Host adaptation mediated by intergenic evolution in a bacterial pathogen

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Abstract

Bacterial pathogens evolve during the course of infection as they adapt to the selective pressures that confront them inside the host. Identification of adaptive mutations and their contributions to pathogen fitness remain a central challenge. Although mutations can either target intergenic or coding regions in the pathogen genome, studies of host adaptation have focused predominantly on molecular evolution within coding regions whereas the role of intergenic mutations remains unclear. Here, we address this issue and investigate the extent to which intergenic mutations contribute to the evolutionary response of pathogens to host environments, and if intergenic mutations have distinct roles in host adaptation. We characterize intergenic evolution in 44 lineages of a clinically important bacterial pathogen, *Pseudomonas aeruginosa*, as they adapt to their hosts. We identify 88 intergenic regions in which parallel evolution occur. At the genetic level, we find that mutations in these regions under selection are located primarily within regulatory elements upstream of transcriptional start sites. At the functional level, we show that these mutations both create or destroy regulatory interactions in connection to transcriptional processes, and are directly responsible for evolution of important pathogenic phenotypes including antibiotic sensitivity. Importantly, we find that intergenic mutations are more likely to be selected than coding region mutations, and that intergenic mutations enable essential genes to become targets of evolution. In summary, our results highlight the evolutionary significance of intergenic mutations in creating host-adapted variants, and that intergenic and coding regions have different qualitative and quantitative contributions to this process.

Significance

Pathogens adapt to their host during infection, but the contribution and function of non-coding intergenic sequences to adaptation is poorly understood. Here, genome-wide identification of adaptive mutations within intergenic regions demonstrates that these sequences constitute an important part of the genetic basis for host adaptation. We find that intergenic mutations are abundant relative to adaptive mutations within coding sequences, and can contribute directly to evolution of pathogen relevant traits. Importantly, we find that intergenic mutations modify expression of essential genes and thus make contributions that are functionally distinct from coding mutations. These results improve our understanding of the evolutionary processes *in vivo*, and can potentially assist in refining predictions of pathogen evolution, disease outcome, and antibiotic resistance development.

Introduction

Bacterial pathogens evolve during infection as they adapt to the environment inside the host (1). Since the bacterial phenotypes selected *in vivo* may have profound impact on disease severity and progression (2, 3) and response to antibiotic therapy (4), identification and analysis of the full range of beneficial genetic changes that underlies host adaptation is of importance.

Although adaptive mutations may potentially change the sequences of either coding regions or non-translated intergenic regions to affect protein function or expression, respectively, studies of pathogen adaptation during infection of host tissues have focused predominantly on molecular evolution within coding regions whereas the role of adaptive mutations in intergenic regions has received comparably less attention. The shortage of systematic, genome-wide analyses of intergenic evolution in bacterial pathogens is surprising given the fact that these regions are home to a large number of functional elements required for expression of virulence and resistance determinants *in vivo*, and that intergenic regions are maintained by purifying selection in many bacterial species (5-7). Moreover, *cis*-regulatory mutations are known to play an important role in phenotypic evolution in eukaryotic organisms (8). Overall, it remains unclear to what extent intergenic mutations contribute to the evolutionary response of pathogens to the host environment, and if intergenic mutations have a qualitative distinct role in host adaptation.

There are clear, albeit few, examples of intergenic regions which evolve under selection within the host. For example, evolution of a novel regulatory interaction between the virulence regulator SsrB and the promoter of the *sfrN* gene was shown

to result in enhanced within-host fitness in *Salmonella* Typhimurium (9). In *Pseudomonas aeruginosa*, evolution of the intergenic region of the *phuR-phuSTUVW* genes during host colonization was shown to increase expression of the Phu heme uptake system and improve the ability of the pathogen to acquire iron from hemeoglobin (10). In *Mycobacterium tuberculosis*, evolution of ethambutol resistance has been linked to acquisition of mutations within the *empAB* promoter region that enhance expression of enzymes essential for the synthesis of cell wall arabinogalactan (11). These and other examples point towards an evolutionary significant role of mutations in intergenic regions in connection to bacterial pathogenesis, and justify a broader analysis of this type of mutations.

