The High Mutational Sensitivity of ccdA Antitoxin Is Linked to Codon Optimality

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Abstract

Deep mutational scanning studies suggest that synonymous mutations are typically silent and that most exposed, nonactive-site residues are tolerant to mutations. Here, we show that the ccdA antitoxin component of the Escherichia coli ccdAB toxin–antitoxin system is unusually sensitive to mutations when studied in the operonic context. A large fraction (∼80%) of single-codon mutations, including many synonymous mutations in the ccdA gene shows inactive phenotype, but they retain native-like binding affinity towards cognate toxin, CcdB. Therefore, the observed phenotypic effects are largely not due to alterations in protein structure/stability, consistent with a large region of CcdA being intrinsically disordered. E. coli codon preference and strength of ribosome-binding associated with translation of downstream ccdB gene are found to be major contributors of the observed ccdA mutant phenotypes. In select cases, proteomics studies reveal altered ratios of CcdA:CcdB protein levels in vivo, suggesting that the ccdA mutations likely alter relative translation efficiencies of the two genes in the operon. We extend these results by studying single-site synonymous mutations that lead to loss of function phenotypes in the relBE operon upon introduction of rarer codons. Thus, in their operonic context, genes are likely to be more sensitive to both synonymous and nonsynonymous point mutations than inferred previously.

Key words: codon preference, saturation mutagenesis, translational efficiency, ribosome pausing, protein and RNA levels.

Introduction

Mutations that lead to changes in the protein’s amino acid sequence are often expected to alter the protein structure, stability, and/or activity and are referred to as missense mutations. Unlike missense mutations, synonymous mutations alter only the DNA (and mRNA) sequence without affecting the amino acid sequence because of the degeneracy of the genetic code (Crick et al. 1961). Synonymous mutations were classically believed to be silent in their effects on protein function. While this is often true, there have been several pioneering studies that show prominent effects of synonymous mutations on protein function (Komar et al. 1999; Kimchi-Sarfaty et al. 2007; Sander et al. 2014). Synonymous mutations are also reported to be associated with over 50 human diseases (Sauna and Kimchi-Sarfaty 2011). Some of the mechanisms known to be involved in the altered phenotypes, observed upon introduction of multiple synonymous mutations, are due to the effects on protein expression and folding (Buhr et al. 2016; Rodnina 2016), transcription (Zhao et al. 2021), as well as mRNA stability due to changes in secondary structure or altered mRNA decay rates (Presnyak et al. 2015; Hanson and Coller 2018). Such effects are often found to be brought about by the bias due to unequal codon usage (Sharp et al. 1986; Karlin et al. 1998; Quax et al. 2015) and tRNA abundance (Gorochowski et al. 2015) as well as effects on translation initiation (Li et al. 2012), elongation or termination (Rodnina 2016).

Most deep mutational scanning (DMS) studies involving parallel high-throughput investigation of phenotypic fitness in large number of single-site mutants of proteins have revealed synonymous mutations to be largely neutral (Hietpas et al. 2011; Jiang et al. 2013; Melamed et al. 2013; Roscoe et al. 2013; Wu et al. 2014), with the exception of studies such as in ß-lactamase where a very small number of synonymous mutants (∼2%) display <50% of wild-type (WT) activity (Firnberg et al. 2016) and in Saccharomyces cerevisiae Hsp90 where one out of fifteen synonymous mutations tested shows significant loss of fitness (Fragata et al. 2018). Several low throughput studies have investigated phenotypic fitness effects of small sets of synonymous and nonsynonymous (missense) single-nucleotide or single-codon mutants and attempted to elucidate molecular mechanisms responsible for the observed patterns of
distribution of fitness effects (DFEs) (Sanjuán et al. 2004; Carrasco et al. 2007; Domingo-Calap et al. 2009; Lind et al. 2010; Peris et al. 2010; Cuevas et al. 2012; Schenk et al. 2012; Bailey et al. 2014; Agashe et al. 2016; Kristofich et al. 2018; Lebeuf-Taylor et al. 2019). These studies either use adaptive evolution experiment to screen for emerging beneficial mutants or use site-directed mutagenesis (SDM) to construct a small subset of single-site mutants to study their fitness. Several single-site synonymous mutants have been identified to exhibit lower fitness than WT with reduced growth rates, while others have higher fitness with improved growth rates in various bacterial genes under selection pressure (Lind et al. 2010; Schenk et al. 2012; Bailey et al. 2014; Agashe et al. 2016; Kristofich et al. 2018). A number of studies in viruses also identify small numbers of synonymous mutants to be deleterious or even lethal (Sanjuán et al. 2004; Carrasco et al. 2007; Domingo-Calap et al. 2009; Wu et al. 2014), as analyzed and detailed in an useful review on synonymous mutants in laboratory evolution experiments (Bailey et al. 2021). However, most of these DFE studies show deleterious synonymous mutations to have smaller effects than nonsynonymous mutations. A study of DFE in two ribosomal proteins reveals a large fraction of mutants to be weakly deleterious and fitness effects of synonymous and nonsynonymous mutants to be comparable (Lind et al. 2010). While some of the studies cite altered expression levels (higher transcript levels) or mRNA stability alterations causing the observed synonymous mutational effects, the molecular basis of altered fitness in synonymous mutants has not been elucidated in most of the above described cases. For nonsynonymous mutations, the large effects of amino acid changes on protein structure, stability and function may often overshadow and obscure the mutational effects on mRNA transcript levels, structure, stability, and translation.

Mutational effects on protein function have been studied extensively in mono-cistronic proteins or in isolated, heterologous expression systems. Studies of bacterial genomes however reveal a high occurrence of operonic systems comprising of functionally related protein coding genes proximally spaced on a DNA stretch, under a shared promoter (Jacob and Monod 1961; Yanofsky 1981; Balážsi et al. 2005; Price et al. 2006; Zhang et al. 2012). Operonic genes are typically separated by less than 20 base pairs (Moreno-Hagelsieb and Collado-Vides 2002). Operons exhibit characteristic tightly regulated mRNA expression from a common promoter, followed by translation of the gene products from the same mRNA molecule. The translation re-initiation efficiency of genes clustered in operons is often dependent on the space between genes (Chenla et al. 2020). Consequently, the DNA and mRNA sequence features are expected to play an important role in gene expression and regulation in operons. Moreover, transcription and translation are known to be spatiotemporally coupled in prokaryotes (Byrne et al. 1964; Stent 1964; Miller et al. 1970). In tightly regulated operonic systems, downstream functions are often acutely dependent on the relative levels of gene products of the operonic genes. Thus, such operonic systems provide a sensitive and relatively unexplored readout to study phenotypic effects of synonymous mutations.

Bacterial type II toxin–antitoxin (TA) systems, where the antitoxin and the toxin proteins are expressed as part of a single operon, serve as an attractive model to study co-regulation of gene expression as well as transcriptional and translational coupling (Masachis et al. 2018). These TA systems usually code for an upstream labile antitoxin protein responsible for negating the toxicity of the downstream toxin protein, the latter can cause growth arrest or cell death (Goeders and Van Melderen 2014; Deter et al. 2017). The Escherichia coli F-plasmid borne ccd operon encodes a TA system that comprises of a labile 72 residue CcdA antitoxin, that prevents killing of the bacterial cells by binding to the 101-residue toxin component, CcdB under the control of a single auto-regulated promoter (Miki et al. 1984; Tam and Kline 1989). Both genes are co-expressed in low amounts in F-plasmid bearing E. coli cells, and their expression is autoregulated at the level of transcription (Afif et al. 2001). If the cell loses F-plasmid, the labile CcdA is degraded by the ATP dependent Lon protease, releasing CcdB from the complex to act on its DNA gyrase target. Free CcdB poisons gyrase by forming a CcdB-gyrase-DNA ternary complex and induces double-stranded breaks in DNA (Bahassi et al. 1999; Van Melderen 2002). This eventually leads to cell death (Van Melderen et al. 1996). Therefore, mutations which disrupt CcdA antitoxin function or lower the CcdA:CcdB ratio in vivo, can lead to cell death. We therefore carried out saturation mutagenesis studies of the antitoxin ccdA, from the F plasmid encoded CcdAB TA system in E. coli to understand how point mutations, especially synonymous mutations, might affect gene function and regulation in such an operonic context.

We find that the labile antitoxin CcdA in the operonic context shows considerably higher mutational sensitivity than most proteins studied to date (Gupta and Varadarajan 2018). Several synonymous point mutations in CcdA lead to loss of antitoxin function and significant reduction in cell survivals, in a manner dependent on the E. coli codon preference. Our data suggests that reduction in optimality of the codons upon mutation results in decreased levels of CcdA protein, likely by altering the translation efficiency. The reduced CcdA:CcdB protein ratio in vivo in such mutants, results in heightened toxic effects on bacterial cells. Moreover, the low CcdA:CcdB ratio also results in upregulation of ccd transcription, further amplifying the effect of mutation, resulting in cell death. Improved translation initiation of the downstream toxin CcdB, upon mutations in the ccdA gene, especially in the ribosome binding site (RBS) of CcdB, also appears to contribute to increased toxicity in cells. The ccd and likely most other TA operons are highly sensitive genetic circuits that can be used to probe effects of mutations on gene function, in vivo. We observe that a large fraction (~80%) of single codon variants of the ccdA gene (both
synonymous as well as nonsynonymous mutations) results in partial or complete loss of antitoxic function, these are referred to as inactive phenotypes.

