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Regular paper

A search for the *in trans* role of GraL, an *Escherichia coli* small RNA*

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Small RNA are very important post-transcriptional regulators in both, bacteria and eukaryotes. One of such sRNA is GraL, encoded in the *greA* leader region and conserved among enteric bacteria. Here, we conducted a bioinformatics search for GraL's targets *in trans* and validated our findings *in vivo* by constructing fusions of probable targets with *lacZ* and measuring their activity when GraL was overexpressed. Only one target's activity (*nudE*) decreased under those conditions and was thus selected for further analysis. In the absence of GraL and *greA,* the *nudE::lacZ* fusion's β-galactosidase activity was increased. However, a similar effect was also visible in the strain deleted only for *greA*. Furthermore, overproduction of GreA alone increased the *nudE::lacZ* fusion's activity as well. This suggests existence of complex regulatory loop-like interactions between GreA, GraL and *nudE* mRNA. To further dissect this relationship, we performed *in vitro* EMSA experiments employing GraL and *nudE* mRNA. However, stable GraL-*nudE* complexes were not detected, even though the detectable amount of unbound GraL decreased as increasing amounts of *nudE* mRNA were added. Interestingly, GraL is being bound by Hfq, but *nudE* easily displaces it. We also conducted a search for genes that are synthetic lethal when deleted along with GraL. This revealed 40 genes that are rendered essential by GraL deletion, however, they are involved in many different cellular processes and no clear correlation was found. The obtained data suggest that GraL's mechanism of action is non-canonical, unique and requires further research.

Key words: GraL, GreA, sRNA, sRNA targets, synthetic lethal genes

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Abbreviations: EMSA, electrophoretic mobility shift assay; IPTG, isopropyl β-D-1-thiogalactopyranoside; ppGpp, guanosine-3′,5′-bis-diphosphate; ppGpp^0 strain, strain devoid of ppGpp; sRNA, small RNA; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

Table S1. Strains used in this study.

Strain	Genotype	Source / Reference
MG1655	F- *λ- ilvG- rfb*-50 *rph*-1	Guyer et al., 1981
CF15615	MG1655 Δ*lacZΔrelAΔspoT* (ppGpp^0)	Vinella et al., 2012
CF15617	MG1655 Δ*lacZ*	Vinella et al., 2012
ECMZ1501	CF15615*ΔgreA*::cat	This work
ECMZ1502	CF15615 *ΔgreA*::cat/ pRC7	This work
ECMZ1503	CF15615 *ΔgreA*::cat / pRC-GraL	This work
ECMZ1504	CF15615 *ΔGraL ΔgreA*::cat	This work
ECMZ1505	CF15615 *ΔGraL ΔgreA*::cat/ pRC7	This work
ECMZ1506	CF15615 *ΔGraL ΔgreA*::cat/ pRC-GraL	This work
ECMZ1507	CF15615*ΔgreA*::cat / pRC7 pHM1873	This work
ECMZ1508	CF15615*ΔgreA*::cat / pRC-GraL pHM1873	This work
ECMZ1509	CF15615*ΔGraL ΔgreA*::cat/ pRC7 pHM1873	This work
ECMZ1510	CF15615*ΔGraL ΔgreA*::cat/ pRC-GraL pHM1873	This work
ECMZ1601	CF15617 *ΔgreA*::cat	This work
ECMZ1602	CF15617 *ΔgreA*::cat / pRC7	This work
ECMZ1603	CF15617 *ΔgreA*::cat / pGraL	This work
ECMZ1604	CF15617 *ΔGraL ΔgreA*::cat	This work
ECMZ1605	CF15617*ΔGraL ΔgreA*::cat / pRC7	This work
ECMZ1606	CF15617*ΔGraL ΔgreA*::cat / pRC-GraL	This work
ECMZ1607	CF15617 *ΔgreA*::cat/ pRC7 pHM1873	This work
ECMZ1608	CF15617 *ΔgreA*::cat / pRC-GraL pHM1873	This work
ECMZ1609	CF15617*ΔGraL ΔgreA*::cat / pRC7 pHM1873	This work
ECMZ1610	CF15617*ΔGraL ΔgreA*::cat / pRC-GraL pHM1873	This work
MD98	CF15617 / pGraL pRS414-*PrpoS::lacZ*	This work
MD99	CF15617 / pScr pRS414-*PrpoS::lacZ*	This work
MD102	CF15617 / pGraL pRS414-*PgadB::lacZ*	This work
MD103	CF15617 / pScr pRS414-*PgadB::lacZ*	This work
MD107	CF15617 / pHM1883 pRS414-*PrpoS::lacZ*	This work
MD108	CF15617 / pHM1883 pRS414-*PgadB::lacZ*	This work
MD112	CF15617 / pGraL pRS414-*PpanD::lacZ*	This work
MD113	CF15617 / pScr pRS414-*PpanD::lacZ*	This work
MD114	CF15617 / pHM1883 pRS414-*PpanD::lacZ*	This work
MD115	CF15617 / pGraL pRS414-*PyhdV::lacZ*	This work
MD116	CF15617 / pScr pRS414-*PyhdV::lacZ*	This work
MD117	CF15617 / pHM1883 pRS414-*PyhdV::lacZ*	This work
MD118	CF15617 / pGraL pRS414-*PydcC::lacZ*	This work
MD119	CF15617 / pScr pRS414-*PydcC::lacZ*	This work
MD120	CF15617 / pHM1883 pRS414-*PydcC::lacZ*	This work
MD121	CF15617 / pGraL pRS414-*PnudE::lacZ*	This work
MD122	CF15617 / pScr pRS414-*PnudE::lacZ*	This work
MD123	CF15617 / pHM1883 pRS414-*PnudE::lacZ*	This work
MD124	CF15617 / pGraL pRS414-*PflgA::lacZ*	This work

