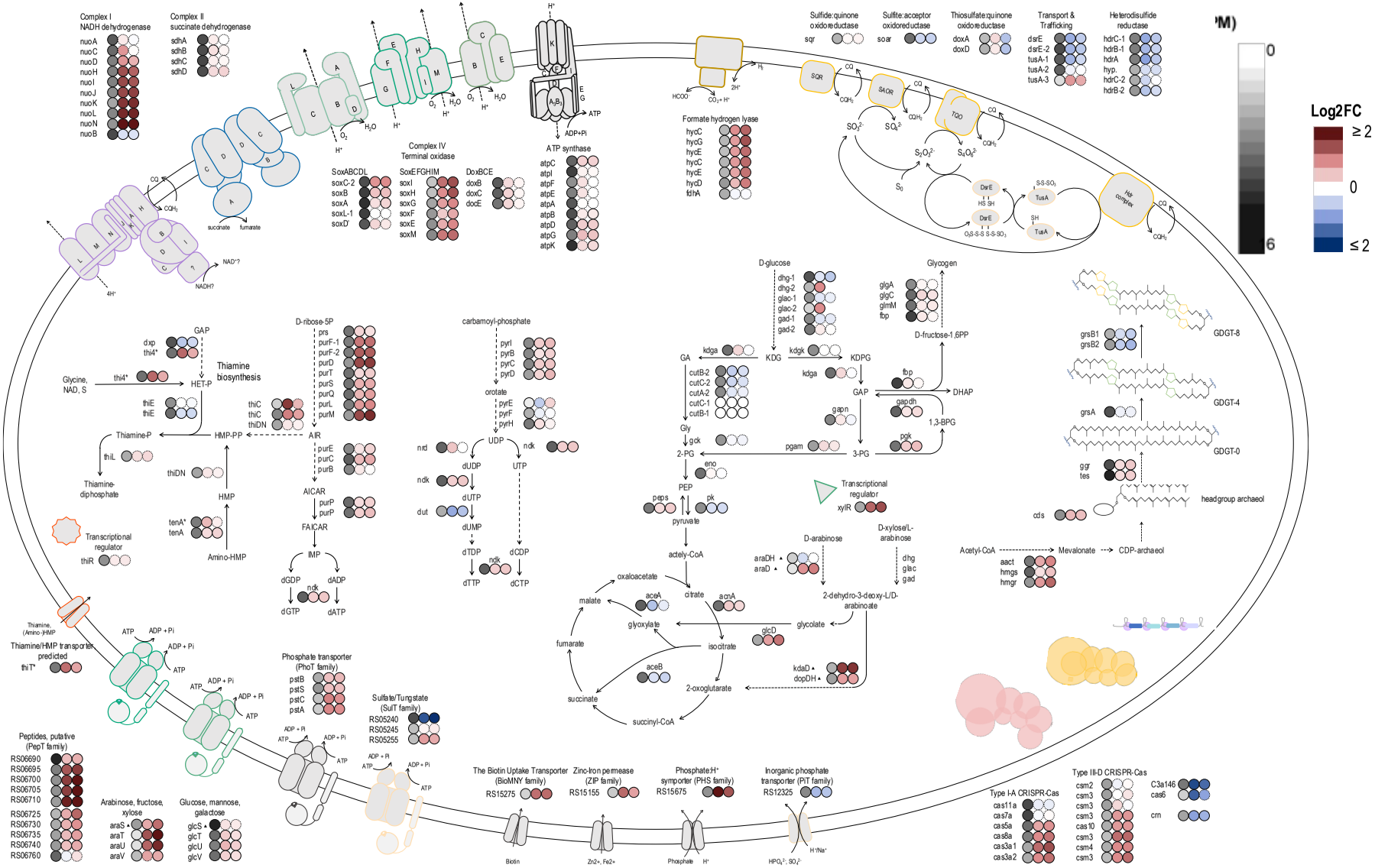

Complex I NADH dehydrogenase
nuoA
nuoC
nuoD
nuoH
nuoI
nuoJ
nuoK
nuoL
nuoN
nuoB
Complex II succinate dehydrogenase
sdhA
sdhB
sdhC
sdhD
Complex IV Terminal oxidase
SoxABCDL
soxC-2
soxB
soxA
soxL-1
soxD
SoxEFGHIM
soxI
soxH
soxG
soxF
soxE
soxM
DoxBCE
doxB
doxC
doxE
ATP synthase
atpC
atpI
atpF
atpE
atpA
atpB
atpD
atpG
atpK
Formate hydrogen lyase
hycC
hycG
hycE
hycC
hycE
hycD
fdhA
Sulfide:quinone oxidoreductase
sqr
Sulfite:acceptor oxidoreductase
soar
Thiosulfate:quinone oxidoreductase
doxA
doxD
Transport & Trafficking
dsrE
dsrE-2
tusA-1
tusA-2
tusA-3
Heterodisulfide reductase
hdrC-1
hdrB-1
hdrA
hyp.
hdrC-2
hdrB-2
Log2FC
≥ 2
0
≤ 2
D-glucose
dhg-1
dhg-2
glac-1
glac-2
gad-1
gad-2
Glycogen
glgA
glgC
glmM
fbp
D-fructose-1,6PP
GDGT-8
GDGT-4
GDGT-0
grsB1
grsB2
grsA
ggr
tes
cds
headgroup archaeol
Acetyl-CoA
Mevalonate
CDP-archaeol
aact
hmgs
hmgr
Thiamine biosynthesis
GAP
dxp
thi4*
Glycine, NAD, S
thi4*
HET-P
thiE
thiE
Thiamine-P
thiL
Thiamine-diphosphate
HMP-PP
thiDN
HMP
tenA*
tenA
Amino-HMP
Transcriptional regulator
thiR
D-ribose-5P
prs
purF-1
purF-2
purD
purT
purS
purQ
purL
purM
thiC
thiC
thiDN
AIR
purE
purC
purB
AICAR
purP
purP
FAICAR
IMP
dGDP
dADP
ndk
dGTP
dATP
carbamoyl-phosphate
pyrI
pyrB
pyrC
pyrD
orotate
pyrE
pyrF
pyrH
UDP
ndk
UTP
nrd
dUDP
ndk
dUTP
dut
dUMP
dTDP
ndk
dCDP
dTTP
dCTP
KDG
kdga
kdgk
KDPG
kdga
GAP
fbp
DHAP
gapdh
1,3-BPG
gapn
pgk
pgam
3-PG
eno
2-PG
gck
Gly
cutB-2
cutC-2
cutA-2
cutC-1
cutB-1
GA
PEP
peps
pk
pyruvate
actely-CoA
citrate
acnA
isocitrate
aceB
2-oxoglutarate
succinyl-CoA
succinate
fumarate
malate
oxaloacetate
aceA
glyoxylate
glcD
glycolate
