

Basal ppGpp signalling by SpoT integrates metabolism with acid resistance

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Running title: Basal ppGpp integrates metabolism and acid resistance

16 Abstract

17 Bacteria maintain low basal levels of the alarmone ppGpp during steady-state growth, yet how this
 18 basal state is established and why it matters physiologically remain poorly understood. Here, we show
 19 that basal ppGpp represents an actively maintained regulatory state that coordinates metabolic
 20 homeostasis with stress resistance. Using a targeted perturbation of SpoT regulation in *Escherichia*
 21 *coli*, we uncover a sharp requirement for a sub-basal yet non-zero ppGpp pool to sustain growth in
 22 minimal medium and survival under extreme acid stress. Disruption of this basal state leads to
 23 misallocation of metabolic flux into arginine biosynthesis, depletion of glutamate, and collapse of the
 24 glutamate-dependent Gad acid resistance system. Through intragenic suppressor analysis, enzymatic
 25 perturbations, and protein-level feedback measurements, we further demonstrate that SpoT
 26 intrinsically tunes basal ppGpp through a distributed intramolecular regulatory network coupled to
 27 negative feedback control of SpoT abundance. This regulatory logic stabilizes ppGpp within a narrow
 28 physiological window, below the threshold of canonical stringent response activation. The
 29 requirement for SpoT-dependent basal ppGpp regulation is conserved in pathogenic *Salmonella* and
 30 *Shigella*. Together, our findings establish basal ppGpp as a distinct and actively regulated signaling
 31 regime that integrates metabolism and stress preparedness beyond acute starvation responses.

32

33 **KEYWORDS:** basal ppGpp, SpoT, stringent response, metabolic homeostasis, acid resistance, Gad
 34 system

35 Introduction

36 Bacteria inhabit environments that fluctuate rapidly and often impose multiple, simultaneous
 37 stresses. Successful survival under such conditions requires tight coordination between core
 38 metabolic processes and stress-protective responses in a manner that preserves essential resources
 39 while maintaining viability. For enteric bacteria in particular, the ability to balance nutrient
 40 availability with resistance to extreme acidity - such as that encountered during gastric passage - is
 41 a key determinant of ecological fitness and pathogenic potential^{1,2}. Despite extensive study of
 42 individual stress-response pathways, how bacteria integrate metabolic homeostasis with acid
 43 resistance remains incompletely understood.

44 A central regulator of bacterial metabolic adaptation is the stringent response, a conserved signaling
 45 network mediated by the alarmone guanosine tetra- and pentaphosphate (ppGpp)³⁻⁶. Canonically,
 46 ppGpp is produced in large bursts upon amino acid starvation, when uncharged tRNAs activate the
 47 ribosome-associated enzyme RelA in *Escherichia coli*⁷⁻¹¹. This acute increase in ppGpp rapidly
 48 remodels transcription, repressing stable RNA synthesis while redirecting metabolic flux toward
 49 amino acid biosynthesis¹²⁻¹⁶. In parallel, ppGpp directly targets numerous metabolic enzymes,
 50 rebalancing nucleotide and amino acid pools¹⁷⁻²¹. This RelA-driven stringent response has therefore
 51 been widely viewed as an emergency program that facilitates rapid bacterial adaptation to acute
 52 nutrient stress.

53 Once this acute response has restored metabolic balance and growth resumes, ppGpp levels decline
 54 but do not return to zero. In contrast to the well-characterized high-amplitude stringent response,
 55 much less is known about the physiological role of these low, basal levels of ppGpp that persist
 56 during adapted growth²². Basal ppGpp is believed to be primarily controlled by SpoT, a
 57 bifunctional enzyme that both synthesizes and hydrolyzes ppGpp²³ and responds to diverse

environmental cues distinct from amino acid starvation²⁴⁻³⁰. Genetic and physiological studies have established that SpoT activity is essential for viability, virulence, and stress adaptation across many bacterial species^{3,31,32}. However, because SpoT's hydrolase activity is required to counterbalance RelA-mediated ppGpp synthesis, direct analysis of SpoT regulatory function has been challenging. As a result, basal ppGpp has often been treated as a passive background signal rather than an active regulatory regime with distinct physiological roles.

