Limited specificity of molecular interactions incurs an environment dependent fitness cost in bacteria

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22 Abstract

23 Reliable operation of cellular programs depends crucially on the specificity of biomolecular 24 interactions. In gene regulatory networks, the appropriate expression of genes is determined through 25 the specific binding of transcription factors (TFs) to their cognate DNA sequences. However, the large 26 genomic background likely contains many DNA sequences showing similarity to TF target motifs, 27 potentially allowing for substantial non-cognate TF binding with low specificity. Whether and how non-28 cognate TF binding impacts cellular function and fitness remains unclear. We show that increased 29 expression of different transcriptional regulators in Escherichia coli and Salmonella enterica can 30 significantly inhibit population growth across multiple environments. This effect depends upon (i) TF 31 binding to a large number of DNA sequences with low specificity, (ii) TF cooperativity, and (iii) the 32 ratio of TF to DNA. DNA binding due to the limited specificity of promiscuous or non-native TFs can 33 thus severely impact fitness, giving rise to a fundamental biophysical constraint on gene regulatory 34 design and evolution.

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36 Introduction

37 Biology at all levels crucially depends on the timely recognition and interaction between cognate 38 biomolecules (Box 1A). The importance of specificity of molecular encounters in the cell is highlighted 39 by the intricate mechanisms that ensure appropriate, and thus specific interactions, a classic example 40 being kinetic proofreading in the loading of amino acids onto tRNAs (1). In gene regulation, 41 transcription factors (TFs) determine the expression of genes at the right time and place by binding to 42 their respective operator sites in a highly specific manner. Additionally, non-cognate interactions can 43 occur due to non-specific TF-DNA interactions or due to specific binding (i.e. recognition of a TF-44 specific DNA motif) at non-cognate sites (Box 1). These interactions are not necessarily detrimental, 45 as non-specific DNA binding was found to speed up the TF search process through sliding along the 46 DNA (called "facilitated diffusion") (2,3). Upon encounter of the target sequence, conformational 47 changes can then increase binding specificity (2). In prokaryotes, many transcriptional regulators 48 appear to be non-specifically bound to DNA most of the time (3,4) and this state likely plays an 49 important role in gene regulation (5-7).

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51 These non-specific TF-DNA interactions are likely weak and transient. However, non-cognate TF-52 DNA interactions can also arise from recognition of a certain DNA motif or conformation, which makes 53 them more specific and therefore stronger (Box 1). As TFs have to search for their 'correct' DNA 54 targets among an extensive genomic background, they might encounter many non-cognate sites with 55 sufficient target sequence similarity, thus potentially trapping TF molecules (2,8). TF binding to such 56 non-cognate sites could thus incur substantial fitness consequences for the cell through i) high-57 specificity binding to few DNA sites with high similarity to the target sequence ; or ii) low-specificity 58 binding to many sites across the genome that accrues to a large overall effect. While in i) the fitness 59 effect is more likely to stem from regulatory interference, in ii) also changes in chromosome structure 60 could play a role. In support of the latter, growth arrest has been found with overexpression of one of 61 the nucleoid-associated proteins (NAPs) of E. coli involved in DNA organization, H-NS (9), which 62 binds DNA preferentially at AT-rich and curved regions (10). 63

64 In eukaryotes, which have shorter operator sites, low-specificity non-cognate binding of TFs seems to 65 be an integral part of gene regulatory function and evolution (11,12). On the other hand, in a recent 66 theoretical study that explored the impact of non-cognate binding we suggested that genomic low-67 specificity sites impose the existence of a global biophysical constraint, termed "crosstalk" (13). Some 68 forms of TF cooperativity and combinatorial regulation can limit this problem (13), but additional 69 mechanisms, such as out-of-equilibrium proofreading mechanisms, may be at work as well in 70 eukaryotes (14,15). In prokaryotes, the importance of non-cognate binding with low specificity has 71 rarely been investigated, although it has been acknowledged in regulatory interference (16), however 72 experiments exploring its fitness impact in general are lacking entirely.

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74 Results

75 Experimental setup

We wanted to test the consequences of non-cognate TF binding on bacterial fitness by expressing TFs lacking known cognate binding sites in native and non-native host cells. For this purpose, we chose phage repressors, which coexist for long periods at low numbers in bacterial hosts during the lysogenic cycle. We expect these repressors to be adapted to their host's genomic background – as temperate phages likely spend most of their existence as lysogens (17) – but potentially not to that of 81 another host. Therefore, we use repressors from different lambdoid phages, but from the same 82 protein family, three of them native to E. coli (λ cl, 434 cl, HK022 cl) and one to S. enterica (P22 c2) 83 and explore their effects on growth and fitness in both bacterial hosts. A bacterial repressor, Lacl, 84 served as a control in our experiments, as it is known to have only a single cognate target region in E. 85 coli, at the lac operon (18). Further, LacI uses facilitated diffusion for target search (3), which means it 86 should have very weak non-specific binding tendencies that should not affect fitness. Note, that all 87 four phage transcriptional regulators can function as either repressors or activators depending on the 88 promoter they bind to.

89 The five repressors were each cloned under the control of an aTc-inducible promoter (P_{tet}) onto a low 90 copy number plasmid (Fig. 1A). The plasmids were then transformed into E. coli and S. enterica cells, 91 whose genomes do not contain any of the phage operators, nor the three lac operators. The phage 92 repressors bind to their cognate operator sites in the phage genome as a dimer, but they can also 93 bind cooperatively to adjacent operators or form short- and long-distance loops (up to 10kbp for λ CI 94 (20)) involving two to four dimers (21) (Fig. 1A). This type of TF cooperativity was previously found to 95 facilitate non-cognate binding (22). Notably, for λ CI significant non-cognate binding has been 96 predicted theoretically (22,23) and substantiated experimentally (24), whereas for P22 C2 binding was 97 lost at few mismatches with the target motif (24), making it a less likely non-cognate binder (Box 1B). 98 Lacl on the other hand, is a tetrameric protein and cooperativity stems from the fact that one 99 repressor tetramer can bind to two different DNA sites simultaneously, resulting in looping (25), as 100 opposed to cooperativity resulting from binding of two different repressor molecules (Fig. 1A), which 101 Lacl lacks. We compared the impact of potential low-specificity non-cognate interactions of the five 102 different TFs by measuring growth of E. coli and S. enterica in various environments over a 10h 103 interval as a fitness proxy.

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105 **Repressor-induced growth reduction depends on growth medium and induction timing**

106 Reduction in growth was quantified as the normalized difference between growth in the presence and 107 absence of repressor (with 1 indicating complete cessation of growth and 0 representing wildtype 108 growth, see Methods). We observed a wide spectrum of growth behaviors across the five repressors 109 and the two bacterial hosts. For cells grown in minimal media (M9) with glucose (the standard media 110 used if not specified otherwise), the presence of λ CI resulted in a strong reduction of growth in *E. coli*

111 and an even more substantial reduction in S. enterica cells (Fig. 1B-D, Table S1). 434 CI also 112 reduced growth in both hosts, though less than λ CI, and interestingly more in its native host, *E. coli* (Fig. 1B). P22 C2 on the other hand, showed no effect in its native host S. enterica, while stopping 113 growth completely when expressed in E. coli (Fig. 1D, Table S1). There was no significant impact on 114 115 growth in either host with HK022 CI or with Lacl (Fig. 1B), which was expected for the latter - at least 116 in E. coli. Further, no growth defect was seen with our other controls: cells with only plasmid 117 backbone or the control plasmid expressing a fluorescence marker instead of a repressor (Fig. S1A). Thus, four different repressors stemming from the same TF family, but likely having different modes of 118 119 DNA recognition (26), showed a broad spectrum of growth effects in the two different bacterial host 120 species. We explored these growth effects and their causes further by focusing on the two best-121 characterized ones, λ CI and P22 C2, which are known to have different propensities for binding at 122 DNA sequences far away from their target motif (24) (Box 1B). 123 124 As a next step, we varied the environmental conditions in which bacteria carrying λ CI or P22 C2 were 125 grown. In rich media (LB), growth inhibition was abolished almost entirely in E. coli for both

repressors, and substantially reduced with λ CI expressed in *S. enterica* (Fig. 2A, Table S1). Minimal media supplemented with casamino acids and glucose resulted in intermediate growth reductions between rich and poor media (Fig. S2A, Table S1). P22 C2 did not affect growth in *S. enterica* in any of the conditions (Fig. 2A, Table S1), which is why this combination is generally not discussed further.

131 Next, we tested the dependence of the growth reduction on repressor concentration and induction 132 timing. In E. coli, decreasing the concentration of either repressor showed a gradual recovery of normal growth (Fig. 2B, Table S2). Conversely, even low expression of λ Cl in S. enterica resulted in 133 134 strong growth reductions (Fig. 2B, Table S2). The concentrations used here (see Methods for 135 measurement details) range from 0.5-5 fold of those achieved under physiological lysogen conditions (27). Surprisingly, the induction time point was also an important determinant for λ CI-induced growth 136 137 reduction, not however for P22 C2-induced ones: while λ CI induction in early- and mid-exponential 138 growth (as opposed to induction during the lag phase) had progressively smaller effects on growth in 139 E. coli and S. enterica (Fig. 2C, S2B,C, Table S3), this was not the case for P22 C2 in E. coli, where 140 growth was always halted ~2h after repressor induction (Fig. S2D, Table S3). Overall, we found a

strong dependence of repressor-induced growth reduction on environmental conditions and repressorconcentration.