MD125	CF15617 / pScr pRS414-*PflgA::lacZ*	This work
MD126	CF15617 / pHM1883 pRS414-*PflgA::lacZ*	This work
MD127	CF15617 / pGraL pRS414-*PykgR::lacZ*	This work
MD128	CF15617 / pScr pRS414-*PykgR::lacZ*	This work
MD129	CF15617 / pHM1883 pRS414-*PykgR::lacZ*	This work
MD130	CF15617 / pGraL pRS414-*PyjfP::lacZ*	This work
MD131	CF15617 / pScr pRS414-*PyjfP::lacZ*	This work
MD132	CF15617 / pHM1883 pRS414-*PyjfP::lacZ*	This work
MD133	CF15617 / pGraL pRS414-*PxanP::lacZ*	This work
MD134	CF15617 / pScr pRS414-*PxanP::lacZ*	This work
MD135	CF15617 / pHM1883 pRS414-*PxanP::lacZ*	This work
MD136	CF15617 / pGraL pRS414-*PrsxC::lacZ*	This work
MD137	CF15617 / pScr pRS414-*PrsxC::lacZ*	This work
MD138	CF15617 / pHM1883 pRS414-*PrsxC::lacZ*	This work
MD139	CF15617 / pGraL pRS414-*PrzoQ::lacZ*	This work
MD140	CF15617 / pScrpRS414-*PrzoQ::lacZ*	This work
MD141	CF15617 / pHM1883 pRS414-*PrzoQ::lacZ*	This work
MD142	CF15617 / pGraL pRS414-*PyjiX::lacZ*	This work
MD143	CF15617 / pScr pRS414-*PyjiX::lacZ*	This work
MD144	CF15617 / pHM1883 pRS414-*PyjiX::lacZ*	This work
MD150	CF15615 / pGraL pRS414 PpanD::lacZ	This work
MD151	CF15615 / pHM1883 pRS414 PpanD::lacZ	This work
MD152	CF15615 / pGraL pRS414-*PydcC::lacZ*	This work
MD153	CF15615 / pHM1883 pRS414-*PydcC::lacZ*	This work
MD154	CF15615 / pGraL pRS414-*PnudE::lacZ*	This work
MD155	CF15615 / pHM1883 pRS414-*PnudE::lacZ*	This work
MD156	CF15615 / pGraL pRS414-*PflgA::lacZ*	This work
MD157	CF15615 / pHM1883 pRS414-*PflgA::lacZ*	This work
MD158	CF15615 / pGraL pRS414-*PykgR::lacZ*	This work
MD159	CF15615 / pHM1883 pRS414-PflgA::lacZ	This work
MD160	CF15615 / pGraL pRS414-*PxanP::lacZ*	This work
MD161	CF15615 / pHM1883 pRS414-*PxanP::lacZ*	This work
MD162	CF15615 / pGraL pRS414-*PgadB::lacZ*	This work
MD163	CF15615 / pHM1883 pRS414-*PgadB::lacZ*	This work
MD164	CF15615 / pGraL pRS414-*PyjfP::lacZ*	This work
MD165	CF15615 / pHM1883 pRS414-*PyjfP::lacZ*	This work
MD169	CF15615 / pGraL pRS414-*PrpoS::lacZ*	This work
MD170	CF15615 / pHM1883 pRS414-*PrpoS::lacZ*	This work
MD171	CF15615 / pGraL pRS414-*PrsxC::lacZ*	This work
MD172	CF15615 / pHM1883 pRS414-*PrsxC::lacZ*	This work
MD173	CF15615 / pGraL pRS414-*PrzoQ::lacZ*	This work
MD174	CF15615 / pHM1883 pRS414-*PrzoQ::lacZ*	This work
MD175	CF15615 / pGraL pRS414-*PyjiX::lacZ*	This work
MD176	CF15615 / pHM1883 pRS414-*PyjiX::lacZ*	This work
MD177	CF15615 / pGraL pRS414-*PyhdV::lacZ*	This work
MD178	CF15615 / pHM1883 pRS414-*PyhdV::lacZ*	This work