**Results**

Unexpectedly High Mutational Sensitivity in the ccdA Antitoxin Gene, in its Native Operonic Context

In the ccd operonic context, mutations in the 72 residue antitoxin CcdA that impair its antitoxic function are expected to promote cell death (fig. 1A). To understand the consequences of mutations in ccdA on bacterial growth, the ccd operon consisting of the promoter, ccdA and ccdB genes (fig. 1B) was cloned into the pUC57 plasmid. Restriction digestion sites introduced in the construct for ease in cloning resulted in a few changes with respect to the WT sequence of the ccdAB operon, at certain positions in the noncoding region upstream of the CcdA gene (supplementary fig. S1, Supplementary Material online). A site-saturation mutagenesis (SSM) library of CcdA was created in this operonic construct using NNK primers (supplementary fig. S2, Supplementary Material online). The library comprised of both synonymous and nonsynonymous mutants of CcdA, along with ∼10% WT population arising owing to the cloning strategy (see Materials and Methods). In order to retrieve the full ccdA library in its operonic context, it was cloned and constructed in an E. coli strain (Top10 gyr) resistant to CcdB toxicity. This Top10 gyr strain has a point mutation in DNA-gyrase that prevents CcdB binding and toxicity (see Supplementary Methods online, Supplementary Material online). Following selection in an E. coli (Top 10) strain that is sensitive to the CcdB toxicity, the operonic ccdA mutant library was subjected to deep sequencing. The enrichment score (ES) of each CcdA mutant was computed from the available sequencing reads after selection (obtained from plasmid isolated from the sensitive strain) versus before selection (obtained from plasmid isolated from the resistant strain), relative to corresponding reads for WT CcdA (supplementary fig. S2 and Supplementary Methods online, Supplementary Material online). The ESseq score is thus defined as the relative ratio of fraction of mutant reads obtained after selection to that obtained before selection, normalized to WT reads. Normalization to WT reads allows facile estimation of the enrichment of the individual mutants with respect to WT. Low enrichment is indicative of reduced survival of a mutant with respect to WT. The ESseq score can therefore be used as a measure of the relative activity of mutants, which in turn is related to but not quantitatively identical to their fitness, as discussed below.

Relative fitness of mutants of microbial genes is routinely estimated from their competitive growth rates (Lind et al. 2010; Zwart et al. 2018; Lebeuf-Taylor et al. 2019) or from the change in ratio of sequencing reads of mutant versus WT over time (Hietpas et al. 2011; Roscoe et al. 2013; Fragata et al. 2018; Flynn et al. 2020). Laboratory evolution experiments of variant populations over several generations are unsuitable for accurate estimation of fitness effects in deleterious and lethal mutants. In an all-against-all competition over long timescales, mutants with higher growth rates tend to enrich exponentially over time and wipe out the slow growing or growth–deleterious variants completely from the population. This is beneficial in adaptive evolution experiments where the aim is to screen for improved variants or new function. However, in studies that aim to identify and investigate the fitness effects of both deleterious and beneficial variants, growth rate based screening can be disadvantageous. Unlike many previous studies that have investigated DFE of nonessential protein coding genes (Lind et al. 2010; Bailey et al. 2014; Lebeuf-Taylor et al. 2019), CcdA antitoxin function is essential for survival of cells containing the CcdAB operon. We observed that a large number of ccdA mutants that showed an inactive phenotype in our plate-based screen, failed to grow in liquid culture when propagated from colonies, indicating a near complete loss-of-function. This prevents estimation of relative fitness effects of ccdA mutants from a conventional growth based assay in liquid culture. Therefore we designed a phenotypic screen of the ccdA mutant library that relies on quantifying mutants in terms of the colonies that appear after transformation. Plasmid pools used for deep sequencing were directly purified from colonies scraped from the plates.

The deep sequencing was performed in three biological replicates, which showed a high correlation (Pearson correlation coefficient = 0.98 approximately; supplementary fig. S3, Supplementary Material online) among themselves. The ESseq was calculated and assigned only to mutants having a minimum of twenty reads in the resistant strain, for a given replicate. The ESs averaged over three replicates were used to classify mutants based on their activity. The WT CcdA has an ESseq value of 1. Of 2272 possible NNK single codon CcdA mutations (71 positions × 32 codons), scores could be assigned to 1,528 mutants. A drastic reduction in cellular growth in the sensitive strain (after selection samples) indicated a substantial prevalence of mutants exhibiting an inactive phenotype in the library. We observed large fractions of both synonymous and nonsynonymous single site mutants in ccdA to reduce antitoxic activity in the operonic context (fig. 1C). CcdA mutants with lower enrichment are expected to have decreased antitoxic activity, thus failing to rescue CcdB mediated cell death. The low incidence of active mutations demonstrates the extreme sensitivity of the ccdAB operon to mutations in ccdA. We classified mutants having ESseq values ≤ 0.1 as inactive. Surprisingly, ∼80% of all CcdA mutants have ESseq values ≤ 0.1 thus displaying an inactive phenotype (fig. 1C and E). A phenotypic assay was conducted for a subset of CcdA mutants by individual transformation in resistant (control) and sensitive (selection experiment) strains, and dilution plating of the single mutants. While the estimated colony forming units (CFUs) for all constructs were similar to that of WT in the resistant
**Fig. 1.** High mutational sensitivity of ccdA antitoxin gene in its operonic context in *Escherichia coli* (A) A schematic overview of the CcdAB TA module and the molecular mechanisms by which CcdB toxin triggers growth arrest in cells (top), how antitoxin CcdA rescues growth arrest by binding and sequestering CcdB (middle), and how mutations in CcdA can affect the antitoxic functions in the CcdAB TA system and promote CcdB mediated cell toxicity (bottom). (B) Schematic representation of ccdAB operon cloned in pUC57 vector. (C) Distribution of the mutational effects for synonymous and nonsynonymous single-site ccdA mutants, in the operonic context. ES\textsubscript{seq} values for the ccdA mutants in the library are inferred through deep sequencing. A low ES\textsubscript{seq} value is indicative of low antitoxic activity of the corresponding ccdA mutant, relative to WT (ES\textsubscript{seq} = 1, log\textsubscript{10}ES\textsubscript{seq} = 0). The median values for synonymous and nonsynonymous mutant subsets are shown in red and blue dashed lines, respectively. WT ES\textsubscript{seq} value is shown in black dashed line. The fractional frequency of mutants = number of mutants with a particular range of ES\textsubscript{seq}/total number of mutants. (D) Distribution of the inferred fitness values for synonymous and nonsynonymous single-site ccdA mutants. The WT fitness and median values for synonymous and nonsynonymous mutant subsets are shown in dashed lines. (E) Phenotypic landscape for different residues in CcdA inferred from deep sequencing data. For each residue position in CcdA, the fraction of mutants showing inactive (ES\textsubscript{seq} ≤ 0.1), marginally active (0.1 < ES\textsubscript{seq} < 0.7), active (0.7 ≤ ES\textsubscript{seq} ≤ 1.5) and hyperactive phenotypes (ES\textsubscript{seq} > 1.5) is plotted. Only positions which have data for more than 10 mutants available have been plotted here. A large number of mutants across the length of ccdA display a loss of a function phenotype.
strain, the CFU/mL varied over a range of \(10^6\) across mutants in the sensitive strain and showed good agreement with the deep sequencing derived mutational scores (supplementary fig. S4, Supplementary Material online; Table 1). Phenotypic assays of individual single mutants with ES\(_{\text{seq}}\) values less than 0.1 confirmed that they show significant growth defects (significantly lower numbers of colonies after transformation) in the sensitive strain. Even when a few colonies are obtained for these inactive colonies after transformation) in the sensitive strain. Significant growth defects (significantly lower numbers of colonies after transformation) in the sensitive strain.

The distribution of ES\(_{\text{seq}}\) scores for ccdA synonymous mutants (median value of 0.03) is typically higher than that for nonsynonymous mutants (median value of 0.004) (fig. 1C). To compare our results with previous investigations of fitness distributions of synonymous versus nonsynonymous mutants (Bailey et al. 2021), we have converted ES\(_{\text{seq}}\) scores to fitness (w) (see Methods), using a previously described formula (Bailey et al. 2014; Lebeuf-Taylor et al. 2019). The median fitness values for synonymous and nonsynonymous ccdA mutants are 0.9 and 0.86, respectively (fig. 1D) and are lower than the WT fitness of 1. In contrast to the previous investigations of mutational effects on microbial growth rate in liquid culture, the current study measures the growth of mutants in terms of colonies appearing on plates after transformation. As discussed above, several ccdA mutants with ES\(_{\text{seq}}\) < 0.1 and corresponding \(w < 0.93\) fail to grow in liquid culture. On the other hand, all previously studied microbial gene variants with reported fitness values could be successfully grown in liquid culture for several generations (Lind et al. 2010; Lebeuf-Taylor et al. 2019; Bailey et al. 2021) implying that the identified inactive ccdA mutants are more deleterious than these previously documented cases of fitness defects. Therefore, the fitness (w) measure in the current study provides the upper limit of fitness estimations in ccdA mutants and should not be directly compared with those in the previous studies.