One reason for the paucity in genome-wide analysis of intergenic evolution is probably related to the inherent difficulties in inferring function directly from the sequence within intergenic regions and, consequently, to differentiate adaptive mutations with functional effects from neutral mutations that have been fixed by chance. Here, we harnessed the combination of parallel evolution and functional genomics to identify intergenic regions under selection in the genome of the opportunistic pathogen *Pseudomonas aeruginosa* during the process of host adaptation in multiple cystic fibrosis patients. Our study reveals that adaptive intergenic mutations represent an egregiously underappreciated aspect of host adaptation in *P. aeruginosa*, and that intergenic and coding region mutations contribute differently both qualitatively and quantitatively to this process.

Results

Parallel evolution in intergenic regions in P. aeruginosa.

To investigate the contribution of intergenic mutations to bacterial adaptation to the host environment, we considered data from seven studies (12-18) in which multiple clonal *P. aeruginosa* isolates have been sampled and sequenced during the course of infection in subjects with cystic fibrosis (CF). In CF infections, the host environments in individual subjects represent parallel selective conditions by which evolution is directed, and identification of parallel genetic evolution in bacteria from independent infections is strongly suggestive of positive selection at these loci (16).

Here, we focused our analysis exclusively on intergenic regions in which mutations were acquired during infection, and included only intergenic regions also present in the PAO1 reference genome. In total, we identified 3,489 mutations (2,024 SNPS and 1,465 indels) in the intergenic regions of the 44 different *P. aeruginosa* clone types included in our data set (Table S1). Since the majority of regulatory elements in the bacterial genome range between 5-30 bp in length (19), we identified intergenic regions under positive selection by only considering mutations found in parallel in different clone types and distributed within a window of less than 30 bp (Materials and Methods). Applying these criteria, we found 63 intergenic regions with parallel genetic evolution (Figure 1).

Since certain *P. aeruginosa* clone types are transmissible and can form clinic-specific outbreaks among patients (16), we also analyzed if distinct intergenic mutations had accumulated in parallel among clonal isolates within each of the 44 clone type. We identified 41 intergenic regions in which three or more distinct mutations (less than 30 bp apart) had accumulated in isolates of the same clone type (Figure 1).

Interestingly, 16 of these regions are also represented among the 63 regions identified in our analysis of parallel mutations between clone types providing further support for the importance of these mutations in adaptation of *P. aeruginosa* to the CF environment (Figure 1). In total, we identify 88 intergenic regions that evolved under the pressure of natural selection within the hosts. The connection between these 'pathoadaptive' regions and their flanking genes identify genetic systems with importance for pathogen adaptation and thus provide insight into the selective forces that operate on the pathogen.

Intergenic mutations frequently target promoter sequences.

We next analyzed the genomic distribution of the identified intergenic mutations. Non-translated intergenic regions are distributed across the genome in three possible orientations: 1) upstream of two genes, 2) downstream of two genes and 3) upstream of one gene and downstream of one gene, where the latter may include regions with no promoter and within an operon (Figure 2a). We found an over-representation of mutations upstream of two genes among the pathoadaptive regions (Fisher's exact test, $P = 0.010$, $n = 88$, Figure 2b). This bias towards selection of intergenic mutations upstream of genes suggest that the majority of intergenic mutations target potential *cis*-regulatory elements such as the core promoter, transcription factor binding sites, ribo-regulators, or translational elements, and consequently influence protein expression levels by affecting transcriptional or post-transcriptional processes.

To further explore this hypothesis, we analyzed the complete set of 88 pathoadaptive regions for the presence of known regulatory elements (Materials

and Methods), and mapped the overlap between these putative regulatory sites and the identified adaptive mutations. While bacterial intergenic regions are home to a wide range of regulatory elements many of which are not well characterized, we nevertheless observed 28 regions (32%), in which the cluster of adaptive mutations was positioned within one or more putative regulatory elements. The majority of mutations within these 28 regions target the putative core promoter alone or in combination with other elements (Figure 2c), suggesting that intergenic mutations frequently target sequences important for transcriptional processes. In support of this, we observed that intergenic mutations were more frequently located upstream of known transcriptional start sites (TSS) (37 cases) than downstream (10 cases) (Table S6).

Pathoadaptive intergenic mutations change transcriptional activity of genes involved in host interaction, metabolism, and antibiotic susceptibility.