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144 Increased repressor expression leads to severe fitness reduction

145 As the severe growth reductions we observed made it difficult to determine meaningful growth rates in our system, we determined the fitness effect of repressor expression in direct competition 146 147 experiments, which reflect all growth differences between the competitors. As a 'neutral' competitor, 148 we used cells expressing LacI from a plasmid construct that contained an additional YFP-venus 149 marker (Fig. 3A). The venus marker resulted in a minor fitness cost (selection coefficients for cells 150 without the marker were 0.05 (E.coli) and 0.09 (S.enterica), see Methods), meaning that an increase 151 in fluorescence (i.e. LacI-carrying cells) indicates an even more pronounced benefit of the LacI-152 carriers than measured. 1:1 mixtures of cells with phage repressor- and Lacl-carrying plasmids were 153 grown in minimal media (Fig. 3A, Methods) and fluorescence was compared between cell mixtures 154 grown without the repressors (no fitness difference; baseline fluorescence) and cell mixtures induced 155 for repressor expression (potential fitness cost of phage repressors over Lacl) (28). In accordance 156 with the experimental results from Fig. 1B-D, expression of repressors led to a significant increase in 157 Lacl-expressing cells, except for competitions with P22 C2 in S. enterica (Fig. 3B,C, Table S4). 158 Growth reductions translated directly into fitness costs as the competition assays were even able to 159 capture the gradual increase in growth reduction with increasing repressor concentration for λ CI in S. 160 enterica (Fig. S3, Table S4).

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Growth reduction is caused by cooperative, low-specificity binding distributed across the genome

Given the surprisingly detrimental growth effect of the two repressors in several environments, we set out to determine its cause. Transcriptional repressors are DNA-binding proteins and could therefore interfere with the cellular program through DNA binding at various non-cognate sites (29). To determine the role of TF binding in the observed growth reductions, we used the fact that λ CI is one of the best-studied TFs, and thus an exhaustive range of mutants for most of its functions exist. As neither of these mutants have been characterized for any of the other repressors, we only performed these experiments with λ CI.

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172 Specifically, we tested the expression of a mutant that cannot form dimers (30) (as λ CI only binds 173 DNA in its dimeric form (31)), as well as of a mutant defective in DNA binding (32), and found that 174 normal growth (Fig. 4A, Table S3) and fitness (Fig. S3) were almost completely restored in E. coli as 175 well as in S. enterica cells. Similar results for a λ CI mutant defective in cooperativity between 176 repressor dimers (Fig. 4A, Table S3) suggest an important contribution from DNA looping or some 177 other form of repressor oligomerization. This is intriguing as λ CI cooperativity and oligomerization are 178 thought to increase binding specificity (25,33), but likely lead to a general increase in binding strength, 179 particularly in the absence of specific sites. We ruled out that repressor misfolding or aggregation was 180 responsible for our observations by over-expressing a chaperone gene (tig) together with the 181 repressors (Fig. S1B).

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183 Hence, the ability to bind DNA, potentially in a cooperative and motif-dependent manner, seems to be 184 central to repressor-mediated growth effects. We tested this hypothesis by combining λ CI 185 cooperativity with the binding specificity of another repressor, using chimeric TFs. Specifically, we 186 replaced the DNA binding helix of λ CI (see Methods) with: i) that of another phage repressor, 434 CI, 187 which showed some growth defect; and ii) the bacterial repressor, Lacl, which showed no growth 188 defect as a wildtype protein (Fig. 1B, Table S5). It has been reported that changes in the geometry of 189 434 CI cooperativity strongly interfere with its binding affinity and the structure of the TF-DNA complex 190 (21,34), which indeed in our experiments resulted in rescue of growth with the λ -434 CI chimera. In 191 contrast, with the λ CI-Lacl chimera the growth reductions were even stronger than with λ CI, leading 192 to growth arrest in S. enterica in rich and minimal media (Fig. 4B, Table S5). This opposing behavior 193 of Lacl and λ CI-Lacl strongly supports our hypothesis that Lacl binding affinity and basepair bias are 194 conducive to low-specificity binding (Box 1B), but it is lacking the strong intermolecular cooperativity 195 and oligomerization potential of λ CI (Fig. 1A). The chimera, however, combines these attributes, 196 leading to strong interference with cell growth.

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198 In order to determine if the non-cognate binding effects involved (i) a few essential, or (ii) many

199 distributed, regions of the chromosome we performed ChIP-sequencing for λ CI in *E. coli* and *S.*

200 *enterica*. In *E. coli* the data did not reveal strong peaks for any genomic site, but rather indicated weak

201 binding at numerous sites all over the chromosome (Fig. 5A, S4A, Table S6). Note that all of the 202 regions plotted in Fig. 5A are significantly enriched in the presence of λ CI, but they only appear at a 203 more lenient cutoff than typically used for strong binding (see Methods). In S. enterica we found both, 204 distributed weak binding as well as a broad peak (indicating substantial binding in several adjacent 205 genes). Interestingly, this broad peak corresponds to prophage regions on the genome that seem to 206 provide binding hotspots for λ CI (Fig. 5B, S4B, Table S6). As such a binding hotspot was absent in E. 207 coli, but λ CI still showed a growth defect, we did not consider this finding necessary to qualitatively 208 explain our results (although it could account for the stronger growth defects seen in S. enterica). 209 Further, none of the apparent peaks for either genome encoded a gene that is essential or obviously 210 beneficial in minimal media conditions.

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212 Using a simple thermodynamic model to predict λ CI binding across the bacterial genome we found a 213 surprisingly high degree of correlation with the number of reads from ChIP-sequencing (Fig. 5C,D), 214 especially given that these models generally perform poorly for low affinity sites (35). Even more 215 surprising, we found a comparable prediction (Fig. 5C,D, S5) with an energy matrix that conserved 216 only the overall λ CI preference for the basepair composition (see Methods). This result could not be 217 explained by nucleotide composition bias in the ChIP-sequencing experiments (Fig. S6), but shows 218 that a large part of the correlation between predicted binding and ChIP-sequencing reads can be 219 explained by the overall genomic basepair bias. Correct basepair composition bias in the DNA 220 sequences could provide λ CI with sufficient recognition pattern to bind with low specificity. In 221 agreement with this hypothesis, the GC bias of the stronger λ CI cognate operators (O_{R1} , O_{R2} , O_{L1} and 222 O_{L2}) is 52.94%, which is very close to that of the S. enterica genome (52.2%) and only slightly higher than that of the E. coli genome (50.8%). However, the residual sequence-dependent contribution 223 224 beyond the basepair composition bias is still highly significant in *E. coli* and weakly significant in S. 225 enterica (as determined by Monte-Carlo permutation tests for significance; Fig. 5C,D, S7). Overall, 226 our results indicate substantial non-cognate binding due to sequence-dependence and basepair bias, 227 which has been also reported, for example, for NAPs (9,10). Non-cognate binding is facilitated by 228 repressor oligomerization (22,36), and distributed over the thousands of low-specificity λ CI binding 229 sites, known to be present in the E. coli genome (29). These findings agree with previous studies on 230 non-cognate binding of λ CI and other prokaryotic TFs (2,3,23,37).

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232 Low-specificity binding leads to arrest of cell division

The distributed non-cognate DNA binding demonstrated by the ChIP-sequencing data for λ CI is in 233 agreement with the observation that increasing concentrations of repressor gradually increases the 234 235 magnitude of the growth reduction seen in Fig. 2B. Additionally, the dependence on growth media and 236 induction timing indicates that DNA concentration - or rather the ratio between repressor and DNA -237 might play a role. If cell doubling time is slower than the time needed for DNA replication and cell 238 division (~60min. in E. coli (38) and ~50min. in S. enterica (39), which is close to our observed 239 doubling time in minimal media: ~63min. and ~58min. respectively), each bacterial cell contains on 240 average only one chromosome. At faster growth, replication cycles are overlapping and daughter cells 241 inherit 2-8 origins at birth, together with partially replicated chromosomes (38). Hence, the richer the 242 medium and the faster the growth, the more DNA will be available (~2-fold for rich versus minimal 243 media in our experiments) to titrate away potentially detrimental non-cognate binding TFs. In 244 agreement with previous studies (40), the number of proteins at fast and slow growth were very 245 similar (see Methods: Protein quantification), leading to a decreased protein concentration in rich 246 media as cells become larger (this is particularly true for proteins expressed from plasmids (40)). 247 Similarly, cells that are induced during the lag or early-exponential phase (after 1-2 doublings) will only have on average one chromosome as they did not inherit partially replicated chromosomes from 248 249 their mothers and grandmothers yet.

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251 Thus we set out to test the titration hypothesis by introducing a high copy number plasmid carrying 252 four cognate λ CI binding sites into *E. coli* cells with inducible λ CI (Fig. S8A), which should reduce the number of free λ CI dimers available for non-cognate binding by about one half (see Methods). 253 254 Although the expression of λ CI was still detrimental, growth was ~20% faster than for cells without 255 the operators (Fig. S8B). Hence, titration of λ CI alleviates the growth reduction – likely even more so 256 if additional chromosomal DNA is present (e.g. at faster growth), which provides many more potential 257 binding sites with low specificity (29). For P22 C2, which is more discerning in its DNA binding targets 258 (24), partially replicated chromosomes would provide less titration, thus explaining why later induction 259 does not rescue growth. The titration phenomenon is reminiscent of growth bistability in drug resistant 260 bacterial cells, which is caused by feedback between the growth rate and the speed of counteracting

toxic agent (41). In our system, the repressors can be seen as 'toxic agents', which are 'counteracted' by dilution if cells manage to start growing, or are growth-arrested if they are not able to dilute
the repressors fast enough.