kdaD
dopDH
2-dehydro-3-deoxy-L/D-arabinoate
D-arabinose
araDH
araD
D-xylose/L-arabinose
dhg
glac
gad
Transcriptional regulator
xylR
Thiamine, (Amino-)HMP
Thiamine/HMP transporter predicted
thiT*
ATP
ADP + Pi
Phosphate transporter (PhoT family)
pstB
pstS
pstC
pstA
Sulfate/Tungstate (SulT family)
RS05240
RS05245
RS05255
Peptides, putative (PepT family)
RS06690
RS06695
RS06700
RS06705
RS06710
RS06725
RS06730
RS06735
RS06740
RS06760
Arabinose, fructose, xylose
araS
araT
araU
araV
Glucose, mannose, galactose
glcS
glcT
glcU
glcV
The Biotin Uptake Transporter (BioMNY family)
RS15275
Biotin
Zinc-Iron permease (ZIP family)
RS15155
Zn2+, Fe2+
Phosphate:H+ symporter (PHS family)
RS15675
Phosphate
H+
Inorganic phosphate transporter (PiT family)
RS12325
HPO4 2-, SO4 2-
H+/Na+
Type I-A CRISPR-Cas
cas11a
cas7a
cas5a
cas8a
cas3a1
cas3a2
Type III-D CRISPR-Cas
csm2
csm3
csm3
csm3
csm3
csm4
csm3
C3a146
cas6
crn

Figure 7. Response in metabolism to deletion of type III-B CRISPR-Cas system. First circle from left to right represents log2-transformed TPM values as an approximation for expression levels in wildtype control, indicated by color bar from white (not expressed) to black (highly expressed). Log2FC in transcript levels found in KO1 and KO2 are represented in that order by the two following circles, indicated by color scale from dark blue (strongly downregulated) to dark red (strongly upregulated). Significant change (padj ≤ 0.001) is indicated by solid outlines around circles. Genes with predicted binding sites for transcription factors ThiR or XylR are indicated by star or triangle symbols, respectively.