To further explore the potential relationship between intergenic mutations and transcription, we quantified the effects of a subset of intergenic mutations on transcription of downstream genes. To this end, we constructed transcriptional fusions of both wild-type and mutant intergenic alleles with the luciferase reporter (*luxCDABE*) genes and integrated single copies of the fusions at the neutral *attB* site (10) in the chromosome of *P. aeruginosa* PAO1. We measured the transcriptional activity of 25 different intergenic regions in which pathoadaptive mutations were located upstream of either one or two genes. This selection resulted in a total of 32 transcriptional fusions, which represent 33% of all possible fusions within the complete set of 88 pathoadaptive regions. In addition, for one of the intergenic

169 regions (*ampR*//*ampC*), we tested two alleles each with different mutations (Table
170 S7 and Figure S1).
171 Measurements of *lux* expression during exponential growth in Luria-Bertani (LB)
172 medium and ABTGC minimal medium revealed significantly altered expressions in 16
173 of 34 tested fusions in at least one of the two conditions (Student *t* test, $P < 0.05$,
174 Figure 3). Altered expression was in most cases moderate (<3-fold change) and
175 ranged between -3.1 to 22.1 fold changes for the mutant allele compared to that of
176 wild type (Figure 3). Interestingly, 10 of these 16 fusions exhibited altered
177 expressions only in either LB or ABTGC minimal medium, but not in both conditions,
178 which suggest that many adaptive intergenic mutations alter transcriptional levels
179 while not interfering with conditional control mechanisms.
180 Overall, our results reveal that a substantial fraction of the intergenic mutations are
181 associated with functional (transcriptional) effects despite the fact that we recorded
182 these effects in the non-native PAO1 genetic background (*i.e.* with removal of
183 potential epistatic effects from the additional mutations found in the clinical isolate)
184 and in a narrow range of conditions, which most likely mean that we are not
185 capturing the full spectrum of functional effects connected to the intergenic
186 mutations.
187 Several of the 16 fusions with altered expression relate to genes that encode
188 proteins with known functions in bacteria-host interactions, cellular metabolism, and
189 antibiotic resistance. For example, *cerN* expresses a ceramidase involved in
190 utilization of host produced sphingolipids (20), *exsC* expresses a protein involved in
191 positive regulation of the type III secretion system (21), and PA4837 is the first gene
192 in an operon (PA4837-34) involved in expression of a metalophore system essential

193 for survival in airway mucus secretions (22, 23). Other genes are known to play a role
194 in pyrimidine and aromatic amino acid metabolism (*pyrC* and *hmgA*, respectively).
195 Finally, two genes are linked to antibiotic resistance *rluC* (24) and *ampR* (25). Seven
196 genes encode proteins of unknown functions and their role in relation to host
197 adaptation remains unclear.
198 Interestingly, expression changes were observed in both directions (seven mutant
199 alleles resulted in increased expression, and nine mutant alleles resulted in
200 decreased expression) (Figure 3), suggesting that pathoadaptive intergenic
201 mutations may equally well either create or destroy regulatory interactions.
202
203 *Mutations upstream of ampR and ampC enhance resistance to several antibiotics*
204 Next, we explored the direct effects of intergenic mutations on the physiology of the
205 pathogen. As resistance towards antibiotics is a common phenotype that emerges
206 during CF infections, we selected the mutations found in the two alleles of the
207 *ampR//ampC* intergenic region for further study. Mutations in this intergenic region
208 resulted in enhanced expression of the global antibiotic resistance regulator AmpR,
209 but had no direct effect on expression of the AmpC β-lactamase (Figure 3). We
210 introduced these mutations in the genome of *P. aeruginosa* PAO1 through allelic
211 replacement (Materials and Methods). Since a SNP mutation (G7A) was present at
212 the start of *ampC* gene in one of the alleles, we also made an allelic replacement of
213 this mutation alone in the PAO1 genome to separate the effects caused by the
214 intergenic mutations (Figure S1). For each strain and their isogenic wild type, we
215 measured the Minimal Inhibitory Concentration (MIC) of various β-lactam antibiotics
216 such as imipenem, ceftazidime and ampicillin from carbapenem, cephalosporin, and