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265 The titration hypothesis together with our ChIP-sequencing results implies that the overall ratio of 266 chromosomal DNA to repressor proteins is a crucial factor determining the growth effects. This 267 suggests that non-cognate binding might interfere with global cellular functions, like DNA replication 268 or cell division, which we investigated using fluorescence microscopy of *E. coli* cells expressing λ CI. 269 First, we imaged cells expressing a SeqA-GFP fusion protein, which is an indicator of replication fork 270 progression (42). λ CI-expressing cells generally formed long filaments, suggesting an inhibition of 271 cell division in these cells, even though the numerous fluorescent dots revealed ongoing replication 272 (Fig. 6A, S9). Most filamentous cells showed low induction of the stress response promoter PsulA 273 (Fig. 6B, S10), which is unlikely to induce sufficient self-cleavage (i.e. inactivation) of the repressor 274 molecules (43) - particularly because λ CI becomes a poor substrate for self-cleavage at higher 275 concentrations (44) - or to inhibit cell division substantially (45). Cell division can also be hindered by 276 the presence of DNA at mid-cell (46). Fluorescence microscopy of cell membrane and DNA shows 277 that in many filamentous cells DNA is located mid-cell, often on top of the established division septum 278 (Fig. 6C, S11). This suggests that it is not FtsZ-ring formation, but a subsequent step in the cell 279 division cascade that is disrupted. However, some filamentous cells manage to divide after growing to 280 substantial length, as FtsZ-ring formation starts to occur at quarter points (47). Indeed, 281 overexpression of FtsZ together with λ CI rescues growth entirely (Fig. S12), likely by forming 282 additional, non-central division septa, which could produce viable cells as filamentous cells often contain additional chromosomes, which are distributed across the cell (Fig. 6C). As the ftsZ operon 283 284 region was not enriched among ChIP-sequencing reads, it is unlikely that λ CI interferes with FtsZ 285 expression directly through transcriptional interference.

286

287 Discussion

We investigated the consequences of limited specificity in molecular recognition of DNA by proteins, using four different, but related, phage repressors and a bacterial repressor, which produced a wide range of effects on host cell growth, from high to no fitness costs, and higher costs either in the native

291 or the non-native host (Fig.1B, 4B). Taking advantage of the rich and well-established genetics and 292 biochemistry of the classic bacteriophage repressor λ CI, we found that its fitness cost results from cooperative, low-specificity binding, which interferes with growth by inhibiting cell division. The 293 294 abundance of low-specificity binding sites in eukaryotic genomes has been shown to play a critical 295 role in gene regulation, potentially increasing robustness and specificity (11). Our data, however, 296 support the hypothesis that binding strategies of prokaryotic TFs are under selection to avoid low-297 specificity binding to the genomic background (29) and highlight the fundamental differences in gene 298 regulatory design between prokaryotes and eukaryotes, and therefore differing evolutionary 299 constraints (48).

300

301 For prokaryotes – in contrast to eukaryotes – TF target sites are sufficiently long to allow specific 302 recognition of single operators (48). Mismatches with the preferred target sequence lead to 303 progressive loss of binding, but the speed of this loss can vary substantially between TFs (24). λ Cl, 304 which shows strong operator binding (offset), low mismatch penalties (energy matrix) and strong 305 cooperativity, is likely to be a rather promiscuous binder (Box 1B, (24)) and indeed induced a high 306 fitness cost due to distributed low-specificity binding. For P22 C2, the lower offset and higher 307 mismatch penalties make it a more specific binder (Box 1B, (24)), producing a significant cost only in 308 non-native host cells. Lacl, which shows similar binding characteristics as λ CI, only showed a strong 309 fitness effect when coupled with λ Cl's intermolecular cooperativity. Together, the mutant and chimera 310 experiments (Fig. 4) demonstrate a significant contribution of cooperativity - and likely oligomerization 311 - to the potential for low-specificity binding, which supports the theoretical finding that TF cooperativity 312 does not strongly alleviate crosstalk when it stabilizes cognate as well as non-cognate binding (13). Hence, the lack of intermolecular cooperativity with Lacl could be a sign of its adaptation to be highly 313 314 specific, as it is one of the few single-target regulators in E. coli (18). Binding cooperativity, offset and 315 TF concentration, can all serve to increase non-cognate binding of a TF (independently of the target motif preference) and the particular interplay between these factors has to be tuned by the cell to 316 317 avoid fitness costs due to low-specificity interactions. Therefore, considering low-specificity binding is 318 crucial in choosing TFs for synthetic biological systems in order to avoid global toxicity effects, as well 319 as unwanted TF titration, which can affect target gene regulation (16,49).

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321 The magnitude of the fitness cost depends on a repressor's ability to bind non-cognate DNA at low 322 specificity, as well as on a repressor's relative ratio to the total amount of DNA within the cell. Slow cell growth compounds the effect as cells contain less DNA but accumulate more proteins than at fast 323 324 growth (40). Additionally, stress tolerance could be higher under optimal growth conditions as found in 325 rich media (50). It does not seem likely, however, that media-specific genes are targeted, as ChIP-326 sequencing generally revealed distributed, low-specificity binding all over the chromosome (Fig. 5). 327 Rather, inhibition of cell division seems to result at least partially from nucleoid localization at mid-cell. 328 Clearance of the division site is impeded if sister chromosomes fail to segregate (46,51), which could 329 be caused by the formation of "bridges" between cooperatively bound repressors, holding the sister 330 chromosomes together. Generally, more compact nucleoids are more efficient at preventing the 331 formation of division septa in the same area (52). Intriguingly, a very similar growth effect has been 332 found with H-NS, one of the NAPs responsible for chromosome organization and compaction in E. 333 coli (9,10): H-NS overproduction drastically reduced cell viability, which seemed to be related to the 334 formation of higher-order H-NS oligomers (53) - a state that is favoring its ability to form bridges 335 between DNA regions (54). As cell growth, shape, division and DNA replication are thought to be 336 tightly linked in complex and poorly understood ways (55), a mechanistic explanation of the observed 337 division inhibition is presently not possible, but prior studies on H-NS combined with our findings 338 make the case for the existence of general constraints on DNA-binding proteins.

339

340 Our results suggest that the inherent ability of DNA-binding proteins to occupy non-cognate DNA 341 regions can pose, in addition to potential regulatory interference (16), a substantial challenge for host 342 cell fitness overall – particularly considering facilitating conditions like cellular crowding (56), horizontal gene transfer (57) and mutations that alter the binding specificity of a protein. This 343 344 challenge stems from the fundamental limits to molecular recognition that are set by the biophysics of 345 molecular interactions (13) and could lead to various non-cognate effects on the physiology of the cell. The five transcription factors used here show a variety of non-cognate effects, which could 346 347 indicate different selection pressures that have been acting on their binding and cooperativity 348 characteristics as well as different potential for being tolerated in a particular environment. There 349 might also be different mechanisms underlying the growth phenotype, as suggested by the ChIP-350 sequencing results for λ CI: the additional strong peak region found in S. enterica could indicate an

351 additional effect of high-affinity sites and explain the stronger growth defect as compared to E. coli 352 (especially at low repressor concentrations). A potential for high fitness costs, even in native environments, as seen here with λ CI and 434 CI, can limit the number and binding affinity of 353 354 promiscuous DNA-binding proteins in the cell (13). More specific binders such as P22 C2 might only 355 be detrimental in non-native environments. Hence, the influx of foreign genes through horizontal gene transfer could be considerably impaired through non-cognate binding effects, as both are likely to 356 357 occur under slow growth conditions. As the phage repressors we used originate from temperate phages, interference with host cell growth can limit their potential host range with regard to 358 359 successfully establishing lysogeny. Considering that phage repressor concentrations are kept low 360 during lysogenic cycles, the selection pressure to reduce low-specificity binding might generally be 361 weak, which would explain the diversity in fitness impacts we observed with related phage repressors. 362 More generally, experimentally uncovering the fundamental biophysical constraints imposed by low-363 specificity bindings of TFs is difficult, as TFs with many specific binding targets need to recognize a 364 diversity of sequences and by default affect many cellular functions, while single target TFs are a very 365 few (18). This is what ultimately motivated our choice of focusing our experiments on phage 366 repressors and Lacl.

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We experimentally demonstrated for the first time that low specificity in biomolecular recognition can 368 369 constitute a limiting factor for cellular function and evolution due to the fundamental biophysical 370 constraints on protein-DNA interactions. However, these costs could be counter-balanced by 371 increased TF robustness to target site mutations or higher evolvability, precisely because interactions 372 can be formed at low specificity (24,58). For example, a TF could co-opt regulation of a non-cognate gene - even if only to a small degree - that provides an advantage in a certain environment, which 373 374 can subsequently be refined by evolution. This opens up a wider question about the interplay of costs 375 and benefits of low-specificity molecular interactions, especially when these interactions also serve as 376 drivers of evolution.