| Mutant Name\(^a\) | Mutant Codon | Description | Phenotype\(^b\) | ES\(_{\text{seq}}\) | Highest Culture Dilution With Visible Growth on Plate\(^c\) |
|-------------------|--------------|-------------|----------------|----------------|----------------------------------|
| T8                | ACT          | Synonymous  | Hyperactive    | 2.482          | \(10^5\)                        |
| V9                | GTG          | Synonymous  | Hyperactive    | 16.505         | \(10^6\)                        |
| S11               | TCT          | Synonymous  | Inactive\(^*\) | 0.011          | \(10^3\)                        |
| S11G              | GGT          | Nonsynonymous | Inactive       | 0.002          | \(10^1\)                        |
| S13               | AGT          | Synonymous  | Inactive       | 0.066          | \(10^1\)                        |
| L16               | CTG          | Synonymous  | Active         | 1.286          | \(10^6\)                        |
| L17               | CTG          | Synonymous  | Inactive       | 0.066          | \(10^1\)                        |
| V22               | GTG          | Synonymous  | Inactive       | 0.009          | \(10^1\)                        |
| S25               | TCG          | Synonymous  | Inactive       | 0.002          | \(10^1\)                        |
| V28               | GTG          | Synonymous  | Inactive       | 0.011          | \(10^2\)                        |
| S29               | AGT          | Synonymous  | Inactive\(^*\) | 0.044          | \(10^1\)                        |
| T30S              | TCG          | Nonsynonymous | Inactive\(^*\) | 0.598          | \(10^1\)                        |
| T31               | ACG          | Synonymous  | Inactive\(^*\) | 0.007          | \(10^3\)                        |
| R38               | AGG          | Synonymous  | Inactive\(^*\) | 0.007          | \(10^3\)                        |
| M52I              | ATT          | Nonsynonymous | Hyperactive    | 2.062          | \(10^4\)                        |
| V55               | GTT          | Synonymous  | Hyperactive\(^*\) | 1.624          | \(10^3\)                        |
| V55               | GTG          | Synonymous  | Moderately inactive\(^*\) | 0.486          | \(10^3\)                        |
| V55S              | TCG          | Nonsynonymous | Inactive       | 0.017          | \(10^1\)                        |
| R57               | AGG          | Synonymous  | Hyperactive    | 1.765          | \(10^5\)                        |
| R57A              | GGC          | Nonsynonymous | Hyperactive    | 8.718          | \(10^5\)                        |
| F58L              | CTG          | Nonsynonymous | Active         | 0.73           | \(10^4\)                        |
| G63L              | CTG          | Nonsynonymous | Inactive       | 0.002          | \(10^1\)                        |
| S64               | AGT          | Synonymous  | Inactive       | 0.002          | \(10^1\)                        |
| A66P              | CCT          | Nonsynonymous | Hyperactive    | 5.416          | \(10^6\)                        |
| N69L              | CTG          | Nonsynonymous | Inactive       | 0              | \(10^1\)                        |
| R70               | CGG          | Synonymous  | Hyperactive    | 9.673          | \(10^6\)                        |
| D71               | GAT          | Synonymous  | Inactive       | 0.007          | \(10^1\)                        |
| D71R              | AGG          | Nonsynonymous | Inactive       | 0              | \(10^1\)                        |

\(^a\) Mutants are labeled as WT amino acid identity_ residue position_ mutated amino acid identity in case of nonsynonymous variants and as WT amino acid identity_ residue position in case of synonymous variants.

\(^b\) Mutant phenotype is assigned based on the deep sequencing derived ES\(_{\text{seq}}\) values. These results correlate well with growth of individual mutants on plate, except in case of some mutants, namely S11_TCT, T30S_TCG and V55_GTT where growth on plates and deep sequencing results do not agree. These cases have been marked with an \(^*\).

\(^c\) Highest dilution showing growth is obtained from experiments involving individual transformation of the mutant plasmids and plating of serial dilutions to validate deep sequencing results. Spotting of serial dilutions of culture correlated well with plating results. WT ccdA construct grows till highest culture dilution of \(10^6\).
Inactivating mutations lead us to investigate codon specific effects amongst nonsynonymous CcdA mutants. We found several nonsynonymous mutations at multiple positions having distinct phenotypes for different codons that code for the same mutant amino acid. Since NNK codons were used for mutagenesis and preparation of the SSM library, we could only investigate phenotypes of mutated codons having G/T at the 3rd nucleotide position. Therefore, the phenotype of each synonymous mutant of CcdA is listed in supplementary table S4B.

The mutational effects observed in case of synonymous mutations led us to investigate codon specific effects amongst nonsynonymous CcdA mutants. We found several nonsynonymous mutations at multiple positions having distinct phenotypes for different codons that code for the same mutant amino acid. Since NNK codons were used for mutagenesis and preparation of the SSM library, we could only investigate phenotypes of mutated codons having G/T at the 3rd nucleotide position. Therefore, the phenotype of each synonymous mutant of CcdA is listed in supplementary table S4B.

The central stretch of CcdA (residues 20–55) was especially sensitive to synonymous mutations, with all documented synonymous mutations in this region being highly inactive. Synonymous mutations at the N terminal and C-terminal regions of CcdA resulted in both active and inactive variants. We also observed that most synonymous active mutants were hyperactive in comparison to WT with ESseq > 1.5.

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Growth Defects Caused by ccdA Mutations Are Not Due to CcdB Binding Defects

The growth defects caused by ccdA mutations are only observed in the Top 10 strain that is sensitive to CcdB toxicity and not in the Top 10 gyr strain (supplementary fig. S4B, Supplementary Material online), where the mutated Gyrase renders the strain resistant to CcdB mediated growth arrest. This clearly indicates that the growth defect displayed by ccdA mutations is caused by decreased inhibition of CcdB toxin in the sensitive strain. CcdA is known to rescue growth arrest by binding to CcdB with very high affinity, thus preventing CcdB from binding and poisoning DNA gyrase (Maki et al. 1996; De Jonge et al. 2009). Decreased inhibition could arise because of mutations

![Fig. 2](https://doi.org/10.1093/molbev/msac187)

**Fig. 2.** Inactive phenotype of synonymous mutants and codon specificity effects for nonsynonymous mutants are nonuniformly distributed across the length of ccdA. (A) Distribution of log10ESseq scores for all 62 available single synonymous mutants as a function of residue position. The WT ESseq score of 1 (log10ESseq = 0) is depicted as a black dashed line. Loss of function mutations with low ESseq values are distributed across the length of the ccdA gene. A few mutants show WT like or higher ESseq values and are largely clustered at the terminal regions of the gene. The phenotype of each synonymous mutant of CcdA is listed in table 2. (B) Averaged pairwise absolute differences in the ESseq scores amongst codons as a function of residue position in CcdA. The differences between ESseq values of each pair of synonymous codons encoding the same mutant amino acids are calculated. The average of the absolute differences of all mutant codon pairs for a residue position is estimated as follows: Average ΔESseq = (Σ |ESseqa - ESseqb|) / nC, where a and b are different codons encoding a missense mutation, and n is the total number of such codons at a position that have data for fitness scores. Regions close to the termini show the greatest variation in mutational effects, whereas the central region is largely populated by loss of function mutations.
affecting the folding of CcdA, mutations at residues in direct contact with CcdB, or because the total amount of expressed CcdA has been altered by mutation. In order to examine whether mutations significantly affect CcdA conformation, we characterized the ability of CcdA mutants to bind CcdB, using yeast surface display (YSD) methodology, where CcdA-CcdB binding can be decoupled from the toxic effect of CcdB on bacterial growth. CcdA was fused to S. cerevisiae Aga2p surface protein, displayed on the cell surface and its binding to purified CcdB toxin protein was measured using flow cytometry (fig. 3). Although CcdA shows high mutational sensitivity throughout its length based on the phenotypic study in the operonic context in E. coli, Synonymous mutations have previously been suggested to affect translation rate and cotranslational folding, thus altering protein activity (Krasheninnikov et al. 1988; Komar et al. 1999; Sander et al. 2014). Such folding defects are unlikely in the case of CcdA, since its C-terminal domain, involved in CcdB binding, is intrinsically disordered and remains natively unfolded in unbound conditions. A more extensive DMS study of CcdA mutants in the YSD system indicates that mutations that affect the CcdB-binding affinity of CcdA primarily occur at residues directly involved in contact with CcdB (Chandra et al. 2022). While YSD fails to replicate the in vivo conditions of E. coli and coupling of transcription-translation to protein function, the binding assay on yeast surface clearly shows that most mutations do not alter the inherent CcdB-binding function of the intrinsically disordered CcdA molecule. Further, we find no discernable difference in the distribution of phenotypes or the ESseq values between mutations at CcdB interacting and CcdB noninteracting residues in the C-terminal CcdB binding domain of CcdA, classified based on the available structure of CcdA C-terminal domain bound to dimeric CcdB (De Jonge et al. 2009) (supplementary fig. S5B, Supplementary Material online). These observations indicate that mutational effects on binding affinity to CcdB contribute insignificantly to the mutant phenotypes observed for ccdA in its native operonic context.

We also studied the distribution of mutational phenotypes (for nonsynonymous mutations) at different classes of functionally important residues in the N-terminal domain of CcdA, identified based on the available NMR structure of the DNA bound dimeric CcdA N terminal domain (Madl et al. 2006). We found that mutations in the DNA binding residues in the CcdA N-terminal domain have no distinguishable difference in terms of the growth phenotype, when compared to other noninteracting exposed residues (supplementary fig. S5B, Supplementary Material online). Surprisingly, mutations at buried positions in the N terminal domain show significantly reduced activity and more severe growth defects, especially in the case of mutations to nonaliphatic residues, when compared to any other classes of CcdA residues (supplementary fig. S5C, Supplementary Material online). This suggests that mutations that can disrupt the N-terminal buried core of CcdA can induce increased protein degradation or proteolysis, ultimately producing the observed inactive phenotypes.