217 penicillin classes of β-lactams respectively. For both intergenic alleles, we observed a
218 small but significant increase in the MIC of imipenem and ampicillin (Student *t* test, *P*
219 < 0.01, Figure 4), but not ceftazidime. AmpR regulates β-lactam resistance both
220 through direct activation of AmpC expression as well as via an AmpC-independent
221 manner (25). Irrespectively of the mechanism, our results show that acquisition of
222 intergenic mutations between *ampR* and *ampC* is directly linked to a host-relevant
223 phenotypic alteration (*i.e.* reduced β-lactam susceptibility).
224
225 *Intergenic evolution targets essential genes and contributes more to host adaptation*
226 *than intragenic evolution*
227 Finally, we compared the relative contribution of coding and intergenic mutations to
228 pathogen adaptation. We focused on the large fraction of isolates (n=474) included
229 in this study, in which 52 coding regions were found to be under positive selection
230 during host adaptation (17). In these isolates, we identified 35 pathoadaptive
231 intergenic regions (Materials and Methods, Table S8). Although coding region
232 mutations are numerically dominant over intergenic mutations, normalization to the
233 mutational targets available for intergenic and coding region mutations (89.8% of
234 the *P. aeruginosa* genome contains coding regions), reveal that intergenic regions
235 are 3.7 times more likely to be selected than cording regions and thus appear to play
236 a quantitatively more prominent role in host adaptation (Figure 5a).
237 We also analyzed qualitative differences between coding and intergenic mutations
238 by determining the presence of essential genes among the pathoadaptive coding and
239 intergenic regions. By cross-referencing the 35 pathoadaptive intergenic regions to
240 the list of 445 genes previously shown to be essential for survival of *P. aeruginosa*

241 PAO1 in CF sputum environment (26), we found that 7 of the 38 genes located
242 immediately downstream of the 35 pathoadaptive intergenic regions are essential
243 (Table S8). Two of these genes (*pyrC* and PA5492) showed altered expression as a
244 consequence of pathoadaptive mutations in their intergenic region, demonstrating
245 that such mutations can indeed modulate expression of essential genes (Figure 3).
246 Importantly, the association between pathoadaptive intergenic regions and essential
247 genes at a level of 18% represents a significant overrepresentation from the normal
248 prevalence of CF sputum essential genes in the *P. aeruginosa* PAO1 genome (Figure
249 5b, Fisher's exact test, $P = 0.029$). In contrast, there were no CF sputum essential
250 genes within 52 pathoadaptive coding regions demonstrating a significant
251 underrepresentation of these genes within adaptive coding regions (Figure 5b,
252 Fisher's exact test, $P = 0.033$).
253

Discussion

In this study, we present evidence that intergenic mutations constitute an important part of the genetic basis for host adaptation in *P. aeruginosa*. Generally, the contribution of intergenic regions to evolution of host-adapted variants has received little attention. However, since the development of predictive models of pathogen evolution and identification of new therapeutic targets (27, 28) rely on understanding the evolutionary response of pathogens to the host environment, identification of the full range of adaptive mutations in both coding and non-coding regions is important. Here, our genome-wide identification of intergenic regions under selection within the host was made possible by combining analysis of parallel evolution across a large number of infected individuals with functional genomics. This approach may be useful for analysis of intergenic regions in connection to host adaptation in other pathogens or niche adaptation in general.

Our identification of pathoadaptive intergenic regions provide insight into the cellular functions targeted by intergenic mutations (Figure 1) and thus point to the selective pressures that confronts the pathogen within its CF hosts. For example, adaptive mutations were found to alter expression of genes such as *cerN* (involved in sphingolipid utilization) (20), *phuR-phuSTUVW* (involved in iron acquisition) (10), and PA4837-34 (involved in zink acquisition) (22), which strongly indicate that metabolic adaptation to better exploit the available nutrients in the host is an important evolutionary driver. Similarly, we observed that mechanisms to tolerate antibiotics and other inhibitors in the host are also frequent targets of intergenic molecular evolution. Similar functional categories have been found in studies focusing on