377

378 METHODS

379 Plasmids and strains

The phage repressors λ CI, P22 C2, 434 CI, HK022 CI or the bacterial repressor LacI were cloned under the control of a $P_{LtetO-1}$ promoter in a low copy number kan^R plasmid (pZS)(59). The plasmids were then transformed into either MG1655 derived *E. coli* cells, which are deleted for the *lac* operon

- 383 (strain BW27785, CGSC#: 7881)(60), or into LT2 derived S. enterica cells with a tetracycline cassette
- inserted in the P22 attachment site (LT2 attP22::*tetRA*). In control experiments the phage repressor
- on the plasmid was replaced by a fluorescence marker gene (*gfp*). The low copy number plasmids
- used (pZS21) are under stringent replication control, which is linked to chromosome replication (61).
- 387 Titration of λ CI was tested by transforming *E. coli* cells containing the pZS21- λ *cI* plasmid with a
- 388 compatible, high-copy number pZE plasmid (50-70 copies)(59), which carries the natural λ CI
- 389 operators O_{R1}, O_{R2}, O_{L1} and O_{L2}, i.e. 200-280 operators per cell although the copy number of this
- 390 plasmid's ori was originally documented at 25-30 copies per cell (62), hence there might only be
- about 100-120 operators per cell. At 25ng aTc induction, there are about 500 λ CI dimers per cell (see
- below), which reduces the number of dimers available per cell either by half or by one fifth.
- 393 For the competition experiments we introduced a constitutive fluorescent marker *venus-yfp* (63) into
- the low-copy pZS plasmid carrying *lacl* (for a detailed description see below).
- In order to test for misfolding of repressor proteins, we used a high copy number plasmid containing a
- 396 chaperone gene (*tig* (64)), which is native to *E. coli* and *S. enterica*, under the control of a P_{Lac} 397 promoter from the ASKA(-) library (65).
- 398 To monitor induction of the stress response, we used a strain with a fast-maturing yellow fluorescent
- 399 protein (YFP)(63) fused to the promoter of *sulA* (*PsulA-yfp*), which was placed on the chromosome
- 400 using lambda red recombineering (66). *SulA* is strongly upregulated as a part of the stress response
- 401 (67). The *PsulA-yfp* strain was then transformed with the pZS21- *λ cl* plasmid. We checked induction
- 402 of the reporter by exposing cells to UV light for 30 seconds.
- 403 We used a SeqA-GFP translational fusion under the control of the natural *seqA* promoter to monitor
- 404 replication as SeqA binds hemi-methylated GATC sequences in the wake of the advancing replication
- 405 fork, marking newly synthesized DNA (42). In order to avoid unnaturally high seqA expression that
- 406 could influence replication progression, the fusion protein was inserted into the HK022 attachment site
- 407 on the *E. coli* chromosome using CRIM plasmids (68).
- 408 Additional expression of FtsZ, the major cell division protein, was performed by cloning *ftsZ*
- 409 downstream of λ *cl* as a transcriptional fusion (i.e. putting it also under the control of aTc induction).
- 410

411 λ CI mutants

- 412 Based on previous studies, we cloned three different λ repressor mutants into the same low copy
- 413 number plasmid (pZS) under the control of a *P*_{LtetO-1} promoter: (i) a repressor mutant that is defective
- 414 in its ability to bind DNA (N52D)(32); (ii) a repressor mutant that cannot form dimers (S228N)(30) and
- 415 hence not bind DNA effectively anymore; and (iii) a mutant that can dimerize but not form higher-order
- 416 oligomers, i.e. that cannot bind cooperatively (Y210N)(30). Mutations were introduced using site-
- directed mutagenesis. The function and stability of the proteins has been shown previously (30,32),
- 418 as well as that their production levels are not different from wildtype repressor (30).
- 419
- 420 λ CI chimeras
- 421 Chimeric λ CI repressors were constructed based on literature describing a chimeric λ CI-434
- repressor (69) and a chimeric 434-P22 repressor (70) by changing the recognition (i.e. DNA binding)

423 helix of λ CI to either the one of the 434 CI repressor or of the Lacl repressor. This was done by

- introducing the following changes for the λ CI-434 chimera: G44T, S46Q, G49E, A50Q; and for the λ CI-Lacl chimera: G44S, Q45Y, S46Q, G49S, A50R. (Note, that Q45 was not changed in the λ CI-434
- 426 chimera because both repressors contain the amino acid Q at this position.)
- 427

428 Growth measurements

- All cells were grown overnight at 37°C in M9 medium supplemented with 0.2% glucose and 50µg/ml
 kanamycin (except specified differently). Cultures were used to dilute (1:100) 6 replicates without
 inducer and 6 replicates with 25ng aTc in 96 well plates and were grown at 37°C under shaking at
 220 rpm. Populations were measured (OD₆₀₀) every 30min or every 60min using Biotek H1 plate
 reader for 10h. Population growth was also measured in LB, or M9 medium supplemented with 0.5%
- 434 Casamino acids and either 0.5% glycerol or 0.2% glucose. Where indicated inducer concentration
- 435 was changed to 1, 2, 3 and 4ng aTc which was chosen in a way that the concentration range was
- 436 covered as uniformly as possible, given that the *P*_{LtetO-1} promoter has a very steep induction curve
- 437 (59) and the induction time was varied from the inoculation time point (0h) to 2h or 4h post-
- inoculation (early- and mid-exponential phase). The chaperone gene was induced using 1mM IPTG
- and Fis-GFP was expressed by adding 0.1mM IPTG to the medium.
- 440 Growth reduction was measured as the normalized difference between the areas under the growth
- 441 curves (from the point of induction for 8.5h, i.e. including lag phase but not stationary growth) in the
- 442 presence and absence of repressor $\left(\frac{AUC_{no \ repressor} AUC_{repressor}}{AUC_{no \ repressor}}\right)$. Hence, a ratio of 0 corresponds to
- wildtype growth even in the presence of repressor and a ratio of 1 corresponds to no growth at all in
- the presence of repressor. We used the area between the growth curves as opposed to growth rates,
- because growth rates were hard to define under conditions with strong growth reductions: growth did
- no longer show an exponential increase and the calculated rate was strongly dependent on the
- specific time points used for determination. Furthermore, growth was generally slowing down over
- time, and the repressor binding effects were not strictly limited to the 'exponential' growth phase.
- Hence, a maximal growth rate would not capture all the effects and we chose to use a growth
- 450 measure that integrates over all of the growth phases and gives an impression of the general
- 451 decrease in fitness.
- 452

453 DNA quantification

The relative amount of DNA within the cells in rich and minimal media was determined using the Wizard® Genomic DNA Purification Kit. We grew cells in LB or M9 with glucose to an OD of 0.2-0.3 and extracted genomic DNA using the purification kit. The amount of DNA was quantified by nanodrop and normalized by OD, which gave a 2-fold higher amount of DNA in rich media than in minimal media. This is an estimate of the minimal difference in DNA concentration as cells are slightly bigger in LB than in M9- (Fig. S11).

460

461 Protein quantification

462 Repressor concentration was determined using the Promega Nano-Glo HiBiT Lytic Detection System. 463 The HiBiT peptide tag was attached at the N-terminal (which is involved in DNA binding) using a (GGGS)₂ linker (sequence: GGTGGTGGTTCTGGTGGTGGTTCT) to assure accessibility of the tag 464 465 for interaction with the detection reagent. Briefly, cells induced (1ng or 25ng aTc) for expression of wildtype repressor or repressor with the HiBit tag were grown to early exponential phase in minimal 466 467 media with glucose or rich media (LB), pelleted and frozen. Cells were resuspended in media and supplemented with 0.1 culture volume of PopCulture Reagent (Sigma Aldrich), 10⁻³ culture volume 468 Benzonase Nuclease (Sigma Aldrich) and 0.5*10⁻³ culture volume lysozyme (Sigma Aldrich). Cells 469 470 were lysed for 30min at room temperature and then kept on ice. A protein standard (Promega) was 471 added to the non-tagged cells as a known reference of protein concentration to luminescence output. 472 Samples were mixed 1:1 with HiBit enzyme mixture and measured in white plates after shaking (in the 473 dark) for 15minutes. Dilution series of tagged repressor and protein standard were measured in a 474 Tecan platereader (Spark 10M) with an integration time of 1.5 seconds. 475 Repressor protein numbers in minimal media with glucose gave about 500 dimers per cell at 25ng aTc and about 50 dimers per cell at 1ng aTc induction (as compared to ~125 dimers of λ Cl in 476 477 lysogenic cells (27)). Similarly, we found around 500 dimers per cell at 25ng aTc in rich media. Note,

478 that the fitness reduction seen for λ CI concentrations at >1ng aTc induction (Fig. 2B) does not

directly translate to lysogenic cell fitness as it is masked by phage induction, superinfection andsuperinfection exclusion (71) effects.

481

482 Competition assays

In order to distinguish between strains carrying a phage repressor (showing growth reduction) or the bacterial repressor Lacl (not showing growth reduction), we used the pZS plasmid carrying *lacl* (under the control of $P_{LtetO-1}$), together with a constitutively expressed fluorescence marker (*venus*) cloned in the opposite direction upstream of $P_{LtetO-1}$. Venus was expressed from a mutated version of P_R, which abolishes λ CI binding affinity in the O_{R1} operator (72,73) (the O_{R1} sequence used is:

488 TGCCTTAATACTGGATA) – and does not contain O_{R2} or O_{R3} - and is therefore constitutive

489 ('P_{constitutive}'). The fluorescence marker was placed on the Lacl-carrying plasmid because Lacl only had

490 a minor growth effect in *S. enterica* and none in *E. coli* (Fig. 4B, Table S5), which did not lead to

491 filamentation and hence did not affect fluorescence due to cell morphology changes. The lack of

492 growth reduction due to Lacl expression agrees with previous findings that GaIR (and possibly other

493 members of the GaIR family like LacI) seem to have evolved to have fewer low-specificity sites across

the chromosome (18,29). There was only a slight fitness cost due to the presence of the fluorescence

495 marker on the plasmid (leading to a selection coefficient of 0.05 and 0.09 in *E. coli* and *S. enterica* for

the Lacl-carrying plasmid without the marker over the one with the marker), which however only

497 strengthens our findings that LacI-expressing cells increase in competition with phage repressor-

498 expressing cells despite this cost.