MD186	CF15615 / pScr pRS414-*PrpoS::lacZ*	This work
MD189	CF15615 / pScr pRS414-*PnudE::lacZ*	This work
MD192	CF15615 / pScr pRS414-*PgadB::lacZ*	This work
MD223	CF15617 (λ PnudE::lacZ)/ pGraL	This work
MD224	CF15617 (λ PnudE::lacZ)/ pScr	This work
MD226	CF15617 (λ PnudE::lacZ)/ pHM 1883	This work
MD241	CF15615 (λ PnudE::lacZ)/ pGraL	This work
MD242	CF15615 (λ PnudE::lacZ)/ pScr	This work
MD244	CF15615 (λ PnudE::lacZ)/ pHM 1883	This work
MD245	CF15615 ΔGraL ΔgreA::cat / pRS414-*PnudE::lacZ*	This work
MD246	CF15615 ΔgreA::cat / pRS414-*PnudE::lacZ*	This work
MD247	CF15615 / pRS414-*PnudE::lacZ*	This work
MD248	CF15615 (λ PnudE::lacZ)	This work
MD251	CF15615 ΔGraL ΔgreA::cat (λPnudE::lacZ)	This work
MD253	CF15615 ΔgreA::cat (λPnudE::lacZ)	This work
MD254	CF15617 ΔGraL ΔgreA::cat / pRS414-*PnudE::lacZ*	This work
MD255	CF15617 ΔgreA::cat / pRS414-*PnudE::lacZ*	This work
MD256	CF15617 / pRS414-*PnudE::lacZ*	This work
MD278	CF15615 (λ *PnudE::lacZ*) / pGraL	This work
MD279	CF15615 (λ*PnudE::lacZ*) / pHM1873	This work
MD280	CF15615 ΔGraLΔgreA::cat (λ *PnudE::lacZ*) / pGraL	This work
MD281	CF15615 ΔGraL ΔgreA::cat (λ*PnudE::lacZ*) / pHM1873	This work
MD282	CF15615 ΔgreA::cat (*λPnudE::lacZ*) / pGraL	This work
MD283	CF15615 ΔgreA::cat (λ*PnudE::lacZ*) / pHM1873	This work

References:

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Vinella D, Potrykus K, Murphy H, and Cashel M. (2012) Effects on growth by changes of the balance between GreA, GreB, and DksA suggest mutual competition and functional redundancy in *Escherichia coli*. *J Bacteriol.* 194:261-73. https://doi.org/10.1128/JB.06238-11