278 pathoadaptive mutations within coding regions (14, 16, 17), suggesting that key
279 selective pressures such as nutrient availability and antibiotic stress can be mitigated
280 both by intergenic and coding region evolution in *P. aeruginosa*.
281
282 At the functional level, we show that intergenic evolution predominantly targets
283 transcriptional processes to alter the transcriptional activity of downstream genes.
284 However, we also found evidence of parallel evolution in two intergenic small RNAs,
285 four transcriptional terminators, and several cases of mutations located downstream
286 of transcriptional start sites (Table S5 and Table S6), which suggest that adaptive
287 mutations may also target elements that control protein expression at the post-
288 transcriptional level. Importantly, we have shown here and in a previous study that
289 intergenic mutations can be directly responsible for the evolution of important
290 pathogenic traits such as reduced sensitivity to antibiotics (Figure 4) and increased
291 iron uptake (10). Further studies, in particular of pathoadaptive regions upstream of
292 genes with unknown functions, will most likely uncover new mechanisms central to
293 CF host colonization and pathogenesis, and assist in identifying the full complement
294 of stressors present in the host most of which are currently unknown.
295
296 Our study also reveals important qualitative differences between the intergenic and
297 coding region mutations. A generally accepted model is that intergenic mutations
298 would typically confer local and subtle regulatory effects primarily on the immediate
299 downstream genes, whereas mutations in coding regions - with their potential to
300 inactivate entire pathways – would be more likely to cause systemic changes of the
301 physiology of the cell (8, 29). One prediction from this model is that intergenic

mutations are associated with less antagonistic effects relative to coding region mutations. While this prediction is difficult to test, our observation of enrichment of essential genes for which intergenic evolution occurred is a clear illustration of this point (Figure 5). The finding that intergenic mutations can bypass the deleterious effects of coding region mutations thus allowing essential genes to become targets for evolutionary changes reveals an important aspect of the role of intergenic mutations, and a key functional difference between intergenic and coding region mutations.

From a quantitative perspective, we find that intergenic mutations are more likely to be selected than coding region mutations during CF host adaptation in *P. aeruginosa* (Figure 5). We hypothesize that the relative contribution of coding and intergenic mutations is variable and depends on a set of identifiable factors of either environmental nature (e.g. niche complexity) or intrinsic to the bacterial pathogen (e.g. genome size, and the number of transcriptional regulatory systems and essential genes encoded in the genome). Although the precise factors that influence the relative contribution of the two types of mutations may be difficult to disentangle, we speculate that in the case of *P. aeruginosa* CF infections, a major contributing factor is the composition of the adaptive environment in the host. The CF host niche is characterized by a complex combination of multiple stressors that must be mitigated for successful bacterial colonization (30). In such environments, mutations in intergenic regions that tune expression levels while maintaining responsiveness to environmental and host derived cues may result in less pleotropic effects than mutations that change protein structure or function (29). Further

studies of *P. aeruginosa* adaptation in other infections and host environments such as chronic wounds and ulcerative keratitis (31) are required to identify factors that may influence the relative contribution of intergenic and coding region evolution.

Our documentation of the evolutionary significance of intergenic mutations was obtained in the particular genetic and ecological context of *P. aeruginosa* adaptation to the cystic fibrosis airway niche. Nevertheless, our systematic study provides insight into the contribution and functionality of intergenic versus intragenic mutations, which is of broader relevance in connection to bacterial evolution in natural environments. This is supported by recent observations indicating that other contexts may also promote intergenic evolution. For example, our results resonates well with results showing that adaptive intergenic mutations contribute to innovation of novel metabolic functions in laboratory-evolving *Escherichia coli* (32), evidence of a signal of positive selection in *Mycobacteria tuberculosis* intergenic regions (6), and the suggestion that intergenic evolution may mitigate detrimental fitness effects associated with acquisition of novel genetic material (33). We suggest that adaptive mutations in intergenic regions represent an important but underappreciated aspect of bacterial evolution not only in connection to host colonization but also niche adaptation in other natural environments.

Materials and Methods

Bacterial strain and growth conditions

Luria-Bertani (LB) and ABT minimal medium supplemented with 1% glucose and 1% casamino acids (ABTGC) (10) were routinely used for growth of *Escherichia coli* and *P. aeruginosa* strains. *E. coli* CC118 λpir was used for maintenance of recombinant plasmids supplemented with 8 µg/ml of tetracycline. *P. aeruginosa* PAO1 strain was used for phenotypic investigation of intergenic mutations. For final marker selection of *P. aeruginosa,* 50 µg/ml of tetracycline was used.