Table 2. Mutational Sensitivities of all Available Synonymous Mutant Codons at Each Residue Position of CcdA Library in Operonic Context.

| Residue Position | Synonymous Mutant Codons |
|------------------|--------------------------|
| 8                | AGG TTT TAA            |
| 9                | CCG TGA ACT            |
| 10               | TGA TCG TCT            |
| 11               | AGT TCG ACT            |
| 12               | GAT TCG TCT            |
| 13               | AGT TCG ACT            |
| 14               | CTT TTG ACT            |
| 15               | GCT TCT ACT            |
| 16               | GTG GTT ACT            |
| 17               | AAG ACT ACT            |
| 18               | GAT ACT ACT            |
| 19               | AGG ACT ACT            |
| 20               | AGG ACT ACT            |
| 21               | AGT TCG ACT            |
| 22               | GGT ACT ACT            |
| 23               | GCT TCT ACT            |
| 24               | GTT ACT ACT            |
| 25               | AGG ACT ACT            |
| 26               | AGG ACT ACT            |
| 27               | AGT TCG ACT            |
| 28               | GGT ACT ACT            |
| 29               | GCT TCT ACT            |
| 30               | GTT ACT ACT            |
| 31               | AGG ACT ACT            |
| 32               | AGT TCG ACT            |
| 33               | GGT ACT ACT            |
| 34               | GCT TCT ACT            |
| 35               | GTT ACT ACT            |
| 36               | AGG ACT ACT            |
| 37               | AGG ACT ACT            |
| 38               | AGG ACT ACT            |
| 39               | AGG ACT ACT            |
| 40               | AGG ACT ACT            |
| 41               | AGT TCG ACT            |
| 42               | GGT ACT ACT            |
| 43               | GCT TCT ACT            |
| 44               | GTT ACT ACT            |
| 45               | AGG ACT ACT            |
| 46               | AGG ACT ACT            |
| 47               | AGG ACT ACT            |

The codons (synonymous with respect to WT sequence of CcdA) have been colored based on the deep sequencing phenotypic activity (ESseq scores) in operonic context. Dark green depicts hyperactivity (ESseq > 1.5), while light green denotes WT like activity (0.7 ≤ ESseq ≤ 1.5). Yellow and red indicate slightly inactive mutants (0.1 < ESseq < 0.7) and highly inactive mutants (ESseq ≤ 0.1), respectively.

The frequency of different degenerate codons (for any amino acid) in the genome of an organism varies significantly. We investigated the relationship between the

**E. coli Genomic Codon Usage Contributes to Growth Phenotypes of Single Codon Mutants in CcdA**

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The frequency of different degenerate codons (for any amino acid) in the genome of an organism varies significantly. We investigated the relationship between the
phenotypes of mutants and relative codon usage (RCU) (in the \( E.\) \( coli \) genome) of the introduced codon. The distribution of RCU values amongst active and inactive CcdA mutants was significantly different (fig. 4A). It has been suggested that having a higher percentage of frequent codons in a gene leads to higher amounts of expressed protein, across various kingdoms of life (Sharp et al. 1986; Shields and Sharp 1987; Sharp and Devine 1989; Karlin et al. 1998). Decreased levels of CcdA protein in \( vitro \) will result in the presence of unbound CcdB molecules that will poison bacterial DNA Gyrase, resulting in cell death. While there is a large fraction of inactive mutants in the \( ccdA \) single mutant library, we observe that \( \sim 70\% \) of \( ccdA \) mutants displaying an active phenotype harbor frequent codons (based on \( E.\) \( coli \) genome) with similar or higher codon usage frequency (CUF) relative to the WT codon. The observed high mutational sensitivity in the central region of the \( ccdA \) gene might result from the high prevalence of frequent codons in this region (supplementary fig. S6A, Supplementary Material online). Thus, most mutations at these positions lead to the introduction of rarer codons, possibly leading to lower CcdA protein expression and subsequent cell death. On the other hand, the distribution of relative tRNA abundance values among active and inactive mutants was similar (fig. 4B). There was also no statistically significant difference between inactive and active mutant distributions for other sequence features such as relative GC (Guanine-Cytosine) content and number(s) of base changes upon mutation (fig. 4C and D).

We also studied the distribution of these DNA sequence features among the active and inactive variants for the
subsets of synonymous mutations, nonsynonymous, as well as for the nonsynonymous substitutions which showed codon specific effects. Inactive mutations show significantly lower RCU values than active mutants in all subsets consistently (fig. 4E–G).

Codon adaptation index (CAI) is a commonly used parameter that describes the degree of codon bias for a whole gene or sequence and helps to infer the relative adaptiveness of the sequence (Sharp and Li 1987). While it is routinely used in studies that investigate functional changes in sequences where multiple rarer or more frequent codons have been introduced, a single-site mutation (change of one codon) does not produce a large change in the CAI. CcdA WT gene has a CAI of 0.76 and CAI calculated for single codon changes are in the range of 0.7–0.78. Such small changes are not expected to show any distinguishable effects on relative fitness of mutants. We find that distribution of relative CAI values (mutant CAI/WT CAI) across inactive and active mutants fail to show any significant difference in the Mann–Whitney test (supplementary fig. S7, Supplementary Material online). On the other hand, RCU uses the relative fraction of usage of the synonymous codon encoding a particular amino acid and also takes into account the most common codon (for the WT amino acid). Estimating phenotypic effects due to altered synonymous codon usage fraction at the particular site using RCU helps to reveal that codon usage bias does indeed play a role in affecting the fitness in single-site mutants as described earlier (fig. 4A and E–G).

**Strength of the CcdB RBS Determines CcdA Mutant Phenotype**

A primary sequence element that is known to be a strong determinant of the efficiency of translational coupling is the Shine Dalgarno (SD) sequence. Early studies by Das and Yanofsky in the Trp operon showed that translation initiation at any start site, located near a functional stop codon of the upstream gene may be influenced by the strength sequence and location of the SD region and its spacing from the start codon (Das and Yanofsky 1989). The RBS for the ccdB gene lies within the CcdA coding region (ccdA gene), primarily spanning CcdA residues 70–71. Therefore, mutations in these residues may affect the relative CcdA and CcdB levels by changing the strength of the RBS for CcdB.

An analysis of all available codon substitutions at residues 70 and 71 of CcdA from the deep sequencing data indicated...
that the predicted strength of the RBS for CcdB expression was a significant contributor to the phenotype of these mutants (fig. 4H). Mutants at residues 70 and 71 displaying inactive phenotype were predicted to improve the CcdB RBS strength. The molecular basis of one such mutation, D71R was examined. Introduction of the AGG arginine codon at the 71st position led to highly inactive phenotype, but no inactivity was observed for the other arginine codons. Therefore, it is unlikely that the inactive phenotype observed for the AGG codon is due to effects on protein stability or protein-protein interaction. It is known that the consensus sequence for the RBS, having the maximal strength is 5′ AGGGAGG 3′ (based on the corresponding complementary sequence of 16S rRNA, 5′CCUCCU 3′). Therefore, it is likely that a change from the suboptimal RBS for CcdB present in the ccdAB operon (5′ AAGGAC 3′), to a consensus RBS (as is predicted in case of D71R_AGG mutation), decreases the CcdA:CcdB ratio in the cell, there by leading to cell death. This prediction was consistent with data from quantitative proteomics (fig. 5A).

Mutations in CcdA Lead to Altered Levels of CcdA Proteins and Therefore Modified CcdA:CcdB Ratio in Cell
Codons for inactive CcdA mutants have significantly lower RCU in comparison to active mutants, suggesting that the observed mutational phenotypes might result from alterations in translational rate of the CcdA or CcdB gene products. To further probe protein levels in mutants, we carried out a proteomics study. The extremely low levels of CcdA and CcdB proteins in vivo cannot be accurately characterized using classical methods like SDS-PAGE or Western Blotting. The ratio of CcdA: CcdB in WT was found to be slightly larger than 1 in the proteomics study (fig. 5A). Interestingly, all the five inactive CcdA mutants tested show a lower CcdA:CcdB ratio (fig. 5A). Introduction of rarer codons thus appears to reduce the translation of CcdA, decreasing the CcdA:CcdB ratio of proteins in the cell, causing more severe growth arrest. The inactive D71R_ AGG mutant (CcdB RBS site mutation in ccdA) also exhibits a lower CcdA:CcdB ratio, likely due to increased translation of CcdB protein. Active mutant R57A and CcdAB operon in F-plasmid show comparable protein ratio relative to WT.