Table S2. Oligonucleotides used in this study

Name	Sequence (5' to 3')	Use
KPr67	**ACCTGGAATCGAGCCGTCATACTACGGCGCAACGCCCT ATAAAGTAAACG**TGTGACGGAAGATCACTTCG	Deletion of *greA* and GraL from the chromosome by lambda Red recombination; in bold – sequence complementary to chromosomal region just upstream of GraL; underlined – sequence complementary to *cat* (chloramphenicol resistance gene); to be used together with KPr68
KPr68	**CCTTTTTCCTTTCTTTACAATACATCAACATCTTGAGTATT GGGTAATTC**ACCAGCAATAGACATAAGCG	Deletion of *greA* alone and deletion of *greA* and GraL from the chromosome by lambda Red recombination; in bold - sequence complementary to chromosomal region just downstream of *greA*; underlined – sequence complementary to *cat* (chloramphenicol resistance gene); to be used together with KPr67 or KPr69
KPr69	**CGGGGTGGGTGAAGACTTGCCCTATCAGGAATATTCAAG AGGTATAACAA**TGTGACGGAAGATCACTTCG	Deletion of *greA* alone from the chromosome by lambda Red recombination; in bold – sequence complementary to chromosomal region just upstream of *greA*; underlined – sequence complementary to *cat* (chloramphenicol resistance gene); to be used together with KPr68
KPr81	GCAATGTAACATCAGAGATTTTGAG	Primer used for sequencing of PCR fragments obtained with KPr83
KPr83	ATGGCTCATAACACCCCTTGTATTA	Primer used for RATE-PCR to amplify DNA fragments encompassing kan transposon insertion sites
MDGLUP	ATGAATTCATCAAAATGTGAATTGTAGCTGACCTGGGACT TGTACCCG	pGraL and pRC-GraL plasmid construction; forward primer
MDGLDWN	ATCAAGCTTAAGCAAAAAAATACCGACCCGGGTACAAGTC CCAGGTCAG	pGraL and pRC-GraL plasmid construction; reverse primer
MDGLSUP	ATGAATTCATAAGATATATGTACTAGTGGCTGGGCCCATG TTCAGGGT	pScrplasmid construction; forward primer
MDGLSDWN	ATCAAGCTTAAGCAAAAAAATCGACTGGACCCTGAACATG GGCCCAGCC	pScrplasmid construction; reverse primer
MDNDUP	TGCGAATTCCATCTTACTTAGTCTGTCAGGCGT	*nudE::lacZ* fusion, forward primer
MDNDDOWN	TCGGATCC ATGGTGGGTTTTTGTAATGATTTGCT	*nudE::lacZ* fusion, reverse primer
MDFAUP1	GTCGAATTCCGTCCACGTTGCATGACTT	*flgA::lacZ* fusion, forward primer
MDFAUDOW	CTGGGATCCATCGCCACGCTACGTTTTATTATC	*flgA::lacZ* fusion, reverse primer
MDRCUP3	TCCGAATTCCAGTTGCTGTTTACT TTGTTGGCA	*rsxC::lacZ* fusion, forward primer
MDRCDOWN	AGGGATCCGCAGAGAATAACTTAAGCATGGTGTTC	*rsxC::lacZ* fusion, reverse primer
MDRSUP1	GCGAATTCTGGACGAAGGCGGGATT	*rpoS::lacZ* fusion, forward primer
MDRSDWN4	CGTGGATCCTTCATATCGTCATCTTGCGTGGTAT	*rpoS::lacZ*fusion, reverse primer
MDXPDOWN	CAGGATCC TTTTCTGACTCGAGGGTGGAAA	*xanP::lacZ* fusion, reverse primer
MDXPUP	GAGAATTCAGCAGCAACGTCAGCG	*xanP::lacZ* fusion, forward primer
MDGBUP	CCTGGAATTCTCAATATGACGATCCTGCAGCAT	*gadB::lacZ* fusion, forward primer
MDGBDOWN	GAGGATCCGATTCTGCGATAGTGGAAATAGACTTCG	*gadB::lacZ* fusion, reverse primer

MDYPUP	GCTGGAATTCTTCGCTTTGATTTCTGCTAATGCG	*yjfP::lacZ* fusion, forward primer
MDYPDOWN	CAGGATCCGCAAAATAGCTATACACCAGACTGGA	*yjfP::lacZ* fusion, reverse primer
MDYRUP	GCGGAATTCAGGTCACCAACAACGATATCTTGTATAAC	*ykgR::lacZ* fusion, forward primer
MDYRDOWN	TCGGATCCCTGATTTGCTGTACTTTATTCTCTTTCATTGG	*ykgR::lacZ* fusion, reverse primer
MDRQUP	GCCGAATTCCTCTTGAAAAACTGTGTTCTGACTCTTG	*rzoQ::lacZ* fusion, forward primer
MDRQDOWN	TAGGATCCATGCTTCGCTGCGTCAAG	*rzoQ::lacZ* fusion, reverse primer
MDPDUP	GCCGAATTCTTTACCGAGCAGCGTTCA	*panD::lacZ* fusion, forward primer
MDPDDWN2	CATGGATCCTTCACGCGGTGGAGTTTG	*panD::lacZ*fusion, reverse primer
MDYCUP	AGCGAATTCCTGGAATAAAGAAGATGGCAC	*ydcC::lacZ* fusion, forward primer
MDYCDOWN	CGAGGATCCGGGATAATAGAAATATGTCCCATC	*ydcC::lacZ* fusion, reverse primer
MDYVUP	GCTGAATTCCGTTGCTGGCAATCTTCTT	*yhdV::lacZ* fusion, forward primer
MDYVDOWN	GAC GGATCC AATCTTTTCATGGTGTTACCTCAG	*yhdV::lacZ* fusion, reverse primer
MDYXUP	ATCGAATTCCCGCAGATGGAAGGCTTC	*yjiX::lacZ* fusion, forward primer
MDYXDOWN	CTGGATCCTTGTCGTAGTCTGGAATGCCAAT	*yjiX::lacZ* fusion, reverse primer
MDPr15	TTTAATACGACTCACTATA**G**GGCTTACTTAGTCTGTCAGG CGTGG	*nudE* "long" forward primer, template generation for MAXISCRIPT
MDPr16	TTTAATACGACTCACTATA**G**GGAACCCTCTGAAGGCGGAA TATCA	*nudE* "medium" forward primer, template generation for MAXISCRIPT
MDPr17	TTTAATACGACTCACTATA**G**GGCGTGTCCGATATCGCACA ATAACC	*nudE* " short" forward primer, template generation for MAXISCRIPT
MDPr19	AAACACGCCGCACGCCATTGC	*nudE* reverse primer, template generation for MAXISCRIPT
MDPr20	TAATACGACTCACTATAGGGGCGTTACCCAACTTAATCGC CTTG	control *lacZ* forward primer, template generation for MAXISCRIPT
MDPr21	GGCATCAGAGCAGATTGTACTGAGAGTGCACCA	control *lacZ* reverse primer, template generation for MAXISCRIPT