Acid resistance provides a compelling context in which to examine basal ppGpp regulation. *E. coli* is capable of surviving exposure to pH values as low as 2.5 through multiple acid resistance systems, among which the glutamate-dependent Gad system is the most effective^{2,33,34}. This system uses cytoplasmic glutamate decarboxylases (GadA and GadB) to convert glutamate to γ -aminobutyrate, consuming intracellular protons, while the GadC antiporter exchanges γ -aminobutyrate for extracellular glutamate to sustain the cycle^{35,36}. The Gad system is subject to complex regulation involving transcriptional activators (GadE), global stress regulators (e.g., ppGpp), and growth-phase signals (e.g., RpoS)^{2,36}. Notably, glutamate itself occupies a central position in cellular metabolism, serving as a key nitrogen donor and metabolic hub. How bacteria coordinate glutamate homeostasis with glutamate-dependent acid resistance under nutrient-limited conditions remains poorly understood.

Here, we set out to investigate how SpoT-dependent basal ppGpp signaling contributes to the integration of amino acid metabolism with acid resistance during nutrient-limited growth. As an entry point into this poorly understood regulatory regime, we focused on a strictly conserved histidine residue (H414) within the C-terminal regulatory region of SpoT. Perturbation of this residue resulted in pronounced physiological imbalances, including altered metabolic flux through arginine biosynthesis, depletion of intracellular glutamate, and reduced expression of the glutamate-

dependent Gad acid resistance system. These observations provided a framework for systematically dissecting how SpoT-dependent basal ppGpp supports metabolic and stress-response homeostasis.

Because SpoT homologs are conserved across enteric bacteria, we further asked whether the regulatory principles revealed by this approach extend beyond *E. coli*. Addressing this question allowed us to evaluate whether SpoT-dependent basal ppGpp represents a species-specific phenomenon or a more general regulatory regime. In this context, our study seeks to expand the functional scope of ppGpp signaling beyond RelA-mediated acute starvation responses to include a role in coordinating metabolism and stress resistance during nutrient-limited growth.

Results

Perturbation of SpoT regulation leads to severe growth defects in minimal medium

To examine how SpoT-dependent regulation supports growth under nutrient-limited conditions independently of RelA, we focused on the conserved C-terminal regulatory region of SpoT, which is highly conserved among RelA/SpoT homologs but remains poorly understood mechanistically. Sequence alignment identified a strictly conserved histidine residue (H414 in *E. coli* SpoT) within the TGS domain, corresponding to H432 in RelA (**Extended Data Fig. 1a, 1b**), a residue crucial for sensing amino acid starvation⁸⁻¹¹.

As a genetic probe of this conserved regulatory region, we generated a SpoT H414A substitution in a $\Delta relA$ background. While the $\Delta relA spoT^{wt}$ (hereafter referred to as $spoT^{wt}$) strain grew normally, the H414A mutant ($spoT^{H414A}$) displayed a severe and unexpected growth defect on glucose minimal medium (M9Glc), both on solid and in liquid culture (**Fig. 1a, 1b**). This unanticipated phenotype indicated that SpoT H414 plays a critical role in supporting growth under nutrient-limited conditions and prompted further investigation into its underlying molecular basis.

Loss of SpoT regulation disrupts transcriptional programs for stress and acid resistance

To gain insight into the molecular defects underlying the growth failure of *spoT^{H414A}* cells, we performed transcriptomic analysis of the *spoT^{H414A}* and *spoT^{wt}* strains. Because *spoT^{H414A}* cells failed to proliferate in M9Glc, cultures were incubated under identical conditions until the *spoT^{wt}* strain entered early exponential phase, at which point RNA was harvested from both strains (Extended Data Fig. 1c).

This analysis revealed extensive transcriptional dysregulation in *spoT^{H414A}* cells, with strong repression of stress adaptation and acid resistance genes. Among the most prominently downregulated loci were the glutamate-dependent Gad acid resistance system (*gadE*, *gadX*, *gadW*, *gadA*, *gadB*, *gadC*), as well as additional components of the acid fitness island, including the periplasmic chaperones *hdeAB* (Fig. 2a, 2b). Genes associated with osmotic and general stress responses were similarly reduced; in contrast, genes linked to phage shock and SOS responses were strongly induced (Extended Data Fig. 1d-f), indicating a broad physiological imbalance. The pronounced repression of acid resistance pathways indicated a defect in acid survival and prompted direct physiological testing of *spoT^{H414A}* cells under acidic conditions.