Increased Levels of Operonic mRNA in Inactive Mutants Confirm Decrease in CcdA:CcdB Ratio
The five inactive and one hyperactive mutants of CcdA tested in the proteomic study have relatively reduced and elevated levels of CcdA proteins relative to WT respectively. The ccdAB expression at the transcription level is autoregulated by the CcdA-CcdB protein complex in a CcdA:CcdB ratio dependent manner (Tam and Kline 1989; Afif et al. 2001). When the CcdA:CcdB protein level ≥1, elongated multimeric CcdA-CcdB complexes with alternating dimeric CcdA2 and CcdB2 are formed which

![Image](https://doi.org/10.1093/molbev/msac187)

**Fig. 5.** Decreased CcdA:CcdB protein ratio leads to loss of function phenotype for inactive synonymous CcdA mutants and also increases ccdAB mRNA levels by feedback autoregulation of the operon. (A) Relative levels of CcdA and CcdB peptides in *E. coli* (Top 10 gyr strain) lysates were determined for WT as well as selected single synonymous mutants of CcdA using a quantitative proteomics approach. All synonymous inactive mutants studied have a decreased CcdA:CcdB protein ratio. The inactive nonsynonymous mutant D71R which is predicted to improve CcdB translation due to a stronger CcdB RBS, also displays a decreased CcdA:CcdB protein ratio relative to WT. (See Table 1 for phenotypic scores of mutants.) F indicates the value for F-plasmid. Mutants showing active and inactive phenotypes in the operonic study are marked in green and red, respectively. (B) Inactive synonymous mutants show higher levels of ccdAB mRNA, while active mutants have lower or WT comparable levels of ccdAB mRNA in Top10 gyr *E. coli* cells. The mRNA levels of ccdAB specific transcripts were determined using q-RTPCR. CcdA and ccdB regions were both independently quantitated. Mean Ct represents the threshold cycle for amplification obtained from duplicate samples from two different experiments. Fold change in ccdA and ccdB transcript levels are with respect to the WT Ct values. Error bars indicate estimated standard deviation of the measurements. The mutants are labeled as WT amino acid identity_residue position_mutated amino acid identity in case of nonsynonymous mutants and as WT amino acid identity_residue position in case of synonymous mutants. (C) Schematic model for CcdA:CcdB ratio dependent autoregulation of ccdAB operon transcription as described previously (Vandervelde et al. 2017).
Table 3. Binding Affinities of CcdA Mutants and WT Calculated Using Titration of Yeast Surface Displayed CcdA Molecules With Purified CcdB.

| CcdA     | Kd (nM) | Phenotype |
|----------|---------|-----------|
| WT       | 0.24 ± 0.09 | Active    |
| L17_CTG  | 0.4 ± 0.1  | Active    |
| S25_TCG  | 0.24 ± 0.07 | Inactive  |
| T8_ACT   | 0.31 ± 0.12 | Active    |
| L16_CTT  | 0.32 ± 0.06 | Active    |

The dissociation constants (Kd) were obtained by fitting the fluorescence intensity titration traces to a single-site ligand binding model. In the operonic context in E. coli, the single codon synonymous mutants L17_CTG and S25_TCG display an inactive phenotype, while T8_ACT and L16_CTT have an active phenotype based on the deep sequencing studies.

bind to the ccdAB operator region with high affinity, repressing gene expression. On the other hand, when CcdA:CcdB < 1 (protein level), hetero-hexameric complexes of one CcdA dimer bound to two CcdB dimers (CcdB2-CcdA2-CcdB2) are formed that have low binding affinity to the operator region and leads to a derepressed state of operon, allowing transcription to proceed (Vandervelde et al. 2017).

To study the possible effects of CcdA sequence changes resulting from single codon mutations on gene expression, we quantified ccdAB operon specific RNA using RT-qPCR using both ccdA and ccdB gene specific primers. Relative amounts of ccdA and ccdB encoding transcripts were near identical in WT and were not significantly changed in case of most mutations (ratio of ccdA and ccdB specific mRNA levels ≈ 1) (fig. 5B), as expected for transcription from an operon. However, significant increases in the mRNA levels for both the ccdA and ccdB amplicons were observed in case of all inactive mutants (fig. 5B). This is consistent with the lowered CcdA:CcdB protein levels in the inactive mutants observed in the previous section. A lowered CcdA:CcdB protein ratio will result in derepression of the operon, leading to upregulation in mRNA production (fig. 5C). Analysis of whole genome RNA-seq and ribosome profiling data available for the E. coli genome indeed indicate that while the mRNA levels for most type II TA systems were similar for the toxin and the antitoxin consistent with our observations in ccdAB, the antitoxin protein levels can be up to two-fold higher than the toxin under normal growth conditions (Deter et al. 2017). This indicates that differential translation of the ccdA and ccdB genes and/or differential proteolysis of the two proteins play a vital role in maintaining the TA protein levels in vivo. Altered DNA sequences affecting the relative levels of the TA proteins can thus be detrimental to functions of TA systems.

Investigation of Possible Effects of Ribosomal Pausing on CcdA Mutant Phenotype

An analysis of bacterial genomes has suggested that SD like sequences within the coding region of genes could be potential ribosomal pause sites and is therefore under negative selection (Li et al. 2012; Mohammad et al. 2016). To examine if mutations in CcdA generate such potential pause sites, the difference in interaction energies with the E. coli ribosomal anti-SD sequence between single synonymous mutants and the WT sequence was calculated using the RNAsubopt program in the Vienna RNA package (Gruber et al. 2008). The data indicate that the inactive and active synonymous mutations show no distinguishable difference in anti-SD interaction (supplementary fig. S8A, Supplementary Material online). The extent of ribosome pausing at each codon and the associated effects on in vivo translation speed have been estimated previously (Chevance et al. 2014). In the present study, we did not observe any significant differences in distribution of these relative ribosome stalling values for different codons between synonymous inactive and active mutants of CcdA (supplementary fig. S8B, Supplementary Material online).

Effect of Single Codon Synonymous Mutations in Another TA Module, RelBE

To test if other TA modules are also sensitive to synonymous mutations, the RelBE system was used as a test case. Unlike the plasmidic ccdAB system, relBE is natively a chromosomal TA system, expressed as a part of the relBE operon (Cherny et al. 2007). The RelE toxin is a site-specific, ribosome dependent mRNA endonuclease. Expression of free RelE leads to bacterial growth inhibition. Mild overexpression of RelE is also reported to increase persister cell frequency (Fasani and Savageau 2013). While unrelated in sequence, the RelB protein from E. coli is similar to the CcdA antitoxin in both fold and thermodynamic properties, and inhibits the action of RelE by forming a tight complex. The RelBE complex acts as a transcriptional repressor and autoregulates its own expression (Cherny et al. 2007). Homologs of this system are found in many bacterial species including pathogens like Haemophilus influenzae. An analysis of ribosome profiling data for the E. coli K12 MG1655 strain indicates that the translation efficiency for the relB gene is 3.6-fold higher than the relE gene (Deter et al. 2017), although both the codon and tRNA adaptation indices are similar for both genes, similar to what we observed in the CcdAB system.

To study mutational effects, relBE operon was cloned in pUC57 plasmid. We studied five single codon synonymous mutations, distributed across the length of the relB gene in a native relBE operonic context, including both three and two base substitutions. These mutations were to both more frequent and rarer codons. A control mutation at residue 77 of relB that strengthens the SD sequence of RelE was also studied. This is expected to lead to an increase in RelE levels and inhibition of cell growth (fig. 6). Using a similar screen of cell death versus growth as in the case of CcdAB system, we monitored the growth of single synonymous mutants in the WT (E. coli BW25113) as well as in the toxin deleted (E. coli BW25113 ΔrelE) strain. The two relB variants with rarer mutated codons tested here had a decreased growth in the WT strain (fig. 6B and C). We also found that two mutations of relB with higher codon usage with respect to WT codon display...
improved growth phenotype. The growth defective D77 variant involves introduction of a rarer codon besides strengthening the RBS. The relB mutant phenotypes indicate the existence of a relatively general phenomenon wherein a single-codon change in DNA sequence upon synonymous mutations can have observable phenotypic effects for TA operon systems, where small perturbations in the system can compromise cellular fitness.

Discussion

In type II TA systems, the toxin and antitoxin can form higher order TA complexes which bind to the TA promoter repressing its transcription. Conditional cooperativity (TA protein ratio dependent co-operative interaction of TA complex with the operator DNA) is known to be a dominant mechanism in regulating the transcription of most TA systems (Cataudella et al. 2012). Given the higher rate of degradation for the antitoxin due to its disordered nature, the antitoxin needs to be produced at a higher rate than the toxin, for the cells to be viable. However, mechanisms that can lead to differential levels of the toxin and the antitoxin protein from a common mRNA are not well understood. While protein levels can in principle be regulated at both the transcriptional and translational levels, RNA-seq data from E. coli indicates that mRNA levels for the toxin and the antitoxin are at least two fold higher than the toxin, for six TA pairs for which sufficient data is available (Deter et al. 2017). Ribo-seq data available for TA systems indicate that the protein synthesis rates (calculated from ribosome densities) for the antitoxin are at least two fold higher than the toxin, for six TA pairs for which sufficient data is available (Deter et al. 2017). These observations indicate that there are indeed nucleotide sequence dependent features, which allow regulation at the level of translation, leading to differential synthesis rates for the two genes in the operon. Maintaining a perfect balance between the antitoxin and toxin proteins is essential for proper functioning of TA modules. Small changes in gene expression caused by altered DNA sequences, both in coding or noncoding regions can potentially be detrimental to this balance and result in observable phenotypes. In the present study, we examined mutational effects in the bacterial ccdA antitoxin gene on phenotype, in its TA operonic context, using saturation mutagenesis coupled to deep sequencing. We observe significantly high fractions (\(\sim 80\%\)) of both synonymous and nonsynonymous mutants to show reduced antitoxic functions in the operonic context, leading to reduced survival of the bacterial host cells. An extensive review by Bailey et al (Bailey et al. 2021) summarizes the DFEs of synonymous versus nonsynonymous mutations for twelve different proteins and viral genomes. Studies investigating DFEs in bacterial and yeast proteins have revealed that most mutants (\(>95\%\)) retain at least 50% fitness (growth-rate based) with respect to WT (Lind et al. 2010; Schenk et al. 2012; Lebeuf-Taylor et al. 2019). Similar results are seen in a comprehensive study of Hsp90 (Flynn et al. 2020). Other studies revealed DFE of missense mutants to be bimodal. For bacterial TEM-1 (Firnberg et al. 2016), \(\sim 25\%\) missense mutations showed loss-of-function and another \(\sim 30\%\) showed reduced fitness. For yeast Hsp90, \(\sim 50\%\) missense mutations showed 10–50% reduced fitness (Hietpas et al. 2011). In case of various viral genes/genomes 20–40% mutants show lethal (loss-of-function) and 10–35% show deleterious (reduced fitness) phenotypes (Carrasco et al. 2007; Domingo-Calap et al. 2009; Peris et al. 2010; Wu et al. 2014). Notwithstanding these variations in DFE for missense mutants, all previous studies indicate most synonymous mutants to be near neutral with the exception of cases where only a small number of synonymous mutants displays a range of reduced (20–70%) fitness (Carrasco et al. 2007; Lind et al. 2010; Cuevas et al. 2012; Wu et al. 2014). For
ccdA, we observe that 80% of both synonymous and non-
synonymous ccdA mutants are observed to have ESseq va-
value $\leq 0.1$ (ten-fold lower colonies obtained following
transformation into Top10 E.coli strain relative to WT) in
the present study. The major peak in the distribution of
ESseq scores in ccdA mutants is shifted towards lower va-
ues relative to the WT. While ESseq is not identical to con-
ventional fitness measures, it is still apparent that the
fraction of synonymous (as well as nonsynonymous) muta-
tions showing significantly reduced activity is higher than
those seen in the other systems, some of which are sum-
marized by Bailey et al. Most importantly, we observed
that most inactive ccdA mutants displaying ESseq $< 0.1$
produced 10–1,000-fold lower number of colonies than
WT ccdA when transformed in the CcdB-sensitive E.coli
strain Top10 (but similar numbers of colonies when trans-
formed in toxin resistant strain Top10 gyr to WT). In
the previous DFE studies involving bacterial/yeast growth-rate
based phenotypic investigation, only mutants with com-
parable number of colonies to ancestor/WT sequence re-
trieved after transformation were used for further DFE
investigations (Lind et al. 2010; Bailey et al. 2014;
Lebeuf-Taylor et al. 2019). This indicates that the ccdAB
operonic system under study is apparently more sensitive
to mutations than those previously studied. We also found
the ccdAB operonic system under study was more sensitive
to nonsynonymous mutations than synonymous muta-
tions in the ccdA gene. A reduced severity in fitness defects
in case of synonymous relative to nonsynonymous muta-
tions is also observed in few other cases (Fragata et al.
2018; Bailey et al. 2021), though the overall mutational sen-
sitivity in these studies was found be much lower than ob-
served for CcdA.