499 A single colony for each host strain (*E. coli* or *S. enterica*) – plasmid (pZS21-*lacl*, pZS21- λ *cl*, pZS21-

500 P22 c2, pZS21- λ cl dimerization mutant, pZS21-lacl-P_{constitutive}-venus) combination was picked from a

501 freshly streaked plate and grown overnight in minimal media supplied with 0.2% glucose and 50µg/ml

- 502 kanamycin. Strains containing a phage repressor plasmid were mixed 1:1 with a strain carrying
- 503 pZS21-*lacl*-P_{constitutive}-venus, diluted 1:100 into fresh medium and grown in 96-wellplates for 10h.
- 504 Fluorescence was measured every 30min. and compared between cultures that were induced with
- 505 aTc (at concentrations as indicated, either 1, 2, 3, 4 or 25ng) and cultures that were not induced. This
- 506 means that we compared the abundance of fluorescent cells (i.e. abundance of Lacl-carrying cells)
- 507 between cultures expressing and not expressing the repressor.
- 508 Selection coefficients were calculated using $ln[(R^+t/R^-t)/(R^+0/R^-0)]$, where R^+t and R^-t represent
- 509 fluorescence measurements (as a proxy for relative LacI-expressing cell density) of cells with and
- 510 without inducer aTc (presence or absence of repressor expression) at time t=10h respectively, and
- 511 R⁺₀ and R⁻₀ represent fluorescence measurements at the beginning of the experiment.
- 512

513 Microscope fluorescence measurements

- A Nikon Ti-E microscope equipped with a thermostat chamber (TIZHB, Tokai Hit), 100× oil immersion
- 515 objective (Plan Apo λ, N.A. 1.45, Nikon), cooled CCD camera (ORCA-Flash, Hamamatsu Photonics)
- and LED excitation light source (DC2100, Thorlabs) was used for the microscopy fluorescence
- 517 measurements of PsulA-Yfp and SeqA-Gfp. The microscope was controlled by micromanager
- 518 (https://micro- manager.org). The cells were grown overnight in minimal media with glucose, diluted
- 519 1:100 in fresh media and grown to early exponential phase in the presence of the inducer aTc. YFP or
- 520 GFP fluorescence (where appropriate), RFP fluorescence (for image correction) and phase contrast
- 521 images were taken simultaneously at 3-min time-lapse intervals. Multiple patches of cells were
- 522 monitored in a single experiment. A custom macro of ImageJ (http://imagej.nih.gov/ij/) was used for
- 523 image analysis.
- 524 Imaging of cell membranes and DNA positioning was done using a Leica DMI6000B (inverted)
- 525 microscope with an Andor iXon EM CCD camera (front illuminated, 8x8 square micron pixel size) and
- 526 a 100x 1,47Na Oil HCX Plan Apo objective, giving an effective pixel size of 64nm/pixel. Images were
- 527 acquired using 405(20)nm and 561(10)nm laser excitation for blue (Hoechst) and red (NileRed) dyes
- 528 respectively. The cells were grown overnight in minimal media with glucose or LB, diluted 1:100 in
- 529 fresh media and grown to early exponential phase in the absence or presence of the inducer aTc.
- 530 After addition of both dyes (Hoechst at 10ug/mL and NileRed at 1ug/mL), cells were shaken at room
- 531 temperature for one hour and imaged in drops of the respective growth media. Images were
- 532 deconvolved using Huygens Professional (version 4.5) and further analyzed using ImageJ.
- 533

534 ChIP-sequencing

- 535 To perform ChIP-sequencing experiments, λ CI was cloned with an HA-Tag at the carboxy-terminal 536 end and transformed into both host strains. HA-tagged λ CI showed the same growth phenotype as
- 537 wildtype in both bacterial strains (Fig. S13). Samples from strains grown in the presence or absence
- 538 of λ CI were prepared according to Waldminghaus & Skarstad (2010)(74); library preparation and
- 539 Illumina Sequencing was performed at the VBCF NGS Unit (<u>www.vbcf.ac.at</u>). The obtained data was
- 540 analyzed using Galaxy and RStudio.

541 Peak calling was performed using custom R scripts modified from Santhanam et al. (75). Briefly, the 542 genome was computationally partitioned into non-overlapping shorter fragments, typically spanning a few kbs to account for local biases arising from sequence content and immuno-precipitation (76,77). 543 544 Peak calling was performed within these fragments using partially overlapping (50% overlap) windows of 100bp. For each window, we calculated strand-specific enrichment as the log-ratio of the scaled 545 546 read coverage between the sample and control ChIP-seg experiments while permitting a maximum of 547 5 reads to be mapped to the same genomic coordinates. We calculated strand-wise p-values for 548 enrichment by first resampling scaled read coverage within each fragment and then randomly 549 partitioning them to calculate enrichments. Finally, we identified bound regions to be those 550 with positive enrichment scores on both strands with a Benjamini-Hochberg false-discovery rate of 551 less than 30% as we were looking for binding of low specificity and ChIP-binding data was previously found to be highly informative for a wide range of specificity profiles (78). For the regions that showed 552 significant enrichment in this analysis we plotted the read-depth across the genome in Fig. 5 (A,B) 553 554 and for comparison we plotted the read-depth for not significantly enriched regions in Fig. S4. As 555 control for our ChIP-sequencing procedure and analysis we used antibodies against SeqA, which 556 gave the expected peaks as published previously (74). 557 We calculated the nucleotide composition of the sequences underlying enriched regions in ChIP-seq 558 data for both bacterial species (Fig. S6). In order to test for sequence composition bias in these 559 enriched regions, we sought to test if the sequence compositions of the enriched regions were significantly different compared to the rest of the genome. To this end, we randomly selected 50 560 561 genomic regions with at least 5kbp distance between them. We then calculated the nucleotide 562 composition of these randomly selected regions and by repeating this procedure 1000 times, 563 generated a null distribution for sequence composition of randomly selected genomic regions. 564 Similarly, we calculated the di-nucleotide composition (with 1 bp overlap) of the same randomly 565 selected genomic regions and compared it to that of the enriched regions. 566 The number of reads within 1000bp windows was compared with the predicted binding by calculating binding energy at each genome position (using a sliding window approach) from the λ CI offset (i.e. 567 568 the energy difference between the repressor being bound specifically to an operator and being free in 569 solution (79)) and the energy penalty as given by the λ CI energy matrix (73). Smaller energies result 570 in stronger binding, meaning positive energy penalties decrease binding affinity (note that negative penalties could increase binding over the one seen with λ CI wildtype operator sites). Binding strength 571 572 was calculated using $1/(1+\exp(E-\mu))$, with E being the calculated binding energy, as described above 573 and used in (24), and µ being the chemical potential, which we optimized to give the highest 574 Spearman correlation fit (2.6 in E. coli and 2 in S. enterica). For comparison with the number of ChIPsequencing reads, calculated binding strength was summed over the same genomic 1000bp regions 575 576 (considering binding to both strands). In Fig. 5 (C,D) we plot a non-parametric, non-linear relationship 577 estimate between the predicted binding energy and the ChIP-sequencing reads obtained from a 578 series of conditional medians. To investigate the dependence of the correlation between the affinity

- 579 predictions and the ChIP-sequencing reads on the structural versus the sequence information
- 580 contained in the energy matrix, we repeated the analysis with i) a matrix of the same size that

581 conserves only the ACGT bias of the λ CI energy matrix (each row contains the average value of that 582 row) or ii) matrices that had completely reshuffled entries. For the latter the average correlation was 583 taken over 100 permutations.

- 584 To assess the importance of specific sequence information versus nucleotide (GC) bias, we used a 585 Monte-Carlo permutation test: We calculated the difference between Spearman correlations of ChIP 586 reads with binding prediction using the wildtype energy matrix vs binding prediction using the energy matrix that only conserves λ CI basepair bias, for the true ChIP read assignment, and 10⁴ random 587 read assignments (null distribution). We found an overall strongly significant difference in E. coli and 588 589 lower significance in S. enterica (Fig. 5C,D), even though the effect size was small. This means that 590 while most of the measured ChIP signal can be accounted for by a TF model that predicts binding 591 based on the nucleotide content of genomic fragments alone, there is a small but highly significant residual ChIP binding signal that requires the full binding site preference (energy matrix), not just 592 single nucleotide bias, to be explained. Further, we examined the influence of GC content by 593
- repeating the Monte-Carlo permutation test for genomic sequences of a specific GC %. Here, we
- found only a significant motif contribution for the 49% bin in *E. coli* (Fig. S7).
- Additionally we used the offset and energy matrix for Lacl (25,80) and P22 C2 (81) to predict binding
- and calculate the Spearman correlation with the λ CI ChIP-sequencing reads (Fig. S5). Basepair bias
- 598 of the energy matrices was calculated as the sum of the average A and T preference minus the sum
- 599 of the average G and C preference.
- 600

601 Statistical analysis

- 602 Collected data was tested for normality (Shapiro-test) and subsequently we compared mean OD₆₀₀ or
- fluorescence expression values using t-tests with FDR correction for multiple comparisons in RStudio.
- T-tests were performed for four different time points between cultures grown in the presence and
- absence of inducer aTc (presence or absence of repressors) under indicated conditions. Error bars on
- growth reductions from AUC differences were obtained as 95% confidence intervals through
 bootstrapping by resampling the data at each time point 1000 times.
- 608 Spearman correlation was calculated for the fit between model predictions of binding strength and the
- number of obtained ChIP-sequencing reads per 1000bp window. P-values for WT and basepair bias
- energy matrix predictions were P<.001 and for the average over random matrices P<.01.
- 611

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- 619

620 AUTHOR CONTRIBUTIONS

- 621 C.I., C.F., G.T. and C.C.G. conceived the study together. C.I. designed the experiments and carried
- them out together with C.F. (growth experiments) and T.W. (ChIP-sequencing). C.I. analyzed the data
- 623 with input from F.M.P. and B.S.. C.I. wrote the initial draft of the manuscript and revised it together with
- 624 the rest of the authors.
- 625