SpoT, but not RelA, is required for survival under extreme acid stress

Guided by the pronounced transcriptional repression of acid resistance genes in *spoT^{H414A}* cells, we next examined survival under lethal acid stress. Upon exposure to pH2.5, *spoT^{H414A}* cells exhibited profound acid sensitivity, with viability declining rapidly and no detectable survivors after 60 minutes (Fig. 2c). In contrast, both the parental wild-type strain and the *spoT^{wt}* strain survived acid challenge efficiently, indicating that RelA is dispensable for acid resistance under this condition.

124 Notably, despite the severe acid sensitivity of *spoT^{H414A}* cells, viability under neutral, well-buffered
125 conditions (M9Glc) was preserved (**Extended Data Fig. 1g**), suggesting that SpoT H414 is
126 specifically required to support stress preparedness rather than basal cell viability. Together, these
127 results identify SpoT, rather than RelA, is required to support survival under extreme acid stress in
128 *E. coli*.

129 **Loss of SpoT regulation drives arginine overflow and glutamate depletion**

130 To define the metabolic basis of the growth defect observed in *spoT^{H414A}* cells, we examined
131 transcriptomic changes in central metabolic pathways. While expression of many amino acid
132 biosynthetic genes was modestly reduced, one pathway was prominently induced: arginine
133 biosynthesis was strongly and coordinately upregulated (**Fig. 3a, 3b**). All the nine enzymes of the
134 arginine biosynthetic pathway exhibited significantly increased expression, together with *carAB*,
135 which supplies carbamoylphosphate to this pathway. This pattern indicates a pronounced
136 redirection of metabolic flux toward arginine production.

137 In contrast, genes directing carbamoylphosphate toward pyrimidine nucleotide biosynthesis were
138 downregulated, suggesting selective diversion of this shared intermediate rather than a generalized
139 increase in anabolic metabolism. Because arginine biosynthesis consumes glutamate as a precursor,
140 the coordinated induction of the *arg* operons is predicted to substantially deplete intracellular
141 glutamate pools.

142 In parallel with arginine pathway induction, genes involved in *de novo* purine nucleotide
143 biosynthesis were broadly upregulated (**Extended Data Fig. 2**). Many of these reactions consume
144 glutamine as a nitrogen donor, which is regenerated from glutamate, thereby imposing an
145 additional, indirect demand on glutamate availability. Thus, the combined activation of arginine and

purine biosynthetic programs is expected to exacerbate glutamate depletion, compounding the metabolic imbalance caused by SpoT H414A mutation.

Together, these transcriptional signatures point to a misallocation of metabolic resources upon loss of SpoT H414, with excessive flux into arginine biosynthesis and increased purine nucleotide production collectively depleting glutamate. Given the central role of glutamate in both metabolism and acid resistance, this imbalance offers a unifying explanation for the growth and stress survival defects observed in *spoT^{H414A}* cells and motivated direct tests of glutamate availability in subsequent experiments.

Glutamate depletion contributes to growth defects caused by SpoT perturbation

Prompted by the transcriptomic evidence for glutamate depletion, we tested whether exogenous supplementation could restore growth. Addition of glutamate to M9Glc robustly improved growth of *spoT^{H414A}* cells, substantially shortening the lag phase and increasing final biomass (**Fig. 3c**). In contrast, supplementation with arginine or uracil failed to restore growth, and co-supplementation with glutamate conferred no additional benefit.

Among all 20 amino acids, supplementation with glutamine, asparagine, or proline partially restored growth, consistent with their metabolic proximity to glutamate, but none matched the effect of glutamate itself (**Extended Data Fig. 3a**). Increasing the concentration of glutamate up to tenfold failed to further improve growth (**Extended Data Fig. 3b**), and combining glutamate with glutamine, asparagine, or proline did not enhance regrowth beyond that achieved with glutamate alone (**Extended Data Fig. 3c**). In contrast, supplementation with all 20 amino acids fully restored the growth defect of *spoT^{H414A}* cells to a level exceeding that of the *spoT^{wt}* strain (**Extended Data Fig. 3d**), indicating that glutamate depletion represents a dominant, though not exclusive, metabolic

limitation associated with SpoT H414A dysfunction. Importantly, glutamate supplementation had only minimal effects on the growth of a ppGpp⁰ (*ΔrelA ΔspoT*) strain (**Extended Data Fig. 3e**), indicating that growth rescue requires SpoT-dependent basal ppGpp rather than nonspecific nutritional supplementation.