The ccd operon used in the present experiments differs
slightly in sequence from the one on F-plasmid
(supplementary fig. S1, Supplementary Material online),
resulting in a slightly reduced CcdA: CcdB protein ratio
(fig. 5A). This can be attributed to the presence of a
suboptimal ccdA RBS in the noncoding region
(supplementary fig. S1, Supplementary Material online)
in our WT CcdAB construct. Small decreases in the ratio
cause by mutations have dramatic phenotypic effects.
This results from the very high toxicity of CcdB, the feed-
back regulation present in the system and the additional
amplification resulting from use of a high copy number
pUC plasmid to house the ccd operon. The readout here
is a function of the CcdA:CcdB ratio. While it is very chal-
lenging to measure absolute levels of either protein, we are
able to measure the ratio using proteomics. The data in fig-
ure 5A show, consistent with the observed phenotypes,
that the CcdA:CcdB ratio is $<1$ for the four inactive syn-
nonymous mutants tested, whereas it is $\sim 1.4$ in the WT op-
eron. For reasons that are currently unclear, in the ccd
operonic construct we have used, mutations that enhance
the CcdA:CcdB ratio and result in higher values of ESseq (in-
dicating beneficial variants) are rare, in contrast to other
systems described in Bailey et al (Bailey et al. 2021) and
Flynn et al (Flynn et al. 2020). The present ccd construct
amplifies effects of point mutations and allows detection
of changes that would be missed in other systems where
small changes in expression level do not result in observ-
able phenotypes. Strong mutant phenotypes were also
found for single-site synonymous variants of the relB
gene in RelBE TA operon, also cloned in a high copy
number plasmid. Single codon changes in relB produced
observable changes in phenotype comparable to those
in the ccdAB system. The current study therefore show-
cases the utility of TA operons for providing general
insights into the plethora of mechanisms by which point
mutations can affect phenotype.

The present studies reveal that most single codon sub-
stitutions, including synonymous mutations along the
length of ccdA lead to a loss of function phenotype.
Many nonsynonymous mutations also show codon specif-
ic phenotypes, meaning that significantly different pheno-
types are observed upon introduction of different codons
that encode the same mutated amino acid residue. These
studies demonstrate that there can be strong selection
for specific synonymous codons. This complicates inter-
pretation and use of dN/dS ratios for studying selection
pressure especially in operons. The observed dependence
of the mutant phenotype on RCU of mutant with respect
to WT suggests that single codon mutations in CcdA likely
influence the translation efficiency of the CcdA, thus alter-
ning the relative levels of CcdA and CcdB proteins in the

cell. A high frequency of frequent codons in the central re-


region of ccdA gene suggests that the observed high muta-
tional sensitivity at this region is because any codon intro-
duced upon mutation is expected to be rarer relative
to the WT codon. At the ccdA gene extremities there is lar-
gar variation in codon usages values (supplementary fig.
S6, Supplementary Material online). Hence mutated cod-
ons at these regions may be more or less frequent relative
to WT. Altered DNA sequence can directly affect ribosome
assembly, ribosome stalling, rate of translation, or promote
altered mRNA structure, which in turn can have multifa-
ceted consequences on protein production. The quantita-
tive proteomics study reveals that for loss of function
mutants, the in vivo CcdA:CcdB protein level ratio is lower
than that of WT, resulting in cell death. Codon usage
has previously been found to be a determinant of protein
translation efficiency, accuracy as well as kinetics
(Ikemura 1985; Sørensen et al. 1989; Quax et al. 2015;
Lyu et al. 2020). Most prior studies do not characterize
the change in protein levels associated with single syn-
nonymous codon substitutions. However, total functional
protein levels have been found to increase in case of ben-
ficial single-codon synonymous mutants in TEM-1 by up to
6–7-fold (Zwart et al. 2018) and in ADAMTS13 enzyme by
1.4-fold (Hunt et al. 2019), without detected effects on
transcription levels and protein conformation respectively.
An earlier study of an operonic system has revealed that
synonymous mutations alter relative protein levels of op-
eronic gene products up to 3-fold, causing altered growth
rates (Kristofich et al. 2018). In vivo translation rates of glu-
tamic acid codons (GAA and GAG) were measured to be
significantly different (and correlated to respective codon usage), despite both codons being read by same tRNA (Sørensen and Pedersen 1991). In FAE and CAT enzymes, protein levels were found to be reduced in a number of variants containing multiple synonymous substitutions across the genes (Amorós-Moya et al. 2010; Agashe et al. 2013). A study of introduction of a small number of rarer codons in the most highly expressed genes in E. coli also suggested that codon usage of a gene can affect the translation rate of gene with altered codons as well as of other genes due to perturbation of cellular tRNA supply (Frumin et al. 2018).

The prevalence of large phenotypic variations amongst CcdA mutants exclusively at the N-terminal (first 20 residues) and C-terminal residues (56–72) might also be a result of mutational effects on translation initiation of ccdA and ccdB genes respectively. Mutations near the ccdA gene terminus, at residue positions 70 and 71 (that also constitute the RBS of CcdB) appear to affect translational efficiency of the downstream CcdB gene. Mutants at these positions which are predicted to show improved ribosome binding exhibit a lower in vivo CcdA:CcdB protein ratio and higher toxicity in E. coli. Mutations that decrease the CcdA:CcdB ratio result in de-repression of the operon, this is confirmed by the observed increased ccd mRNA expression levels in these mutants. The elevated operonic mRNA level is expected to amplify the mutational effect on differential translation efficiency of CcdA and CcdB.

Earlier studies which observe a strong influence of codon rarity and codon pair bias on protein level (Quax et al. 2015; Boël et al. 2016) typically used over-expressed proteins. In natural contexts, many bacterial genes are organized in operons to ensure co-regulation of genes involved in similar pathways and functions. In the special case of TA operons, fine tuning of the relative amounts of antitoxin and toxin is essential and directly linked to bacterial fitness. Given the abundance and diversity of these TA systems in many bacterial species, including the pathogen Mycobacterium tuberculosis, it is reasonable to assume that the cell must have evolved multiple mechanisms to ensure their stringent spatial and temporal regulation. We find that these relative protein levels can be perturbed through changes in DNA sequences. Although effects of synonymous mutations are often attributed to co-translational folding defects and altered activity of protein, we find that most of the inactive ccdA mutants are not CcdB binding defective, based on YSD studies. We also observed that single mutations in DNA interacting residues of the CcdA N-terminal domain did not affect growth phenotype more drastically than mutations in other residues in CcdA, indicating no significant loss of DNA binding in such mutants. In contrast however, the heightened inactivity was observed in case of mutations at buried positions in the structured N-terminal domain. This suggests probable disruption of the hydrophobic core in the N-terminal domain, leading to increased proteolysis and decreased CcdA protein level. Besides the amino acid substitution related effects observed in the N-terminal domain, we also observe large phenotypic variation in the ccdA activity amongst a subset of missense mutants coded by different codons in both the N- and C-terminal regions of CcdA that cannot be explained by the codon usage bias or tRNA abundance. Interestingly, these effects occur in spans of about 40 bases which is on the order of the footprint of a single ribosome on mRNA in prokaryotes (Mohammad et al. 2019). Thus, it is possible that the high diversity in phenotype for synonymous codons exclusively at the two termini of ccdA may be caused by altered translation initiation rates for CcdA and CcdB in the operon with point mutations at the N- and C-terminus positions of the ccdA gene respectively.