Table S3. β-galactosidase activities (expressed as Miller units), calculated for data presented in Figure 1 A&B. These specific activities were calculated for curve regions where absolute activities increase linearly with OD_{600}. At least 3 points from each curve where included in the calculations. S.D. is given in parenthesis.

Plasmid/Fusion	Multicopy fusion	Single copy fusion
Vector control	850.29 (+/-58.72)	19.32 (+/-3.59)
pGraL	600.55 (+/-39.74)	16.75 (+/-5.07)
pGraL + 1mM IPTG	585.42 (+/-40.03)	16.76 (+/-2.43)
pScr	764.33 (+/-64.52)	18.07 (+/-3.03)
pScr+1mM IPTG	794.59 (+/-76.29)	18.24 (+/-3.84)

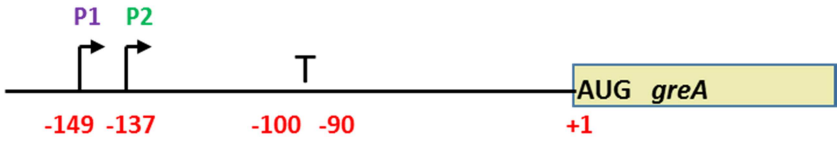
Table S4. β-galactosidase activities (expressed as Miller units), calculated for data presented in Figure 1 C&D. These specific activities were calculated for curve regions where absolute activities increase linearly with OD_{600}. At least 3 points from each curve where included in the calculations. S.D. is given in parenthesis.

Strain/Fusion	Multicopyfusion	Single copyfusion
$ppGpp^0$ (control)	635.22 (+/-17.96)	34.15 (+/-2.18)
$ppGpp^0$*ΔgreA*	1131.65 (+/-33.99)	42.91 (+/-6.30)
$ppGpp^0$*ΔgreAΔgraL*	913.16 (+/-20.62)	42.31 (+/-3.12)

Table S5. β-galactosidase activities (expressed as Miller units), calculated for data presented in Figure 1 E&F. These specific activities were calculated for curve regions where absolute activities increase linearly with OD_{600}. At least 3 points from each curve where included in the calculations. S.D. is given in parenthesis.

Plasmid/Strain	$ppGpp^0$ (control)	$ppGpp^0$*ΔgreA*	$ppGpp^0$*ΔgreAΔgraL*
pGraL	18.46 (+/-1.17)	28.20 (+/-0.90)	27.31 (+/-0.96)
pGraL + 1mM IPTG	19.29 (+/-0.56)	29.99 (+/-2.36)	29.26 (+/-2.38)
pGreA	19.16 (+/-0.42)	19.23 (+/-1.32)	20.10 (+/-2.15)
pGreA + 1mM IPTG	26.42 (+/-3.23)	26.43 (+/-1.22)	26.74 (+/-1.65)

A.



B.

AUCAAAAUGUGAAUUGUAGCUGACCUGGGACUUGUACCCGGGUCGGUAUUUUUUUGCUU

C.

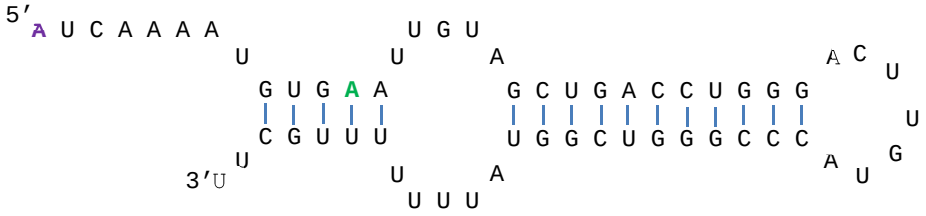


Figure S1. GraL location, sequence and predicted structure. (A) Location of GraL within the *greA* leader region. P1 and P2 promoter start sites are indicated relative to the first AUG codon of *greA*. Termination takes place within the -100 to -90 region of the leader sequence, indicated here with a T. (B) GraL sequence. P1 and P2 transcription start sites are indicated in purple and green, respectively. (C) GraL structure predicted with the Mfold software [M. Zuker (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* **31:** 3406-3415].