626 CONFLICT OF INTEREST

- 627 Authors declare no competing financial interests.
- 628

629 **REFERENCES**

- Hopfield JJ. Biosynthetic Processes Requiring High Specificity. Pnas. 1974;71(10):4135–9.
 Mirny L, Slutsky M, Wunderlich Z, Tafvizi A, Leith J, Kosmrlj A. How a protein searches for its site on DNA: the mechanism of facilitated diffusion. J Phys A Math Theor [Internet]. 2009 Oct 30;42(43):434013. Available from: http://stacks.iop.org/1751-
- 634 8121/42/i=43/a=434013?key=crossref.c3b19cfa3ebb32a80370e612c091028f
- 635 3. Elf J, Li G-W, Xie XS. Probing transcription factor dynamics at the single-molecule level in a living cell. Science [Internet]. 2007 May 25;316(5828):1191–4. Available from:
 637 http://www.sciencemag.org/cgi/doi/10.1126/science.1141967
- Flyvbjerg H, Keatch SA, Dryden DTF. Strong physical constraints on sequence-specific target
 location by proteins on DNA molecules. Nucleic Acids Res. 2006;34(9):2550–7.
- 5. von Hippel PH, Revzin A, Gross CA, Wang AC. Non-specific DNA binding of genome
 regulating proteins as a biological control mechanism: I. The lac operon: equilibrium aspects.
 Proc Natl Acad Sci U S A. 1974 Dec;71(12):4808–12.
- 6. Bakk A, Metzler R. Nonspecific binding of the OR repressors CI and Cro of bacteriophage
 lambda. J Theor Biol [Internet]. 2004 Dec 21;231(4):525–33. Available from:
 https://linkinghub.elsevier.com/retrieve/pii/S0022519304003285
- Kao-Huang Y, Revzin A, Butler AP, O'Conner P, Noble DW, Von Hippel PH. Nonspecific DNA
 binding of genome-regulating proteins as a biological control mechanism: measurement of
 DNA-bound Escherichia coli lac repressor in vivo. Proc Natl Acad Sci U S A [Internet].
 1977;74(10):4228–32. Available from:

650 http://www.pnas.org/content/74/10/4228.short%5Cnhttp://www.pubmedcentral.nih.gov/articlere 651 nder.fcgi?artid=431912&tool=pmcentrez&rendertype=abstract

- 652 8. Gerland U, Moroz JD, Hwa T. Physical constraints and functional characteristics of 653 transcription factor-DNA interaction. Proc Natl Acad Sci U S A. 2002;99(19):12015–20.
- Spurio R, Dürrenberger M, Falconi M, La Teana A, Pon CL, Gualerzi CO. Lethal
 overproduction of the Escherichia coli nucleoid protein H-NS: ultramicroscopic and molecular autopsy. MGG Mol Gen Genet. 1992;231(2):201–11.
- 10. Yamada H, Yoshida T, Tanaka K ichi, Sasakawa C, Mizuno T. Molecular analysis of the
 Escherichia coli has gene encoding a DNA-binding protein, which preferentially recognizes
 curved DNA sequences. MGG Mol Gen Genet. 1991;230(1–2):332–6.
- 660 11. Crocker J, Preger-Ben Noon E, Stern DL. The Soft Touch. In: Current Topics in
 661 Developmental Biology [Internet]. 1st ed. Elsevier Inc.; 2016. p. 455–69. Available from: 662 http://dx.doi.org/10.1016/bs.ctdb.2015.11.018
- Burger A, Walczak AM, Wolynes PG. Abduction and asylum in the lives of transcription factors. Proc Natl Acad Sci [Internet]. 2010;107(9):4016–21. Available from: http://www.pnas.org/cgi/doi/10.1073/pnas.0915138107
- Friedlander T, Prizak R, Guet C, Barton NH. Intrinsic limits to gene regulation by global crosstalk. 2015;
- Cepeda-Humerez SA, Rieckh G, Tkačik G. Stochastic Proofreading Mechanism Alleviates
 Crosstalk in Transcriptional Regulation. Phys Rev Lett. 2015;115(24):1–5.
- 67015.Grah R, Zoller B, Tkačik G. Nonequilibrium models of optimal enhancer function. Proc Natl671Acad Sci U S A. 2020;117(50):31614–22.
- 672 16. Sasson V, Shachrai I, Bren A, Dekel E, Alon U. Mode of Regulation and the Insulation of
 673 Bacterial Gene Expression. Mol Cell [Internet]. 2012;46(4):399–407. Available from:
 674 http://dx.doi.org/10.1016/j.molcel.2012.04.032
- Howard-Varona C, Hargreaves KR, Abedon ST, Sullivan MB. Lysogeny in nature:
 mechanisms, impact and ecology of temperate phages. ISME J [Internet]. 2017 Jul

677		14;11(7):1511–20. Available from: http://dx.doi.org/10.1038/ismej.2017.16
678	18.	Shimada T, Ogasawara H, Ishihama A. Single-target regulators form a minor group of
679		transcription factors in Escherichia coli K-12. Nucleic Acids Res. 2018;46(8):3921–36.
680	19.	Ptashne M. Principles of a switch. Nat Chem Biol [Internet]. 2011 Aug [cited 2014 Jun
681		3];7(8):484–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21769089
682	20.	Priest DG, Cui L, Kumar S, Dunlap DD, Dodd IB, Shearwin KE, Quantitation of the DNA
683	20.	tethering effect in long-range DNA looping in vivo and in vitro using the Lac and repressors
68/		Proc Natl Acad Sci [Internet] 2014:111(1):349-54. Available from:
60 4		http://www.nooo.org/ori/doi/10.1072/ppop.121791711
005	04	http://www.piras.org/cgi/doi/10.1073/piras.isi/roi/rin
686	21.	Donner AL, Carlson PA, Koudeika GB. Dimerization specificity of P22 and 434 repressors is
687		determined by multiple polypeptide segments. J Bacteriol [Internet]. 1997 Feb [cited 2014 Jul
688		8];179(4):1253–61. Available from:
689		http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=178823&tool=pmcentrez&rendertyp
690		e=abstract
691	22.	Pray TR, Burz DS, Ackers GK. Cooperative non-specific DNA binding by octamerizing λ cl
692		repressors: a site-specific thermodynamic analysis. J Mol Biol [Internet]. 1998 Oct [cited 2017
693		Mar 1];282(5):947–58. Available from:
694		https://linkinghub.elsevier.com/retrieve/pii/S0022283698920563
695	23	Bakk A Metzler R In vivo non-specific binding of λ CI and Cro repressors is significant FEBS
696	20.	Lett 2004:563(1=3):66=8
697	24	Ider C Lagator M Tračik G Bollback IP Guet CC Evolutionary potential of transcription
609	24.	factore for gone regulatory rewiring. Nat Ecol Evol. 2019:2(October)
600	25	Vilor IM Soit L DNA looping in gone regulation from the cocombly of macromologular
599	25.	Vilar Jivi, Saiz L. DINA looping in gene regulation: from the assembly of macromolecular
700		complexes to the control of transcriptional noise. Curr Opin Genet Dev [internet]. 2005 Apr
/01		[cited 2014 May 14];15(2):136–44. Available from:
702		http://www.ncbi.nlm.nih.gov/pubmed/15797196
703	26.	Fattah KR, Mizutani S, Fattah FJ, Matsushiro A, Sugino Y. A comparative study of the
704		immunity region of lambdoid phages including Shiga-toxin-converting phages : molecular basis
705		for cross immunity . 2000;223–32.
706	27.	Oppenheim AB, Kobiler O, Stavans J, Court DL, Adhya S. Switches in bacteriophage lambda
707		development. Annu Rev Genet [Internet]. 2005 Jan [cited 2014 May 24];39:409-29. Available
708		from: http://www.ncbi.nlm.nih.gov/pubmed/16285866
709	28.	Schlechter RO, Remus DM, Remus-Emsermann MNP, Constitutively expressed fluorescent
710		proteins allow to track bacterial growth and to determine relative fitness of bacteria in mixed
711		cultures bioRxiv 2020: (Figure 1):1-5
712	29	Chakrabarti I. Chandra N. Raba P. Roy S. High-affinity quasi-specific sites in the genome:
712	25.	How the DNA-binding proteins cone with them Biophys 1 [Interpet] 2011:101(5):1123 0
713		Available from: http://dv.doi.org/10.1016/i.hpi.2011.07.041
714	20	Available from. http://dx.doi.org/10.1016/j.bpj.2011.07.041
/15	30.	whipple FW, Kuldell NH, Cheatham L a, Hochschild a. Specificity determinants for the
/16		interaction of lambda repressor and P22 repressor dimers. Genes Dev [Internet]. 1994 May 15
717		[cited 2014 Sep 22];8(10):1212–23. Available from:
718		http://www.genesdev.org/cgi/doi/10.1101/gad.8.10.1212
719	31.	Weiss MA, Pabo CO, Karplus M, Sauer RT. Dimerization of the operator binding domain of
720		phage lambda repressor. Biochemistry [Internet]. 1987 Feb 10 [cited 2014 Jun 5];26(3):897-
721		904. Available from: http://dx.doi.org/10.1021/bi00377a034
722	32.	Nelson HCM, Sauer RT. Interaction of mutant λ repressors with operator and non-operator
723		DNA. J Mol Biol. 1986;192(1):27–38.
724	33.	Mazumder A, Batabyal S, Mondal M, Mondol T, Choudhury S, Ghosh R, et al. Specific DNA
725		sequences allosterically enhance protein-protein interaction in a transcription factor through
726		modulation of protein dynamics: Implications for specificity of gene regulation. Phys Chem
727		Chem Phys [Internet] 2017:19(22):14781–92 Available from:
728		http://dx.doi.org/10.1039/CZCP01193H
720	3/	Guarage C Raman B Zahariay S Simonesits A Pongor S DNA-mediated assembly of
720	<u>о</u> т.	weakly interacting DNA-hinding protein subunits: In vitro recruitment of phage 424 repressor
721		and vesset GCN/4 DNA-binding domains. Nucleic Aside Res. 2004;22(17):4002-5002
731 727	2F	and yeast GUNY DINA-DINUNY UNITAINS. MULIEN ACIUS RES. 2004,32(17).4992-3002. Maarki S.L. Ouaka S.D. A. Svetame Approach to Macauring the Diading Energy Londocards of
152	55.	Transperintion Factors, Science (80,) Internet 2007 for 40:045/5000,000, 7 August 10 (1997)
/33		Transcription Factors. Science (80-) [Internet]. 2007 Jan 12;315(5809):233–7. Available from:
/34	20	nttp://www.ncbi.nim.nin.gov/pubmed/1/218526
/35	36.	Sarkar-Banerjee S, Goyal S, Gao N, Mack J, Thompson B, Dunlap D, et al. Specifically bound
736		lambda repressor dimers promote adjacent non-specific binding. Saiz L, editor. PLoS One