Identification of pathoadaptive intergenic regions

The dataset used for analysis of intergenic evolution is described in SI Materials and Methods. Presence of independent clonal lineages of *P. aeruginosa* were verified by detection of MLST allele profiles as described in SI Materials and Methods. Pathoadaptive intergenic regions selected across 44 clones were defined as regions containing multiple mutations from isolates of independent clones within a narrow region of less than 30 bp. Similarly, intergenic regions containing multiple distinct mutations from isolates of the same clone within a narrow 30 bp window were also defined as pathoadaptive within that clone. Presences of mutations within narrow windows of pathoadaptive regions were more enriched than what is expected by chance within the dataset. Detailed description is available at SI Materials and Methods. Identification of putative intergenic elements within pathoadaptive intergenic regions is described in SI Material and Methods.

370 *Genetic techniques*

371 Genetic engineering of reporter fusion and allelic replacement plasmids are
372 described in SI Materials and Method. Presence of mutated alleles within reporter
373 fusion and allelic replacement constructs were verified by Sanger sequencing.
374 Reporter fusion plasmid were electroporated into *P. aeruginosa* PAO1 as previously
375 described (34). Constructs with allelic replacements were introduced into *P.*
376 *aeruginosa* by triparental mating using the helper strain *E. coli* HB101/pRK600 (35).
377 The presence of mutated alleles in tetracycline sensitive sucrose resistant colonies
378 isolates were verified by PCR and Sanger sequencing.

379

380 *Phenotype assays*

381 Phenotypic expression of reporter fusion strains were investigated as described in SI
382 Materials and Methods. Continuous measurements of growth (OD_{600}) and
383 luminescence were recorded by Cytation 5 multimode reader (BioTek) at 200 rpm
384 shaking condition and 37 C temperature. Lux expression normalized by cell density
385 for all strains were recorded and compared against reporter fusions containing wild
386 type alleles. Measurements were repeated three times for each reporter fusion
387 strain. Determination of MIC values for ceftazidime, imipenem and ampicillin are
388 described in SI Materials and Methods. MIC values were either measured by
389 standard microdilution method in Mueller-Hinton (MH) broth or using E-test on MH
390 agar plates. Measurements were repeated five times for each strain. Statistical
391 differences between means of replicates for reporter fusion and MIC resistance
392 assays were calculated by two-tailed student *t* test.

393

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402

403 **Author contributions**

404 S.M.H.K and L.J. conceived study and designed research. S.M.H.K. performed
405 research. S.M.H.K and L.J. analyzed data and wrote the manuscript.

406

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508

509 **Figure Legends**

510 **Figure 1:** Pathoadaptive intergenic regions. Regions targeted by mutations involved
511 in host adaptation through parallel evolution across or within clone types. The black
512 squares in the matrix show the intergenic region with parallel mutations in isolates
513 of the respective clone type. The red squares in the matrix show the intergenic
514 region with parallel mutations within isolates of a clone type alone. Squares with
515 striped red color indicate regions in which mutations has been selected both within
516 isolates of that clone type and across other clone types.

517

518 **Figure 2:** Orientation and regulatory elements in intergenic regions. a) Overview of
519 the three different orientations of intergenic regions and the possible location of
520 potential elements within each type. b) Distribution of different orientations of
521 intergenic regions (I-III) within PAO1 genome and the pathoadaptive intergenic
522 regions. Two-tailed Fisher's exact test is performed to analyze over-representation
523 or under-representation of certain orientations within pathoadaptive intergenic
524 regions (n = 88). c) Pie chart demonstrating the distribution of putative intergenic
525 elements targeted by pathoadaptive intergenic mutations among regions where the
526 mutation cluster was within any known element (n = 28).

