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 whether 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 occurs. 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) as well as 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 and thus 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 whether intergenic mutations have a qualitative distinct role in

host adaptation.

There are clear, albeit few, examples of intergenic regions that 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

hemoglobin (10). In *Mycobacterium tuberculosis*, evolution of ethambutol resistance

has been linked to the *empAB* promoter region acquiring mutations 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 *P. 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

to this process both qualitatively and quantitatively.

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 dataset (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 types. 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 suggests 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 (20). 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 regions (*ampR*//*ampC*), we tested two alleles, each with different

mutations (Table S7 and Figure S1).

Measurements of *lux* expression during exponential growth in Luria-Bertani (LB)

medium and ABTGC minimal medium revealed significantly altered expressions in 16

of 34 tested fusions in at least one of the two conditions (Student *t* test, $P < 0.05$,

Figure 3). Altered expression was in most cases moderate (<3-fold change) and the

fold change ranged from -3.1 to 22.1 for the mutant allele compared to that of wild

type (Figure 3). Interestingly, 10 of these 16 fusions exhibited altered expressions

only in either LB or ABTGC minimal medium, but not in both conditions, which

suggests that many adaptive intergenic mutations alter transcriptional levels while

not interfering with conditional control mechanisms.

These results show that a substantial fraction of the intergenic mutations are

associated with functional (transcriptional) effects despite the fact that we recorded

these effects in the non-native PAO1 genetic background (*i.e.* with removal of

potential epistatic effects from the additional mutations found in the clinical isolate)

and in a narrow range of conditions, which most likely means that we are not

capturing the full spectrum of functional effects connected to the intergenic

mutations.

Several of the 16 fusions with altered expression relate to genes that encode

proteins with known functions in bacteria-host interactions, cellular metabolism and

antibiotic resistance. For example, *cerN* expresses a ceramidase involved in

utilization of host-produced sphingolipids (21), *exsC* expresses a protein involved in

positive regulation of the type III secretion system (22) and PA4837 is the first gene

in an operon (PA4837-34) involved in expression of a metalophore system essential

for survival in airway mucus secretions (23, 24). Other genes are known to play a role

in pyrimidine and aromatic amino acid metabolism (*pyrC* and *hmgA*, respectively).

Finally, two genes are linked to antibiotic resistance: *rluC* (25) and *ampR* (26). Seven

genes encode proteins of unknown functions and their role in relation to host

adaptation remains unclear.

Interestingly, expression changes were observed in both directions (seven mutant

alleles resulted in increased expression and nine mutant alleles resulted in decreased

expression) (Figure 3), suggesting that pathoadaptive intergenic mutations may

equally well either create or destroy regulatory interactions.

Mutations upstream of ampR and ampC enhance resistance to several antibiotics

Next, we explored the direct effects of intergenic mutations on the physiology of the

pathogen. As resistance towards antibiotics is a common phenotype that emerges

during CF infections, we selected the mutations found in the two alleles of the

ampR//ampC intergenic region for further study. Mutations in this intergenic region

resulted in enhanced expression of the global antibiotic resistance regulator AmpR,

but had no direct effect on expression of the AmpC β-lactamase (Figure 3). We

introduced these mutations in the genome of *P. aeruginosa* PAO1 through allelic

replacement (Materials and Methods). Since a SNP mutation (G7A) was present at

the start of *ampC* gene in one of the alleles, we also made an allelic replacement of

this mutation alone in the PAO1 genome to separate the effects caused by the

intergenic mutations (Figure S1). For each strain and their isogenic wild type, we

measured the Minimal Inhibitory Concentration (MIC) of various β-lactam antibiotics

such as imipenem, ceftazidime and ampicillin from carbapenem, cephalosporin and

penicillin classes of β-lactams, respectively. For both intergenic alleles, we observed

a small but significant increase in the MIC of imipenem and ampicillin (Student *t* test,

P < 0.01, Figure 4), but not ceftazidime. AmpR regulates β-lactam resistance both

through direct activation of AmpC expression as well as via an AmpC-independent

manner (26). Irrespective of the mechanism, our results show that acquisition of

intergenic mutations between *ampR* and *ampC* is directly linked to a host-relevant

phenotypic alteration (*i.e.* reduced β-lactam susceptibility).