Previous studies on prokaryotic translation also suggest mRNA structure and accessibility near the translation start site play important roles in translation initiation, a rate limiting step of translation (Studer and Joseph 2006; Gualerzi and Pon 2015; Mustoe et al. 2018). Though we find no significant correlation of the phenotype with change in GC content or number of base changes upon mutations in these regions, we suspect that local mRNA structural changes upon mutations in the 5’ end of the ccdA gene affect the translation initiation of CcdA while those in the 3’ end of the ccdA gene affect the translation initiation of CcdB, leading to the large codon-specific phenotypic variation observed at both termini of the ccdA gene. While the RNA structures of the ccdAB transcript predicted by the MFold (Zuker 2003) and RNAfold (Gruber et al. 2008) web servers show that several single codon mutations can affect the predicted mRNA structural architecture of ccdA mRNA and its stability, no consistent difference is observed amongst the structures for mutants displaying neutral, inactive and hyperactive phenotypes. Since mRNA stability, transcription and translation are coupled in prokaryotes, to understand mutational effects of mRNA structure on protein levels and activity, one must determine the in vivo mRNA structure of the mutants which is beyond the scope of the present study.

Materials and Methods
Cloning of WT and Saturation Mutagenesis Library of CcdA
A 986 bp region comprising of the complete ccdAB operon along with its upstream and downstream regulatory regions (based on F-plasmid sequence deposited in GenBank) was synthesized at GenScript (USA) and cloned in the EcoRV site of the pUC57 plasmid vector. A few mutations were introduced in this cloned ccdAB construct relative to F-plasmid sequence, in order to introduce restriction enzyme sites to facilitate cloning of ccdA or ccdB mutant libraries (Supplementary fig. S1, Supplementary Material online). A single-site saturation mutagenesis (SSM) library of ccdA in this operonic construct was then constructed using inverse PCR.
methodology (Jain and Varadarajan 2014) (see Supplementary Methods online, Supplementary Material online).

Sample Preparation for Deep Sequencing and Data Processing

The plasmid library purified from the resistant Top10 gyr strain was considered as the initial unselected library. This was then transformed into the sensitive Top10 strain for selection. We used high efficiency electro-competent cells of Top10 and Top10 gyr strains, having equal efficiencies of $10^9$ CFU/µg of pUC57 plasmid DNA. The detailed method for construction of the Top10 gyr strain is available in Supplementary Methods, Supplementary Material online. Approximately equal numbers of transformants ($\sim 10^8$ in number) from both strains obtained after 14 h of growth at 37°C on LB-Agar plates containing 100 µg/mL Ampicillin, were scraped into 10 mL LB media and directly used for plasmid purification using a Thermo Scientific Plasmid purification kit for both the unselected and selected libraries. These transformations and subsequent plasmid isolation procedures were performed in three biological replicates. The ccdA gene was PCR amplified from each purified plasmid sample, with primers (Sigma-Aldrich) containing condition specific, six-base long Multiplex IDentifier (MID) tags (barcodes). The resulting 270 bp long PCR products containing the full ccdA gene, were pooled, gel-band purified, and sequenced using Illumina Sequencing, on the NovaSeq6000 Platform, at Macrogen, South Korea. The overall quality of the sequencing data was assessed using the FASTQC software. Further analysis was performed using our in-house Perl scripts. Reads were separated into ‘bins’ based on their MID tags. The downstream primer sequence was used to identify forward and reverse reads in each bin. A Phred score cutoff of 20 and a minimum read length cutoff of 75 were used to filter low quality reads. Reads were converted into FASTA format and aligned with the ccdA gene sequence using the WATER program of the EMBOSS package (Rice et al. 2000; Carver and Bleasby 2003). Default values were used for all the parameters except the Gap Opening Penalty, which was increased to 20. For the present CcdA DMS library, we have used a paired end sequencing platform. Forward and reverse reads of the same read pair were merged together if the overlapping regions were identical, and all mismatches between forward and reverse reads were discarded. Only those reads which cover the entire ccdA gene length were considered for further analysis. Reads with insertions, deletions and multiple mutations were omitted, and only mapped single mutants were used for further data analyses. The total number of usable reads including the WT reads in the three replicates were 201938, 478,296, and 100,205, respectively. Since NNK primers were used for construction of mutant library, 2.7% of all constructs are expected to be WT sequence. However, deep sequencing of unselected library (as well as Sanger sequencing of 15 colonies) revealed $\sim 10\%$ of the constructed ccdA library to be WT. We suspect the additional 7.3% arises from carry-over of the WT plasmid used as template in mutagenesis PCR reactions that has not been completely removed by the DpnI digestion and subsequent clean up steps.

We used $\times 200$ theoretical coverage for each mutant for deep sequencing and have achieved high average read counts of each mutant in the unselected library (mean = 263 and median = 101). The average quality score for this final dataset is 36.76 and majority reads have Q score > 35. Assuming a Q score of 35, the probability of a sequencing error is 1/3,162. Assuming that all four bases are equally likely to be assigned an error at a given position, the probability of erroneously observing a specific base substitution at a specific position in a read is 1/12,649. For a given error probability and total number of reads, the probability of getting at least the observed number of reads for a specific mutant codon can be calculated assuming a binomial distribution. This binomial cumulative distribution function can be approximated by a Poisson cumulative distribution function. For 100,000 total reads and a probability $P < 0.05$, the minimum number of reads is 13 for a single base substitution when Q35 score is taken. This means that any single-site mutant (with 1 nucleotide change with respect to WT) with 13 or more reads has a probability of $< 0.05$ of being observed by chance. In present analysis, we have discarded all mutants with less than 20 reads in the unselected library. This read cutoff, combined with high average read number, ensures the accuracy of the ES$_{seq}$ values and the correctness of phenotypic assignments from the DMS data.

Assignment of Mutant Phenotypes Based on the Deep Sequencing Data

Read numbers for mutants at all 71 positions (2–72) in CcdA were analyzed. Mutants with $< 20$ reads in the resistant strain were not considered for analysis. The total number of reads in different conditions was calculated. Read numbers for each mutant at a given condition were normalized to the total number of reads in that condition. Mutational enrichment was defined as the ratio of the normalized reads after selection to the normalized reads before selection, for a given mutant. We further normalized the mutant scores with respect to the WT fitness score to obtain the ES.

Survival score of a mutant $i$
Survival score of a mutant strain. The ESseq scores were calculated for all mutants in the sensitive mid sample. Detailed Methodology is available in the

Monitoring the Relative Levels of CcdA and CcdB Using a Quantitative Proteomics Approach

The relative levels of CcdA and CcdB proteins were quantified from cell lysates using mass spectrometry based quantitative proteomics. All the experiments were carried out in two or more technical replicates except for F plasmid sample. Detailed Methodology is available in the

Estimation of ccd Specific mRNA Levels Using qRT-PCR

For qRNA isolation and quantification, 3 mL of culture was grown for each of the mutants of ccdA transfomed in E. coli Top10 gyr strain. Cells were grown to saturation under shaking conditions at 37°C, 180 rpm, pelleted and total RNA was extracted by the RNAsnap method (Stead et al. 2012). Chromosomal and plasmidic DNA was removed by treatment with 2 units of DNase1 (New England Biolabs) for 2 h at 37°C, followed by Sodium Acetate-ethanol precipitation of RNA. RNA was quantified by nanodrop spectrophotometric estimation, and quality was assessed by agarose gel electrophoresis (samples with A260/A280 = 2, were used for further studies), prior to downstream processing. 2 μg of total RNA was taken from reforming. To this mix, 200 units of SuperScript III Reverse Transcriptase (Invitrogen) were added, along with dNTPs, RT Reaction Buffer and 25 units of Ribonuclease Inhibitor following the manufacture’s protocol. The reaction mix was incubated for 60 min at 37°C. cDNA was directly used for quantitative PCR (Q-PCR) analysis. Q-PCR was set up with ccdA as well as ccdB gene specific primers (250 nM) using the cDNA template and Bio-Rad iQ SYBR Green Supermix (×1) in 20 µL total reaction volume, in a Bio-Rad iQ5 machine. The thermocycle parameters used for the Q-PCR were initial 95°C for 10 min followed by 95°C for 30 s, 56°C for 30 s and 72°C for 30 s in 40 repeat cycles. 16S rRNA was used as the internal positive control for total RNA quantification. Reactions with no reverse transcriptase as well as no template were used as a negative control.

Threshold cycle of amplification in PCR reaction was analyzed and automatically provided by the Bio-Rad iQ5 Optical System Software Version 2.1. All reactions were

Fitness (w) Estimation

Fitness was calculated as previously described (Bailey et al. 2014; Lebeuf-Taylor et al. 2019) using the formula,

\[
\text{Fitness (w)} = \frac{\text{Survival score of WT}}{\text{Survival score of a mutant i}}
\]

ES\textsubscript{seq} is a measure of the enrichment (growth advantage) of a mutant relative to the WT construct in the sensitive strain. The ES\textsubscript{seq} scores were calculated for all mutants in the library that had >20 reads in the resistant strain, separately for each replicate. ES\textsubscript{seq} values used in the work are the average of the three replicates.

For each residue in the CcdA protein, reads for all 32 possible mutant codons (NNK codons) were analyzed in the sensitive versus resistant strain, and phenotypes were assigned in terms of the ES\textsubscript{seq} scores. Positions showing codon specific mutation effects were analyzed separately.

In Vivo Activity of Individual Single-site CcdA Mutants

Selected single-site synonymous mutants of CcdA (in pUC57-ccdB construct) were designed and synthesized from GenScript and sequence confirmed (see Table 1). 40 ng of each plasmid was individually transformed into the sensitive and the resistant strains and plated on LB-amp agar plates in ten-fold serial dilutions and grown at 37°C for 16 h. The number of CFUs for the transformants in Top10 strain obtained in different dilution plates was counted to confirm their activity. For easy readout, serially diluted transformation mix samples were also spotted using 3 µL volumes per spot to visualize differences among mutant phenotypes.