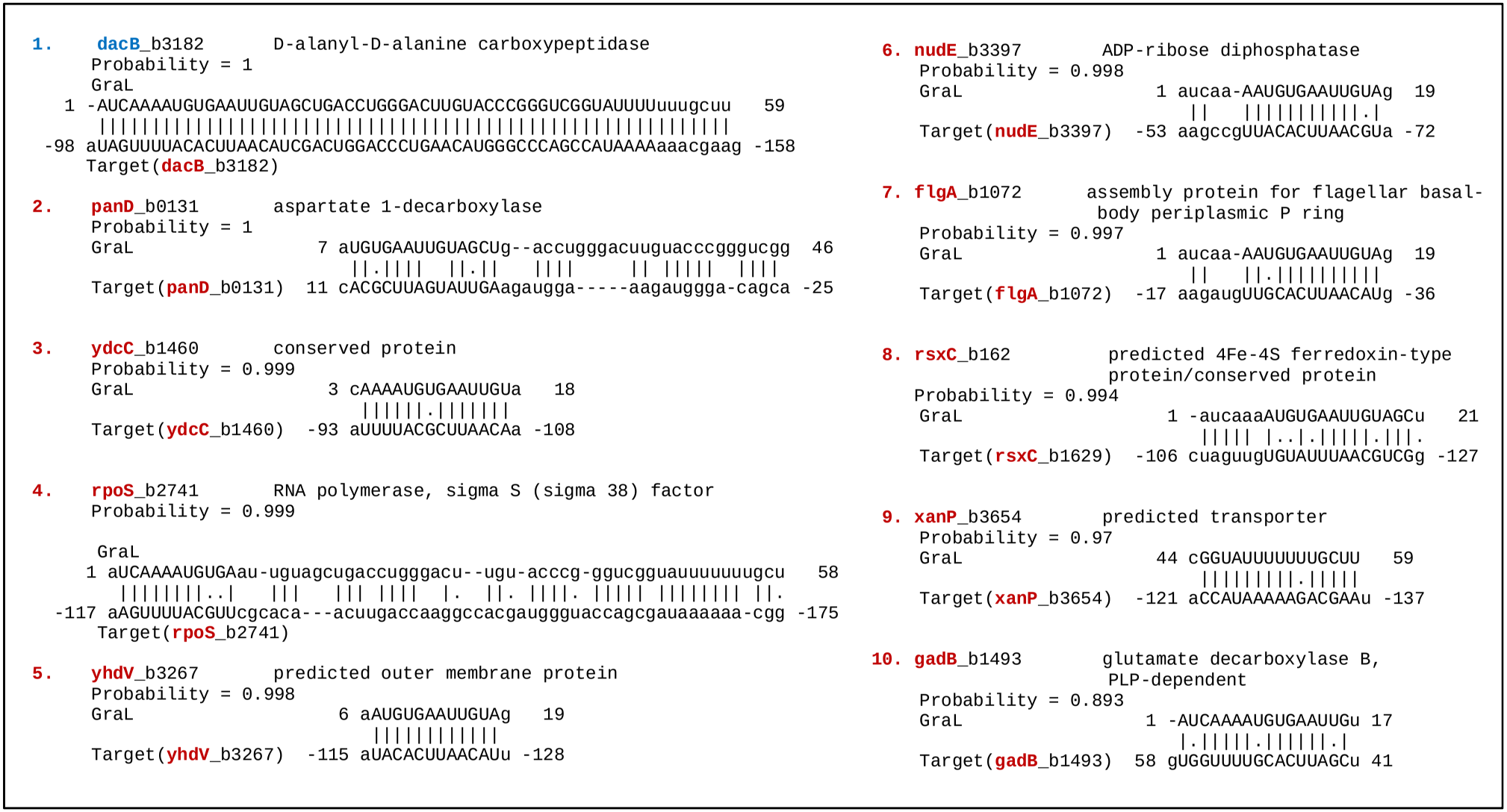


Figure S2. Full-length GraL mRNA targets predicted with the sTarPicker software. (Ying et al., 2011) Top 10 results are presented. All of the targets were confirmed to have upstream untranslated regions within the region predicted to interact with GraL. For example, *rpoS* has a long 5'UTR of 567 nucleotides.
Probability score is based on 1000 classifiers (for e.g. if a given interaction gives 700 positive classifier predictions, the score is 0.7; generally a score above 0.5 is considered as sRNA is interacting with mRNA). Numbers in mRNA refer to the nucleotide position relative to the first AUG codon.
* Please note that the region that was attributed the highest overall score and annotated as *dacB*, lies in the intergenic region between *greA* and *dacB*, and thus corresponds to GraL itself.