Intergenic evolution targets essential genes and contributes more to host adaptation

than intragenic evolution

Finally, we compared the relative contribution of coding and intergenic mutations to

pathogen adaptation. We focused on the large fraction of isolates (n=474) included

in this study, in which 52 coding regions were found to be under positive selection

during host adaptation (17). In these isolates, we identified 35 pathoadaptive

intergenic regions (Materials and Methods, Table S8). Although coding region

mutations are numerically dominant over intergenic mutations, normalization to the

mutational targets available for intergenic and coding region mutations (89.8% of

the *P. aeruginosa* genome contains coding regions) reveals that intergenic regions

are 3.7 times more likely to be selected than cording regions and thus appear to play

a quantitatively more prominent role in host adaptation (Figure 5a).

We also analyzed qualitative differences between coding and intergenic mutations

by determining the presence of essential genes among the pathoadaptive coding and

intergenic regions. By cross-referencing the 35 pathoadaptive intergenic regions to

the list of 445 genes previously shown to be essential for survival of *P. aeruginosa*

PAO1 in CF sputum environment (27), we found that 7 of the 38 genes located

immediately downstream of the 35 pathoadaptive intergenic regions are essential

(Table S8). Two of these genes (*pyrC* and PA5492) showed altered expression as a

consequence of pathoadaptive mutations in their intergenic region, demonstrating

that such mutations can indeed modulate expression of essential genes (Figure 3).

Importantly, the association between pathoadaptive intergenic regions and essential

genes at a level of 18% represents a significant overrepresentation from the normal

prevalence of CF sputum essential genes in the *P. aeruginosa* PAO1 genome (Figure

5b, Fisher's exact test, $P = 0.029$). In contrast, there were no CF sputum essential

genes within 52 pathoadaptive coding regions demonstrating a significant

underrepresentation of these genes within adaptive coding regions (Figure 5b,

Fisher's exact test, $P = 0.033$).

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 discovery of new therapeutic targets (28, 29) 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 provides insight into the

cellular functions targeted by intergenic mutations (Figure 1) and thus points to the

selective pressures that confront the pathogen within its CF host. For example,

adaptive mutations were found to alter expression of genes such as *cerN* (involved in

sphingolipid utilization) (21), *phuR-phuSTUVW* (involved in iron acquisition) (10) and

PA4837-34 (involved in zinc acquisition) (23), which strongly indicates that metabolic

adaptation for a better exploitation of available nutrients in the host is an important

evolutionary driver. Similarly, we observed that mechanisms of the development of

tolerance to 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 pathoadaptive mutations within coding regions (14, 16, 17),

suggesting that key selective pressures such as nutrient availability and antibiotic

stress can be mitigated both by intergenic and coding region evolution in *P.*

aeruginosa.

At the functional level, we show that intergenic evolution predominantly targets

transcriptional processes to alter the transcriptional activity of downstream genes.

However, we also found evidence of parallel evolution in two intergenic small RNAs,

four transcriptional terminators, and several cases of mutations located downstream

of transcriptional start sites (Table S5 and Table S6), which suggests that adaptive

mutations may also target elements that control protein expression at the post-

transcriptional level. Importantly, we have shown here and in a previous study that

intergenic mutations can be directly responsible for the evolution of important

pathogenic traits such as reduced sensitivity to antibiotics (Figure 4) and increased

iron uptake (10). Further studies, in particular of pathoadaptive regions upstream of

genes with unknown functions, will most likely uncover new mechanisms central to

CF host colonization and pathogenesis, and assist in identifying the full complement

of stressors present in the host, most of which are currently unknown.