To directly test whether arginine biosynthesis drives glutamate depletion, we deleted *argA*, encoding the first committed enzyme of the arginine biosynthetic pathway. As expected, the resulting *spoT^{wt} ΔargA* strain failed to grow in M9Glc; this defect was fully rescued by arginine supplementation but not by glutamate, confirming that growth limitation in this background reflects arginine auxotrophy rather than glutamate deficiency (**Fig. 3d**). Similarly, the *spoT^{H414A} ΔargA* strain failed to grow in M9Glc (**Fig. 3d**). Notably, arginine, but not glutamate, largely restored growth in this background, indicating that blocking arginine biosynthesis prevents excessive glutamate consumption and alleviates the growth defect of *spoT^{H414A}*.

Arginine biosynthesis is normally subject to feedback inhibition through ArgA. Conversely, we found that artificial overexpression of a feedback-resistant ArgA variant (ArgA^{H15Y})³⁷ via IPTG induction phenocopied the *spoT^{H414A}* growth defect in M9Glc, and this delayed growth was substantially alleviated by glutamate supplementation (**Fig. 3e**). Together, these results establish that SpoT H414 restrains arginine-driven glutamate consumption, thereby preserving amino acid homeostasis required for growth in minimal medium.

SpoT perturbation compromises glutamate-dependent acid resistance

Because glutamate is the essential substrate for the Gad acid resistance system, we next asked whether glutamate depletion contributes to the acid sensitivity of *spoT^{H414A}* cells. Supplementation of the acid challenge medium with glutamate fully rescued survival during short-term acid exposure

and markedly improved long-term viability (**Fig. 4a**). In contrast, supplementation with arginine or lysine provided only modest protection (**Extended Data Fig. 4a**), consistent with the prominent contribution of the glutamate-dependent Gad system under extreme acid stress.

To determine whether impaired regulation of the Gad system accounted for the acid resistance defect, we ectopically expressed *gadE*, the master transcriptional activator of Gad genes. Even low-level expression of *gadE* significantly restored acid resistance in *spoT^{H414A}* cells, and combined supplementation with glutamate further enhanced survival (**Extended Data Fig 4b**). These interventions did not alter acid resistance in *spoT^{wt}* cells, indicating that the defect arises specifically from SpoT dysfunction. Notably, despite its ability to restore acid survival, ectopic expression of *gadE* failed to improve the growth of *spoT^{H414A}* cells in M9Glc (**Extended Data Fig. 4c**). This functional separation indicates that impaired acid resistance is not sufficient to account for the growth defect, which is more closely associated with glutamate depletion and broader metabolic misallocation.

We further examined acid tolerance across a range of pH values. In addition to catastrophic sensitivity at pH 2.5, *spoT^{H414A}* cells exhibited reduced survival under mildly acidic conditions (pH 4.0-6.0) (**Fig. 4b, Extended Data Fig 4d**), consistent with a broader impairment of multiple acid stress response pathways (**Extended Data Fig 4e**). Notably, these defects occurred despite preserved viability under neutral pH conditions (**Extended Data Fig 1g**), indicating that SpoT-mediated basal regulation selectively primes cells for acid stress rather than broadly supporting growth.

Intragenic suppression reveals intrinsic regulation within SpoT

211 These findings raised a fundamental question of how SpoT activity is regulated to maintain the
 212 physiological state underlying growth and stress resistance. The severe growth and acid resistance
 213 defects of *spoT^{H414A}* cells suggested that H414 plays a central regulatory role in SpoT function. To
 214 determine whether this phenotype reflected impaired regulation by trans-acting protein factors, we
 215 first tested overexpression of established SpoT regulators, including ObgE³⁸, Rsd³⁹, YtfK⁴⁰, and
 216 AcpP²⁵, none of which measurably restored growth of *spoT^{H414A}* cells in minimal medium
 217 (**Extended Data Fig 5**). We further explored the possibility that H414 mediates regulation through
 218 interaction with previously unidentified protein partners by introducing a photo-crosslinkable
 219 unnatural amino acid at position 414 and performing UV-activated crosslinking under the same
 220 growth conditions. Despite robust expression of full-length SpoT proteins, we did not detect
 221 reproducible interactions with additional protein factors (**Extended Data Fig. 6**). Together, these
 222 results suggested that the regulatory function of H414 is unlikely to be mediated by trans-acting
 223 protein partners. We therefore pursued an unbiased genetic approach and isolated spontaneous
 224 suppressor mutants capable of restoring growth of *spoT^{H414A}* cells on M9Glc. After prolonged
 225 incubation (24-72 h), colonies of varying sizes emerged on M9Glc agar (**Fig. 5a**).