527

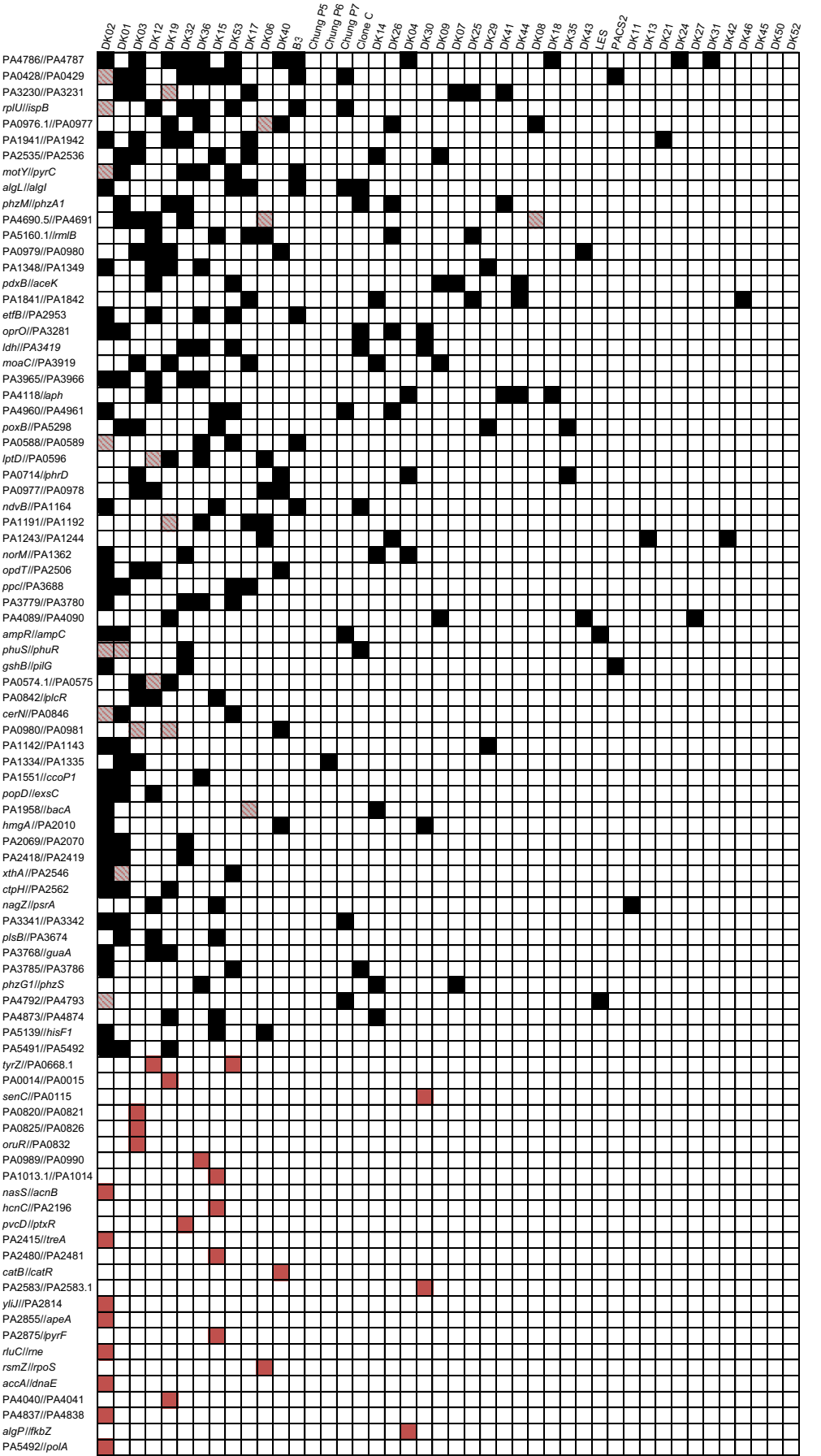
528 **Figure 3:** Intergenic mutations with functional effects on transcription. Expression of
529 *lux* from transcriptional fusions with mutated and wildtype alleles were measured at
530 OD_{600} = 0.15 and normalized by cell density. Transcriptional fusions were examined
531 in Luria-Bertani (LB) and ABTGC minimal media. Mean luminescence was calculated
532 for three biological replicates and the relative fold change of mutant versus wildtype

allele calculated. Statistical analysis of the difference between two means was performed by a two-tailed Student *t* test and the asterisk denotes $P < 0.05$.

Figure 4: Mutations in the intergenic region between *ampC* and *ampR* cause an increased tolerance towards imipenem and ampicillin. The values for Minimal Inhibitory Concentration (MIC) and the constructed mutations in each strain of PAO1 are shown. Mutation G-98A upstream *ampC* derives from isolate DK2-CF173-1995. Three mutations G-38A, C-66T and G-78A upstream of *ampC* originate from isolate DK1-P43-M2-2002. A SNP mutation at the start of *ampC* (G7A) in DK1-P43-M2-2002 was also constructed in laboratory strain PAO1 to isolate the effect of this mutation and the effect of intergenic mutations from DK1-P43-M2-2002. Error bars indicate standard deviation from three different biological replicates. Double asterisk indicate significant difference between mean MIC of the strains (Two-tailed Student *t* test, $P < 0.01$).