777		
/3/		[Internet]. 2018 Apr 2; 13(4):e0194930. Available from:
/38		http://dx.doi.org/10.13/1/journal.pone.0194930
739	37.	Chen Y, Golding I, Sawai S, Guo L, Cox EC. Population Fitness and the Regulation of
740		Escherichia coli Genes by Bacterial Viruses. Waldor M, editor. PLoS Biol [Internet]. 2005 Jun
741		21;3(7):e229. Available from: http://dx.plos.org/10.1371/journal.pbio.0030229
742	38.	Nordström K, Dasqupta S. Copy-number control of the Escherichia coli chromosome: A
743		plasmidologist's view, EMBO Rep. 2006;7(5):484–9.
744	39	Cooper S Buettinger T Replication of decovribonucleic acid during the division cycle of
745	00.	Salmonella typhimurium Bacteriol 1973:114(3):966–73
745	40	Samonena typining and a bacterior. 1973, 114(0):300–73.
740	40.	Runnipp S, Zhang Z, Hwa T. Glowin Kale-Dependent Global Effects on Gene Expression in
747		Bacteria. Cell [internet]. 2009 Dec; 139(7): 1366–75. Available from:
/48		http://dx.doi.org/10.1016/j.cell.2009.12.001
749	41.	Deris JB, Kim M, Zhang Z, Okano H, Hermsen R, Groisman A, et al. The Innate Growth
750		Bistability and Fitness Landscapes of Antibiotic-Resistant Bacteria. Science (80-) [Internet].
751		2013 Nov 29;342(6162):1237435–1237435. Available from:
752		http://www.sciencemag.org/cgi/doi/10.1126/science.1237435
753	42.	Waldminghaus T. Weigel C. Skarstad K. Replication fork movement and methylation govern
754		Send binding to the Escherichia coli chromosome Nucleic Acids Res 2012;40(12):5465-76
755	12	Squar BT, Bass M. Dischard M. Clouvage of the lambde and B22 represented by rock protoin
755	43.	Biol Chem 4000 April 27(0):4460 - 20
750		J Biol Chem. 1982 Apr;257(8):4458–62.
/5/	44.	Phizicky EM, Roberts JW. Kinetics of recA protein-directed inactivation of repressors of phage
758		λ and phage P22. J Mol Biol. 1980;139(3):319–28.
759	45.	Arends SJR, Weiss DS. Inhibiting Cell Division in Escherichia coli Has Little If Any Effect on
760		Gene Expression. J Bacteriol [Internet]. 2004 Feb 1;186(3):880–4. Available from:
761		http://jb.asm.org/cgi/doi/10.1128/JB.186.3.880-884.2004
762	46	Cambridge J. Blinkova A. Magnan D. Bates D. Walker JR. A Replication-inhibited
763		unsequent and the second standard blocks Z-ring formation and cell division independently of
767		Sos and the SimA nucleoid acclusion protation in Eacharishi a coli - Leastarial 2014:106(1):26
704		
705	47	49. Culture of D. Nerdeteine V. Etc. Zring formation without subsequent call division of the replication.
700	47.	Guibrand B, Nordström K. Fisz fing formation without subsequent cen division after replication
/6/		runout in Escherichia coli. Mol Microbiol [Internet]. 2002 Jan 18;36(6):1349–59. Available from:
768		http://doi.wiley.com/10.1046/j.1365-2958.2000.01949.x
769	48.	Wunderlich Z, Mirny L. Fundamentally different strategies for transcriptional regulation are
770		revealed by analysis of binding motifs. Nat Preced. 2008;2(i).
771	49.	Brewster RC, Weinert FM, Garcia HG, Song D, Rydenfelt M, Phillips R. The transcription
772		factor titration effect dictates level of gene expression. Cell. 2014;156(6):1312–23.
773	50.	Pleška M. Qian L. Okura R. Bergmiller T. Wakamoto Y. Kussell E. et al. Bacterial
774		Autoimmunity Due to a Restriction-Modification System, Curr Biol [Internet] 2016 Feb
775		$3.26(3) \cdot 404 = 0$ Available from this '/www.ncbi.nlm. bit nov/pubmed/26804559
775	51	de Roor DA L Advances in understanding E, coli coll fiscion Curra Opin Microbiol
770	51.	de boer - AJ. Advances in understanding E. con cen inssion. Cun Opin Microbiol.
777		2010;13(6):730-7.
//8	J2.	Sun Q, iviargolin vv. Effects of perturbing nucleoid structure on nucleoid occlusion-mediated
//9		toporegulation of FtsZ ring assembly. J Bacteriol. 2004;186(12):3951–9.
/80	53.	Spurio R, Falconi M, Brandi A, Pon CL, Gualerzi CO. The oligomeric structure of nucleoid
781		protein H-NS is necessary for recognition of intrinsically curved DNA and for DNA bending.
782		EMBO J. 1997;16(7):1795–805.
783	54.	Verma SC, Qian Z, Adhya SL. Architecture of the Escherichia coli nucleoid. Vol. 15, PLoS
784		Genetics, 2019, 1–35 p.
785	55.	Amir A. Männik J. Woldringh CL. Zaritsky A. Editorial: The Bacterial Cell: Coupling between
786		Growth Nucleoid Replication Cell Division and Shane Volume 2 Front Microbiol
700		2010/10(Soptombor):1, 3
789	56	Zahat NR Adrivan B. The affects of transcription factor composition on gone regulation. Front
700	50.	
109		Genet. $2013,4(001),1-10$.
/90	57.	Baitrus DA. Exploring the costs of norizontal gene transfer. I rends Ecol Evol [Internet].
/91		2013;28(8):489–95. Available from: http://dx.doi.org/10.1016/j.tree.2013.04.002
792	58.	Friedlander T, Prizak R, Barton NH, Tkačik G. Evolution of new regulatory functions on
793		biophysically realistic fitness landscapes. Nat Commun. 2017;8(1).
794	59.	Lutz R, Bujard H. Independent and tight regulation of transcriptional units in Escherichia coli
795		via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. Nucleic Acids Res [Internet].
796		1997 Mar 15;25(6):1203–10. Available from:

797		http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=146584&tool=pmcentrez&rendertyp
798		e=abstract
799	60.	Datsenko K a, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli K-
800		12 using PCR products. Proc Natl Acad Sci U S A [Internet]. 2000 Jun 6;97(12):6640–5.
801		Available from:
802		http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=18686&tool=pmcentrez&rendertype
803		=abstract
804	61.	Kües U, Stahl U. Replication of plasmids in gram-negative bacteria. Microbiol Rev [Internet].
805		1989 Dec;53(4):491–516. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2687680
806	62.	Hershfield V, Bover HW, Yanofsky C, Lovett MA, Helinski DR. Plasmid ColEl as a molecular
807		vehicle for cloning and amplification of DNA. Proc Natl Acad Sci U S A. 1974;71(9):3455–9.
808	63.	Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A. A variant of vellow fluorescent
809		protein with fast and efficient maturation for cell-biological applications. Nat Biotechnol
810		[Internet]. 2002 Jan;20(1):87–90. Available from:
811		http://www.ncbi.nlm.nih.gov/pubmed/11753368
812	64.	Nishihara K. Kanemori M. Yanagi H. Yura T. Overexpression of trigger factor prevents
813	-	aggregation of recombinant proteins in Escherichia coli. Appl Environ Microbiol [Internet]. 2000
814		Mar:66(3):884–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10698746
815	65.	Kitagawa M. Ara T. Arifuzzaman M. Joka-Nakamichi T. Inamoto E. Tovonaga H. et al.
816		Complete set of ORF clones of Escherichia coli ASKA library (A complete set of E, coli K-12
817		ORF archive): unique resources for biological research. DNA Res. 2005;12(5):291–9.
818	66	Costantino N Court DI Enhanced levels of lambda Red-mediated recombinants in mismatch
819	00.	repair mutants. Proc Natl Acad Sci U S A [Internet] 2003;100(26):15748–53. Available from:
820		http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=307639&tool=pmcentrez&rendertyp
821		e=abstract
822	67	Friedberg FC, Walker GC, Siede W, Wood RD, DNA repair and mutagenesis. American
823	07.	Society for Microbiology Press: 2005
824	68	Haldimann A Wanner BL Conditional-Replication Integration Excision and Retrieval
825	00.	Plasmid-Host Systems for Gene Structure-Function Studies of Bacteria, I Bacteriol [Internet]
826		2001 Nov 1:183(21):6384–93 Available from:
827		http://ib.asm.org/cgi/doi/10.1128/JB.183.21.6384-6393.2001
828	69	Marchetti A Abril-Marti M Illi B Cesareni G Nasi S Analysis of the Myc and Max interaction
829	00.	specificity with λ repressor-HI H domain fusions. J Mol Biol. 1995;248(3):541–50
830	70.	Wharton RP. Ptashne M. Changing the binding specificity of a represser by redesigning an g-
831		helix. Nature. 1985:316(6029):601–5.
832	71.	Lin L. Bitner R. Edlin G. Increased reproductive fitness of Escherichia coli lambda lysogens. J
833		Virol, 1977:21(2):554–9.
834	72.	Flashman SM. NMutational Analysis of the Operators of Bacteriophage Lambda, 1978;73:61-
835		73.
836	73.	Sarai A, Takeda Y. Lambda repressor recognizes the approximately 2-fold symmetric half-
837		operator sequences asymmetrically. Proc Natl Acad Sci U S A [Internet]. 1989;86(17):6513-7.
838		Available from:
839		http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=297874&tool=pmcentrez&rendertyp
840		e=abstract
841	74.	Waldminghaus T, Skarstad K. ChIP on Chip: surprising results are often artifacts. BMC
842		Genomics [Internet]. 2010;11(1):414. Available from: http://www.biomedcentral.com/1471-
843		2164/11/414
844	75.	Santhanam B, Cai H, Devreotes PN, Shaulsky G, Katoh-Kurasawa M. The GATA transcription
845		factor GtaC regulates early developmental gene expression dynamics in Dictyostelium. Nat
846		Commun. 2015;6(May).
847	76.	Jothi R, Cuddapah S, Barski A, Cui K, Zhao K. Genome-wide identification of in vivo protein-
848		DNA binding sites from ChIP-Seq data. Nucleic Acids Res. 2008;36(16):5221–31.
849	77.	Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-based
850		analysis of ChIP-Seq (MACS). Genome Biol. 2008;9(9).
851	78.	Tanay A. Extensive low-affinity transcriptional interactions in the yeast genome. Genome Res
852		[Internet]. 2006 Jun 29;16(8):962–72. Available from:
853		http://www.genome.org/cgi/doi/10.1101/gr.5113606
854	79.	Koblan KS, Ackers GK. Site-Specific Enthalpic Regulation of DNA Transcription at
855		Bacteriophage λ OR. Biochemistry. 1992;31(1):57–65.
050	00	Dema CL. Dellivery NM Ireland WT Kinney ID Dhilling D. Menning DNA seguence to