Figure S3. P2 promoter originating GraL mRNA targets predicted with the sTarPicker software (Ying et al., 2011). Similarly to Figure S1, probability score is based on 1000 classifiers. Numbers in mRNA refer to the nucleotide position relative to the first AUG codon.

* Please note that the region that was attributed the highest overall score and annotated as *dacB*, lies in the intergenic region between *greA* and *dacB*, and thus corresponds to GraL itself.

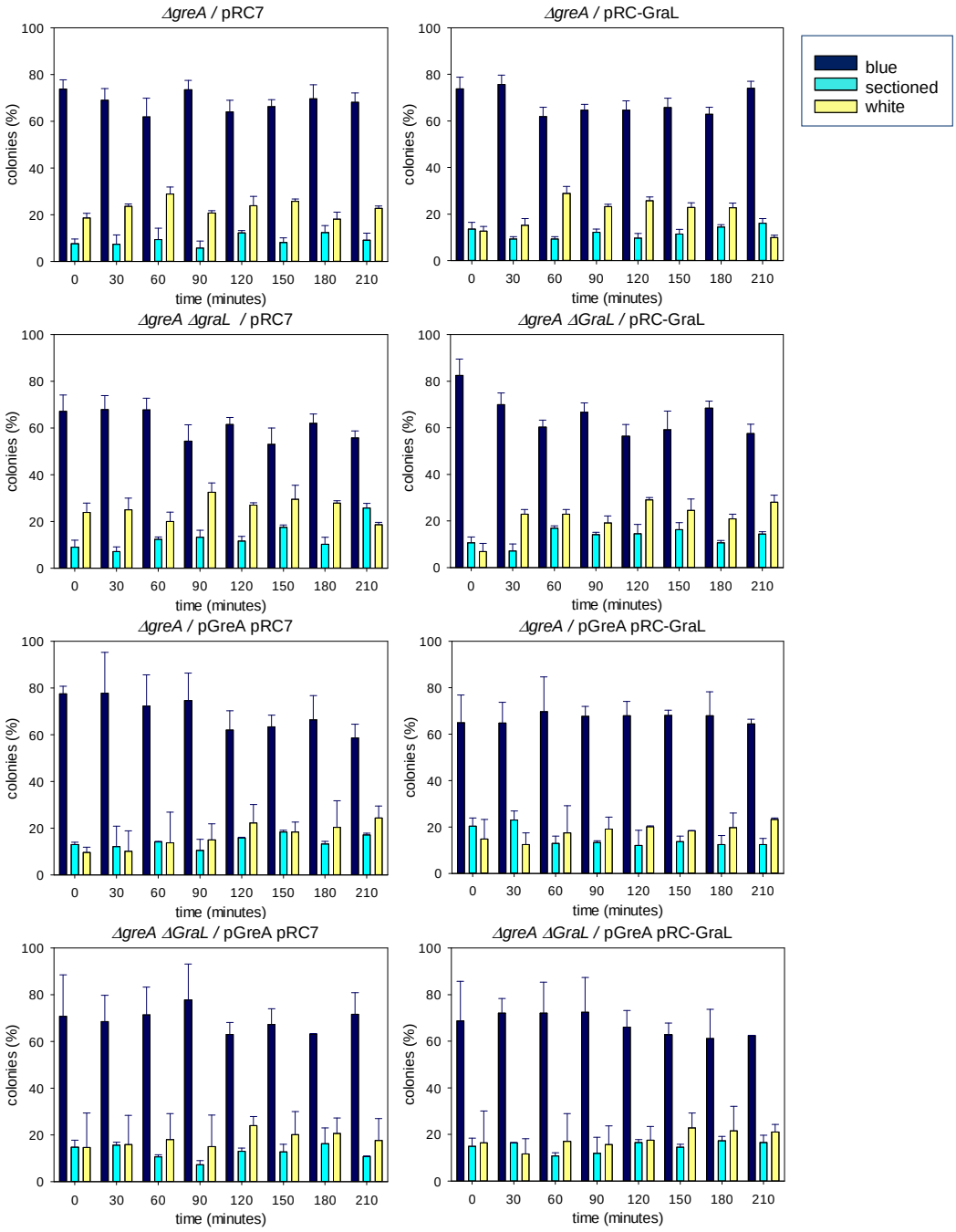


Figure S4. pRC7 and pRC-GraL plasmid maintenance in the ppGpp$^+$ strains. Bacteria were inoculated into 50ml LB and cultured without any antibiotic pressure. Every 30 min, a sample was withdrawn and cells were plated on LB plates with X-gal. The number of completely blue ("blue"), completely white ("white") or sectioned colonies ("sectioned") was being monitored. When present, GreA was overproduced from the pHM1873 (pGreA) plasimd. Relevant strain numbers are listed in Table S1. Experiments were done in duplicate. Error bars represent S.E.M.