Figure 5: Contribution and function of coding and intergenic mutations. 52 coding regions are under positive selection in a selected subset of our dataset comprising of 474 long-term CF adapted isolates of *P. aeruginosa* (17). In the same isolates, we identify 35 intergenic regions under positive selection for adaptive mutations (Table S8). a) To calculate the relative presence of adaptive mutations within coding or intergenic regions, we divided the number of pathoadaptive features by mutational targets available within each category (89.8% coding and 10.2% intergenic). Final relative frequency for selection of each pathoadaptive feature is shown in the barplot. b) 445 genes are essential for survival of *P. aeruginosa* in CF sputum

557 environment (26). The percentage of these essential genes within 52 pathoadaptive
558 genes and 38 genes downstream of 35 pathoadaptive intergenic regions is
559 demonstrated. Asterisk denotes $P < 0.05$ from two-tailed Fisher's exact test. Red
560 dashed line indicates the percentage of CF essential genes in PAO1.
561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PA4118//aph
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norM//PA1362
opdT//PA2506
ppc//PA3688
PA3779//PA3780
PA4089//PA4090
ampR//ampC
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cerN//PA0846
PA0980//PA0981
PA1142//PA1143
PA1334//PA1335
PA1551//ccoP1
popD//exsC
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hmgA//PA2010
PA2069//PA2070
PA2418//PA2419
xthA//PA2546
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PA2875//pyrF
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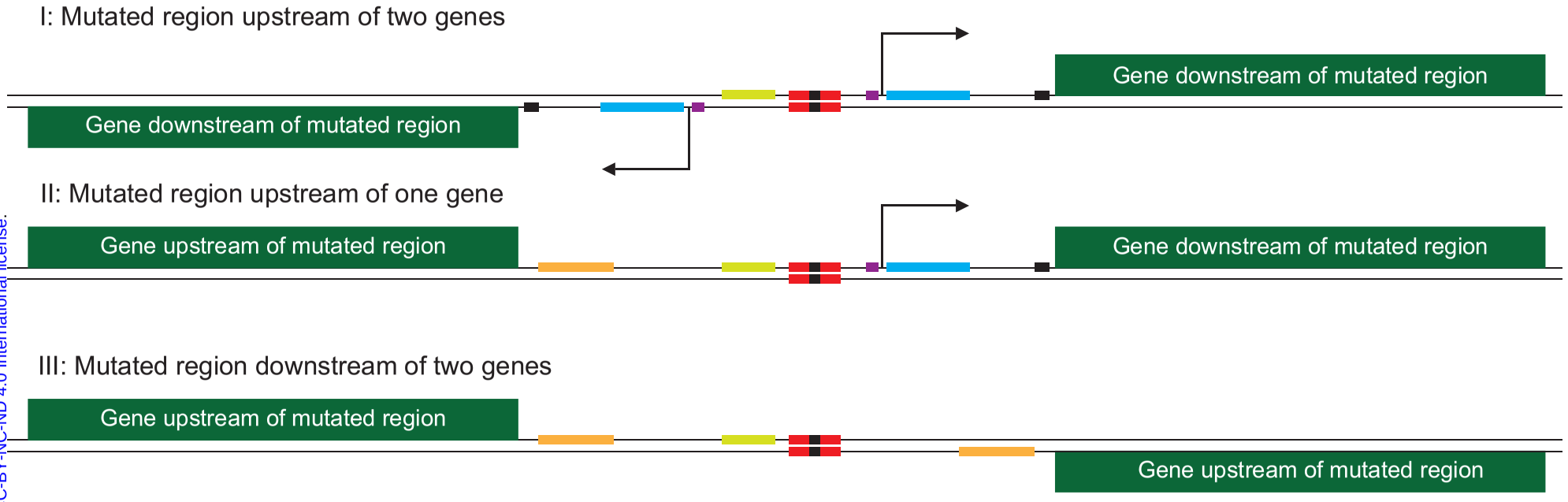

b

	Present	% of total	PAO1 genome	% of total	Enrichment	*P-value*
I	25	28	812	17	1,64	0,010
II	15	17	755	16	1,06	0,771
III	48	55	3115	67	0,82	0,022

c