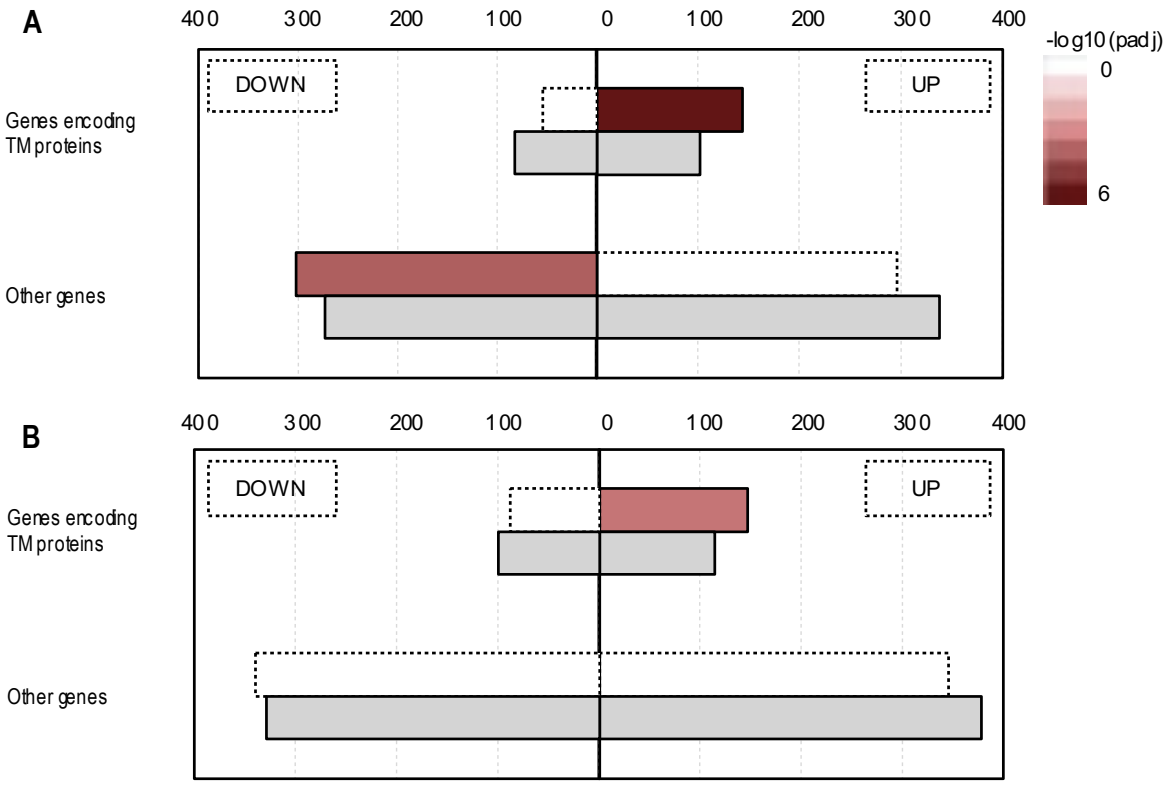


Figure 8. Enrichment analysis of genes encoding transmembrane-domain proteins as predicted for protein products by phobius. for **(A)** KO1 and **(B)** KO2. Left and right side of the plot show results for significantly downregulated and upregulated genes, respectively. Grey bar size represents the number of genes expected for each category based on a hypergeometric test, whereas neighboring bars represent the found number of genes. Categories with padj-value < 0.05 are considered significantly overrepresented and highlighted by solid lines around the bar. Significance levels (-log10 transformed adjusted p-value) are indicated by color bar from white to dark red.