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Supplementary Methods, Supplementary Material online. The ratio of CcdA: CcdB protein levels were determined for each replicate and the mean values with standard deviations have been reported.

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\[
\text{Fitness (w)} = \frac{\text{Survival score of WT}}{\text{Survival score of a mutant i}}
\]

Where total # of reads refers to all single mutants and WT reads identified in a sample.

Supplementary Methods, Supplementary Material online. The ratio of CcdA: CcdB protein levels were determined for each replicate and the mean values with standard deviations have been reported.

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Threshold cycle of amplification in PCR reaction was analyzed and automatically provided by the Bio-Rad iQ5 Optical System Software Version 2.1. All reactions were
carried out in triplicates. The negative control experiments had Ct values in the range of 29–31.

Computational Prediction of mRNA Secondary Structure
The initial 150 bp of the ccdAB transcript were submitted to mFOLD (Zuker 2003) and RNAfold (Gruber et al. 2008) for prediction of secondary structure. All energy parameters were set at default values. Detailed output was obtained in the form of structure plots with reliability information, single strand frequency plots, and energy dot plots. Local mRNA secondary structural elements



Relative codon usage (RCU) = \frac{\text{CUF of mutant codon}}{\text{CUF of most common codon of mutant amino acid}} \div \frac{\text{CUF of WT codon}}{\text{CUF of most common codon of WT amino acid}}

The CAI, CAI for WT CcdA sequence and all single-site mutants were calculated using a Python implementation of CAI (Lee 2018).

The tRNA abundance raw data (Dong et al. 1996) depicts the abundance of tRNA molecules in an E. coli cell encompassing the region of synonymous inactive mutations were further analyzed.

Calculation of Parameters Describing Various DNA Sequence Features
The CUF of E.coli K12 strain (Nakamura et al. 2000) (based on 14 coding gene sequences) was used in the study since the Top10 strain is a derivative of the K12 strain. These values of CUF in K12 was also found to correlate very well (r = 0.98) with the E. coli genome CUF table at the GenScript website (https://www.genscript.com/tools/codon-frequency-table). The CUF fraction was used to calculate the RCU for mutants, using the following formula:

\text{Relative tRNA abundance} = \frac{\text{tRNA abundance of mutated codon}}{\text{tRNA abundance of WT codon}}

The ribosome stalling data (Chevance et al. 2014) describes the observed averaged pausing of ribosome on each codon at the A-site for all genes in E. coli. The ribosomal pausing was estimated by introducing each codon individually into a 6X-His leader sequence of the histidine operonic system and quantified by downstream HisD-LacZ fusion protein (β-lactamase) activity. For the current work, the relative ribosome stalling for each mutation was calculated by dividing the mutant codon pause value by the WT codon pause value.

The relative GC content for mutations was calculated by dividing the fractional GC content of mutant codon by that of WT codon. The number of base changes is the number of positions in a 3 bp codon where the mutant codon varies from the WT codon.

To investigate if there is any significant difference in the distribution of these sequence parameters across the inactive and active classes of mutants, we chose to use the nonparametric Mann–Whitney test since our data is ordinal and does not follow a normal distribution.

Calculation of Anti-SD Interaction Energies for Synonymous Mutants in ccd mRNA
The difference in the interaction energy between single synonymous mutants in ccdA and the WT sequence, with the consensus anti-SD (aSD) sequence (5’ CCUCCUAU 3’), was calculated for a window of eight nucleotides using the RNAsubopt program from the RNA Vienna package 2.4.3 (Gruber et al. 2008). The calculations were performed for ten such windows encompassing the mutant codon. The average energy difference for each mutant across these ten windows was compared for all the available synonymous active and inactive mutants. Such aSD like sequences are known ribosomal pause sites (Li et al. 2012).

The binding energies for different mutants at the RBS of CcdB in the ccdA gene (residue positions 70 and 71) to the aSD sequence (5’ CCUCCUAU 3 ’) was also calculated similarly, to look for possible ccdA mutants (at residue positions 70 and 71) that can alter the translation initiation of CcdB.

YSD of CcdA Library and Single Mutants to Probe CcdB Binding
The CcdA libraries, individually recovered both from the resistant and the sensitive strains, were amplified from the pUC57-ccdAB plasmid vector and recombined into the YSD vector, pETcon (Addgene plasmid # 41,522) between Ndel and Xhol sites, using yeast homologous recombination in S. cerevisiae EBY100 strain. Positive clones, sufficient in number to cover the library diversity, were obtained on selective SDCA plates. The transformant pools obtained were grown in liquid SDCAA media for 48 h and stored in aliquots of 10^6 cells per ml in SDCA media containing 20% glycerol at −70°C. The same methodology was used for cloning WT and individual synonymous
mutants of CcdA into pETcon. The CcdA coding region was cloned as a C-terminal fusion to the yeast Aga2p protein with a C-terminal c-myc tag under control of the GAL promoter. The library was displayed on the yeast surface as described (Chao et al. 2006). Cells were induced and grown in SGCA media containing 2% galactose at 30°C for 16 h. The surface expression of CcdA was monitored by incubation of 10^6 cells with anti-c-myc antibody raised in chicken (1:400 dilution) that binds to the Myc tag at the C-terminus of CcdA. Anti-chicken IgG conjugated AlexaFluor-488 (1:300) was used as the secondary antibody. CcdB binding to the surface expressed CcdA was assessed by incubating cells with a fixed concentration of 2 nM biotinylated CcdB (or varying concentrations between 0.1 pM and 200 nM for titration experiments) and binding of streptavidin conjugated AlexaFluor-633 secondary antibody (1:2,000) to biotinylated CcdB was monitored. All the primary and secondary antibodies were obtained from Invitrogen. Labeled cells were analyzed on a BD FACSARIA III. The titration curves were fit to a single ligand binding model to obtain the dissociation constants as described previously (Rathore et al. 2017; Chandra et al. 2021). Double plots for mean fluorescence intensities for both expression and binding for the CcdA library as well as for single synonymous mutants were analyzed to monitor differences with respect to the WT.

Monitoring the Effect of Single Synonymous Mutations in the RelBE Operon

Design of Mutants
The WT sequence for the RelBE operon from E.coli K12MG1655 strain was retrieved from the NCBI database. The entire transcription unit of 910 bp (Bech et al. 1985) including another gene relF, which is expressed as a part of the same operon, was cloned in the EcoRV site of the pUC57 vector at GenScript. Five different constructs having synonymous mutations in the relB gene were synthesized with a stop codon in the relE toxin gene to ensure normal cell growth even after inactivation of the antitoxin, for ease of cloning. Three base synonymous substitutions are only possible with serine codons. Three base synonymous substitutions with varying codon preferences were made in three of the serine codons, S3, S16, and S28, all at the N-terminal half of the relB coding sequence. One representative two base substitution at position R7 was also tested. A single base substitution at the D77 residue was made to increase the strength of the SD sequence for relE and was used as a positive control mutant. To obtain an easy phenotypic readout, the introduced stop codon in the relE gene was reverted, using the inverse PCR strategy described earlier (Jain and Varadarajan 2014).

Phenotype Testing
Due to absence of a strain that is resistant to toxin action, we employed a toxin deletion strain (E. coli BW25113 ΔrelE) for the propagation of mutants. By virtue of this deletion, the WT chromosomal operon is de-repressed, leading to an excess of antitoxin in the cell, relative to the WT strain (E. coli BW25113 ΔrelE). The inverse PCR products for individual mutants were phosphorylated and ligated, as described previously. Ligation mixes were individually transformed into the E. coli BW25113 ΔrelE strain, to recover the plasmid. Single clones were isolated, and sequence confirmed. Equal amounts of the mutant plasmids (~30 ng) were transformed in E. coli BW25113 WT strain (having chromosomal relBE genes intact) to test their phenotype, transformation mixes were plated on LB-amp agar plates in ten-fold serial dilutions and grown at 37°C for 16 h. The number of CFUs for the transformants in E. coli BW25113 WT strain obtained in different dilution plates was counted. Serially diluted transformation mix samples were also spotted using 2 μL volumes per spot to visualize differences among mutant phenotypes.

Supplementary Material
Supplementary data are available at Molecular Biology and Evolution online.

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Author Contributions
K.G. prepared the SSM library. S.C. carried out all the experiments except for the quantitative proteomics (S.V.M. and H.G.) and the experiments with RelBE system (P.K). S.K. processed the deep sequencing data. A.A helped in acquiring FACs data. R.V, S.C. and K.G. designed the experiments, analyzed the overall data and wrote the manuscript with critical inputs and review from all other authors.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability
The data relevant to the figures in the paper have been made available within the article and in the Supplementary Information section, or are submitted to GitHub. The deep sequencing data are submitted in NCBI SRA database and to be released upon acceptance of manuscript (BioProject ID: PRJNA795874). The deep sequencing read counts, calculated Fitness Scores as well as values for codon usage, tRNA abundance, GC content, number of base changes, ribosome stalling, predicted binding energies for DNA interactions with SD and aSD sequences and all data generated in experiments such as YSD, Q-PCR, proteomics are available in GitHub (https://github.com/rvaradarajanlab/CcdA-Operonic-Data.git). The raw quantitative proteomics data is available at https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/PASS_view?identifier=PASS01727, Password: EV7788uz. All materials generated in this study are available from the lead contact (varadar@iisc.ac.in) without restriction.

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