Our study also reveals important qualitative differences between the intergenic and

coding region mutations. A generally accepted model is that intergenic mutations

would typically confer local and subtle regulatory effects primarily on the immediate

downstream genes, whereas mutations in coding regions - with their potential to

inactivate entire pathways – would be more likely to cause systemic changes of the

physiology of the cell (8, 30). 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 as well as 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 (31). In such environments,

mutations in intergenic regions that tune expression levels while maintaining

responsiveness to environmental and host-derived cues may result in less pleiotropic

effects than mutations that change protein structure or function (30). Further

studies of *P. aeruginosa* adaptation in other infections and host environments such

as chronic wounds and ulcerative keratitis (32) 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 study resonate

well with results showing that adaptive intergenic mutations contribute to

innovation of novel metabolic functions in laboratory-evolving *E. coli* (33), evidence

of a signal of positive selection in *M. tuberculosis* intergenic regions (6) and the

suggestion that intergenic evolution may mitigate detrimental fitness effects

associated with acquisition of novel genetic material (34). 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 strains 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 *E. 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* was verified by

recording of MLST allele profiles of each isolate 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. Mutations within narrow

windows of pathoadaptive regions were more enriched than what is expected by

chance within the dataset. Detailed description is available in SI Materials and

Methods. Identification of putative intergenic elements within pathoadaptive

intergenic regions is described in SI Materials and Methods.

Genetic techniques

Genetic engineering of reporter fusion and allelic replacement plasmids are

described in SI Materials and Methods. Presence of mutated alleles within reporter

fusion and allelic replacement constructs was verified by Sanger sequencing.

Reporter fusion plasmids were electroporated into *P. aeruginosa* PAO1 as previously

described (35). Constructs with allelic replacements were introduced into *P.*

aeruginosa by triparental mating using the helper strain *E. coli* HB101/pRK600 (36).

The presence of mutated alleles in tetracycline-sensitive, sucrose-resistant colony

isolates was verified by PCR and Sanger sequencing.

Phenotype assays

Phenotypic expression of reporter fusion strains was investigated as described in SI

Materials and Methods. Continuous measurements of growth (OD_{600}) and

luminescence were recorded by Cytation 5 multimode reader (BioTek) at 200 rpm

shaking condition and 37 °C temperature. Lux expression normalized by cell density

for all strains was recorded and compared against reporter fusions containing wild

type alleles. Measurements were repeated three times for each reporter fusion

strain. Determination of MIC values for ceftazidime, imipenem and ampicillin is

described in SI Materials and Methods. MIC values were either measured by

standard microdilution method in Mueller-Hinton (MH) broth or using E-test on MH

agar plates. Measurements were repeated five times for each strain. Statistical

differences between means of replicates for reporter fusion and MIC resistance

assays were calculated by two-tailed Student *t* test.

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Author contributions

S.M.H.K and L.J. conceived study and designed research. S.M.H.K. performed

research. S.M.H.K and L.J. analyzed data and wrote the manuscript.

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512

Figure Legends

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

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

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

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

environment (27). The percentage of these essential genes within 52 pathoadaptive

genes and 38 genes downstream of 35 pathoadaptive intergenic regions is

demonstrated. Asterisk denotes $P < 0.05$ from two-tailed Fisher's exact test. Red

dashed line indicates the percentage of CF essential genes in PAO1.

a

I: Mutated region upstream of two genes

Gene downstream of mutated region

II: Mutated region upstream of one gene

Gene upstream of mutated region

III: Mutated region downstream of two genes

Gene upstream of mutated region

Gene downstream of mutated region

Gene downstream of mutated region

Gene upstream of mutated region

Gene downstream of mutated region

Gene upstream of mutated region

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

n = 13
n = 6
n = 4
n = 2
n = 2
n = 1

Core promoter
Core promoter and another element
Transcriptional terminator
Invert repeat
sRNA
Transcription factor binding site
Shine-delgarno sequence