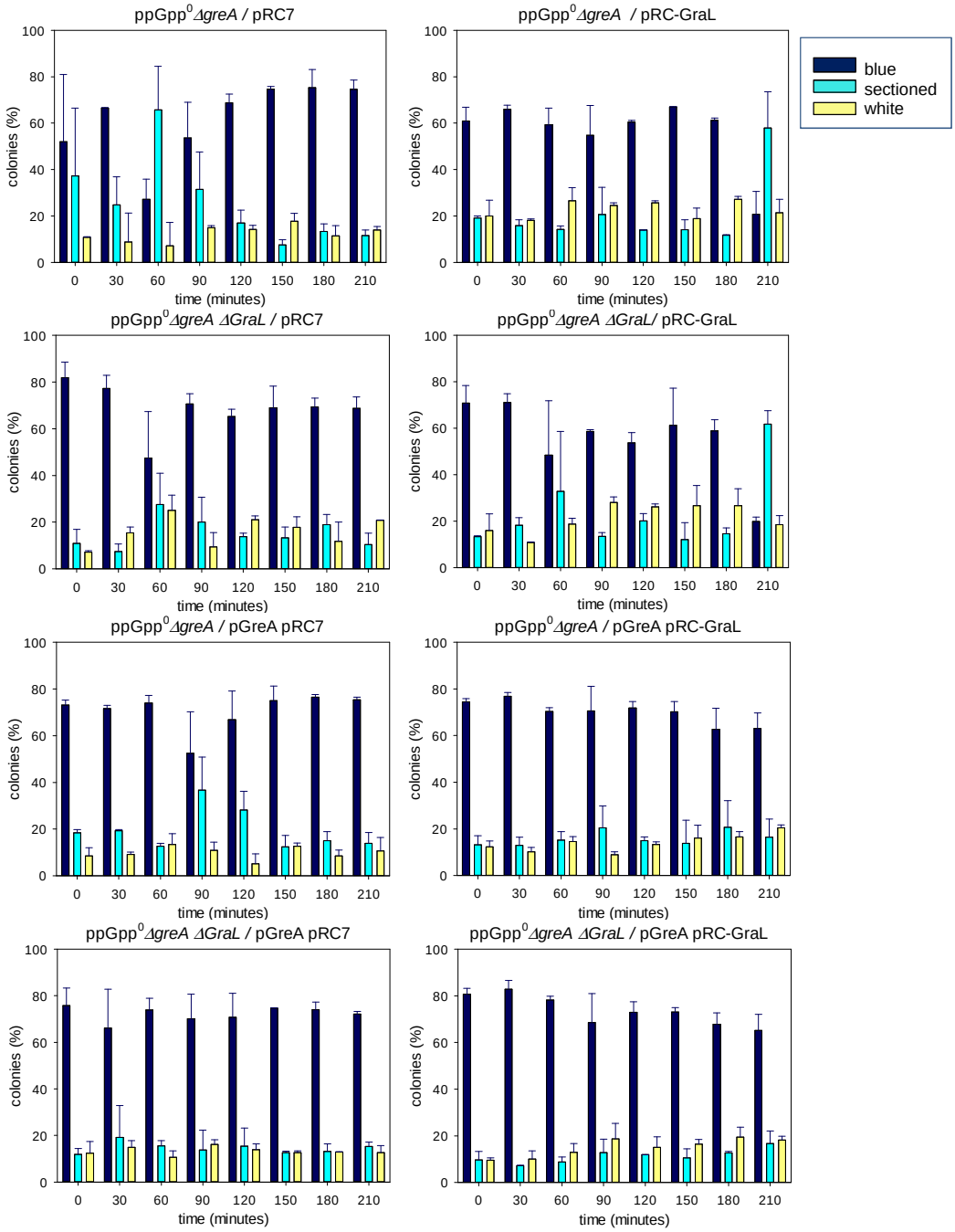


Figure S5. pRC7 and pRC-GraL plasmid maintenance in the ppGpp^0 strains. Bacteria were inoculated into 50ml LB and cultured without any antibiotic pressure. Every 30 min, a sample was withdrawn and cells were plated on LB plates with X-gal. The number of completely blue (“blue”), completely white (“white”) or sectioned colonies (“sectioned”) was being monitored. When present, GreA was overproduced from the pHM1873 (pGreA) plasmid. Relevant strain numbers are listed in Table S1. Experiments were done in duplicate. Error bars represent S.E.M.