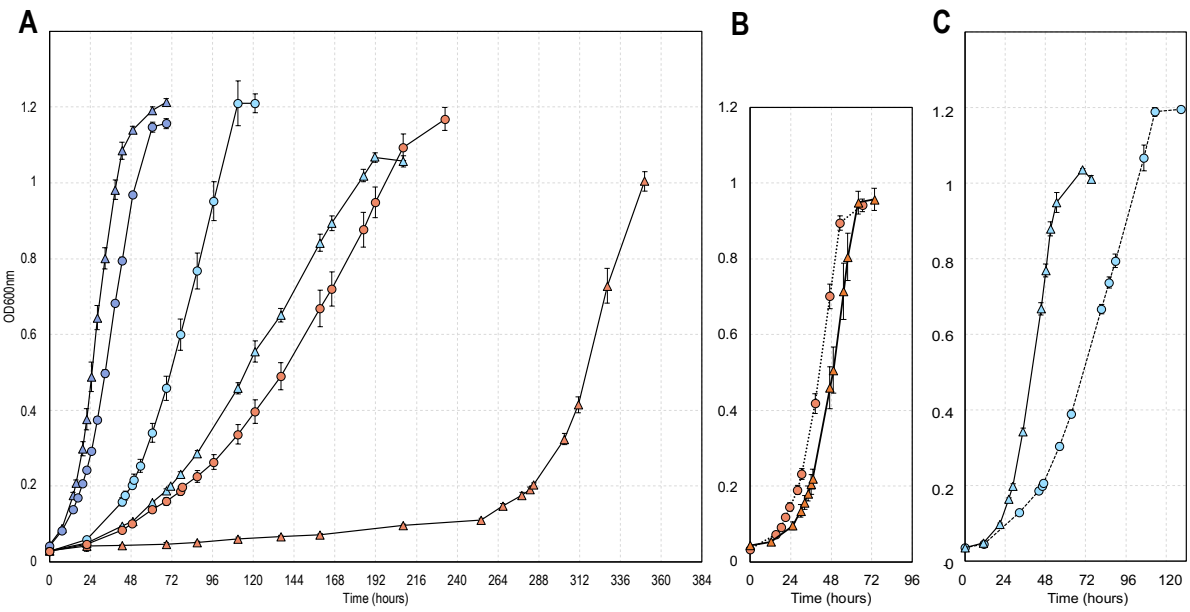


Figure 9. Adaptation Experiment Growth Curves. (A) Switching wildtype control and deletion mutant from complex to defined carbon sources sucrose and glucose causes lag phase during adaptation. Growth was followed by measuring optical density at 600nm. Wildtype control and Ko2 samples are represented by circles and triangles, respectively. Samples are color coded according to media composition as follows: Sucrose-tryptone – blue; sucrose – light blue; glucose – orange. Error bars, mean ± SD (n = 4, i.e. four separate inoculations). **(B)** and **(C)** show growth of KO2 and wildtype in glucose and sucrose media, respectively, after adaptation.

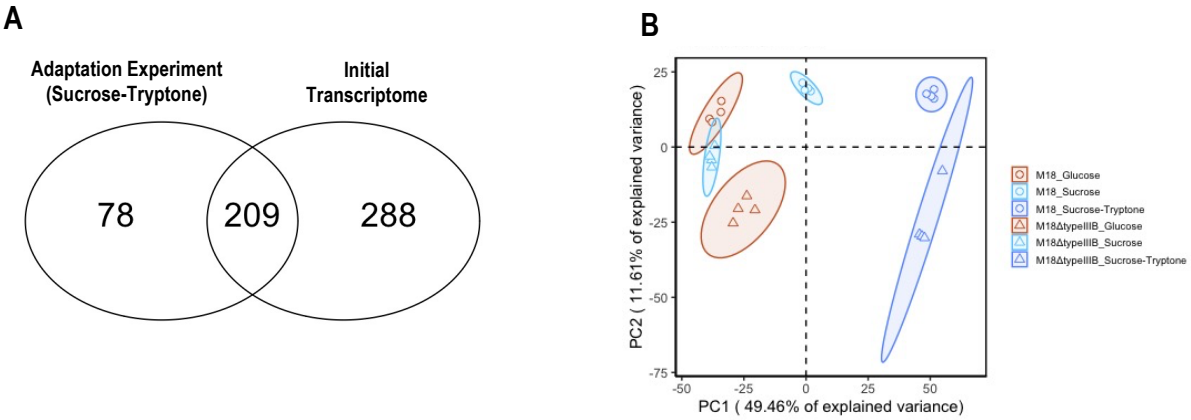


Figure 10. Transcriptomic Analysis of Cultures Adapted to Glucose and Sucrose Medium. (A) Venn diagram showing overlap in significantly upregulated genes between initial transcriptomic dataset and sucrose-tryptone condition of adaptation experiment. **(B)** Principal component analysis of log transformed transcriptomic data from early exponential growth phase. Ellipses indicate 95% confidence interval. Wildtype control and KO2 samples are represented by circles and triangles, respectively. Samples are color coded according to media composition as follows: Sucrose-tryptone – blue; sucrose – light blue; glucose – orange.