- transcription factor binding energy in vivo. PLoS Comput Biol. 2019;15(2):1–29.
- 81. Hilchey SP, Wu L, Koudelka GB. Recognition of Nonconserved Bases in the P22 Operator by
 P22 Repressor Requires Specific Interactions between Repressor and Conserved Bases *.
 1997;272(32):19898–905.



865 Box 1. (A) In order to elicit an appropriate function, TFs have to recognize their cognate DNA sites (operators) among a large background of non-cognate sites, where binding is non-functional and 866 867 potentially interferes with cellular programs. Binding of TFs to cognate or non-cognate sites can occur 868 by recognizing a specific motif on the DNA (with specificity ranging from high to low, depending on the overlap of the target site with the TF's consensus sequence (8)) or through generic, non-specific 869 870 interactions with any DNA sequence. (B) Binding with high specificity usually occurs to a TF-specific DNA consensus motif and the offset gives the binding energy (lower energy = stronger binding) to a 871 872 single operator sequence relative to the unbound state (TF in solution). Random DNA sequences are 873 usually located at the lower end of the sigmoid (gray box) with energy values similar to that of the TF 874 in solution. Basepair mismatches with the operator sequence incur an energy penalty and increase 875 the binding energy (i.e. weaken the binding) based on two features: specific effects of basepair 876 mismatches and overall basepair bias (given here as average AT preference - average GC 877 preference). Hence, for a TF with low offset, which is aided by high cooperativity, and an energy 878 matrix characterized by small mismatch effects, many random sequences will not be far from the rise 879 of the sigmoid, making higher occupancy at low specificity binding sites more likely. The table compares these three criteria for three well-characterized TFs, showing that all of them are fulfilled for 880 881 λ CI, but only partially for P22 C2 and Lacl (24,25,80,81), making λ CI an obvious candidate for 882 substantial low-specificity non-cognate binding.





885 Figure 1. Growth reduction in the presence of repressors in minimal media with glucose.

(A) The experimental model system with repressors being expressed from a plasmid and their binding cooperativity modes are shown. (B) Growth reduction as calculated by the normalized growth difference in the presence and absence of repressor are shown for λ CI, 434 CI, P22 C2, HK022 CI and Lacl in *E. coli* and *S. enterica* cells. Error bars show 95% confidence intervals. (C,D) Curves show mean OD₆₀₀ for *E. coli* (left) or *S. enterica* (right) cells in the presence (color) or absence (black) of (C) λ CI or (D) P22 C2; error bars show standard deviation over 6 replicates (black on color and white on black).



895 Figure 2. Effect of environment on repressor-dependent growth reduction.

Growth reduction as calculated by the normalized growth difference in the presence and absence of repressor are shown for λ CI (blue) and P22 C2 (grey) in *E. coli* and *S. enterica*. Error bars show 95% confidence intervals. (A) Cells were grown in minimal medium with glucose (M9) or rich media (LB) at full induction of repressors. (B) Inducer concentrations for repressor expression were varied from 1 to 25ng (very low to full induction (59)). (C) Induction time points of repressor expression were varied

901 from lag phase (0h) to early- (2h) and mid- exponential phase (4h).

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904 Figure 3. Competition assays reveal fitness cost of repressor expression.

905 (A) Cells containing plasmids with a repressor affecting growth (λ CI or P22 C2) or with a repressor 906 not affecting growth (Lacl) were mixed 1:1 with cells containing a plasmid with the repressor not 907 affecting growth and a separately, constitutively expressed Venus marker (Lacl-Venus). Competition 908 was performed in minimal media with glucose and fluorescence was used as a measure of the 909 relative change in the cells carrying Lacl-Venus. (B,C) Relative fluorescence was calculated for E. coli 910 (crosses) or S. enterica (circles) between induced and non-induced samples of cell mixtures of Lacl-911 Venus together with Lacl (control), λ CI or P22 C2 after (B) 90min or (C) 510min of competition; error 912 bars show relative errors. Selection coefficients (for calculation see Methods) after 10h were 0.27 (E. 913 coli) and 0.29 (S. enterica) for λ CI and 0.67 (E. coli) and -0.06 (S. enterica) for P22 C2. 914



Figure 4. Effect of mutants and chimeric proteins on repressor-dependent growth reduction.
Growth reduction as calculated by the normalized growth difference in the presence and absence of

917 Growth reduction as calculated by the normalized growth difference in the presence and absence of 918 repressor are shown in *E. coli* and *S. enterica*. Error bars show 95% confidence intervals. **(A)** Growth

919 reduction for λ CI wildtype (blue) is compared to a λ CI binding mutant (red), a λ CI dimerization

920 mutant (orange) or a λ CI cooperativity mutant (turquoise) in minimal media. **(B)** Growth reduction of

921 the phage repressor 434 CI and the bacterial repressor Lacl (black) compared to a chimera of the λ

922 CI protein containing the binding specificity of either 434 CI (λ -434 CI) or Lacl (λ CI-Lacl) (blue) is

923 shown for minimal (M9) and rich media (LB).

924



926 Figure 5. Distributed, low-specificity binding of λ Cl across the genomes of *E. coli* and *S.*

927 enterica.

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(A,B) Distributions of ChIP-sequencing reads for the regions found to be significantly enriched in the 928 929 experiment with λ CI over the experiment without λ CI across the (A) *E.coli* or (B) *S. enterica* genome. 930 On the right density plots of enriched read numbers are given for repressor (color) or no repressor 931 (black) experiments (Number of reads for not significantly enriched regions are shown in Fig. S4 for 932 comparison). None of the apparent peaks in (A) or (B) encodes for an essential gene, nor one obviously 933 beneficial in minimal media. (C,D) Fit between binding strength predictions of a simple thermodynamic 934 model using the energy matrix for λ CI binding and the ChIP-sequencing reads across 1000bp windows 935 along the (C) E. coli or (D) S. enterica genome. In (D), 33 out of 4856 data points showed more than 936 3600 reads and were omitted for clarity (the data is available on request). Lower insets show the 937 calculated Spearman correlations using either the wildtype energy matrix, one that only conserves the 938 λ CI basepair bias (Fits are shown in Fig. S5) or one that has completely reshuffled entries (averaged 939 over 100 permutations). Upper insets: Permutation test for the significance of the difference in 940 Spearman correlation between binding predictions using the wildtype energy matrix (first bar in the 941 lower inset) vs prediction with the energy matrix that only conserves λ CI basepair bias (second bar in

the lower inset). Black histograms represent the Monte-Carlo-derived null distribution (10⁴ random
reassignments of ChIP reads to genomic regions), green dot and line show the true excess Spearman
correlation. The correlations for *S. enterica* were not substantially affected by the strong binding peak
in prophage regions shown in (**B**).

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949 Figure 6. Induction of λ CI interferes with cell division but not DNA replication.

- 950 Cells were imaged under the microscope in minimal media with glucose and aTc, either (A,B) on an
- 951 agar pad, or directly (C) in liquid media (see Methods). Fluorescence indicates (A) ongoing replication
- 952 (a SeqA-GFP fusion as a replication fork marker) or **(B)** potential induction of a primary stress
- 953 response promoter (P_{sulA} -yfp reporter). (C) Chromosome positioning within the cell is shown in blue
- 954 (Hoechst dye), relative to the cell membrane in red (NileRed). White arrows indicate an overlap of
- 955 septation spots (thicker red dots) with DNA (blue) for cells containing λ CI.
- 956