226 Analysis of 50 independently isolated suppressors revealed that the strongest suppressors
 227 predominantly carried secondary mutations within the *spoT* gene itself. Among large-colony
 228 suppressors, we identified recurrent substitutions at residues G334 (G334S; supA, 17/50), E364
 229 (E364K; supB, 5/50), and S463 (S463R; sup41, 9/50) as well as a small in-frame deletion within
 230 the synthetase domain ($\Delta V_{200}IKE$; sup17, 3/50) (**Fig. 5b**). Whole-genome sequencing of small-
 231 colony suppressors lacking *spoT* mutations identified a single mutation in *rpoB* (K1262Q), which is
 232 likely similar as the previously described stringent-response suppressors⁴¹ supporting the growth of
 233 ppGpp⁰ strain on minimal media, and therefore these strains were excluded from further analysis.

234 Reconstruction of the identified *spoT* suppressor mutations in a clean background fully
 235 recapitulated growth restoration of *spoT*^{H414A} in M9Glc (**Extended Data Fig 7**), whereas deletion of
 236 *spoT* in the suppressor strains abolished growth (**Fig. 5c**). These results demonstrate that intragenic
 237 mutations within SpoT are both necessary and sufficient to suppress the *spoT*^{H414A} growth defect.

238 We next asked whether suppression of the growth defect was accompanied by restoration of acid
 239 resistance. All four *spoT* suppressor strains exhibited improved survival under lethal acid challenge
 240 (pH 2.5) compared to the parental *spoT*^{H414A} strain, although the degree of rescue varied (**Fig. 5d**).
 241 The suppressor sup41 displayed the most robust acid resistance, followed by supB, supA and sup17.
 242 Supplementation of the acid challenge medium with glutamate further enhanced survival across all
 243 suppressor strains, with particularly pronounced effects in the weaker suppressors supB and sup17
 244 (**Fig. 5d**). These differences suggest that individual suppressor mutations restore SpoT function
 245 through distinct mechanisms that differentially affect glutamate homeostasis and stress
 246 preparedness.

247 Together, these results show that intragenic suppression of SpoT H414A simultaneously restores
 248 growth and acid resistance, reinforcing the link between SpoT activity, metabolic balance, and
 249 stress survival.

250 **Basal ppGpp is actively maintained within a narrow functional window**

251 Given SpoT's dual role as a ppGpp synthase and hydrolase, we next asked whether the growth
 252 defect of *spoT*^{H414A} cells is associated with altered ppGpp homeostasis, and whether suppressor
 253 mutations restore a physiologically relevant ppGpp state. To address this, we measured intracellular
 254 ppGpp levels using ³²P-orthophosphate labeling and thin-layer chromatography¹⁸. As expected,
 255 *spoT*^{wt} cells produced a readily detectable basal ppGpp signal, whereas *spoT*^{H414A} cells accumulated

only marginal, near-background levels of ppGpp (**Fig. 6a**). Notably, ppGpp levels in the suppressor strains remained close to the detection limit and were not substantially elevated relative to *spoT^{H414A}* cells.

This observation raised the possibility that either very small increases in ppGpp are sufficient to restore physiological function, or that ppGpp turnover is highly dynamic in suppressor strains. To distinguish between these possibilities, we genetically perturbed SpoT enzymatic activities. Inactivation of the ppGpp synthetase activity of SpoT by the E319Q substitution in suppressor backgrounds severely delayed or abolished growth recovery in M9Glc (**Fig. 6b**), demonstrating that ppGpp synthesis is required for suppression. Conversely, inactivation of SpoT's hydrolase activity by the H72A/D73A substitution led to robust ppGpp accumulation (**Fig. 6c**) and accelerated growth resumption (**Fig. 6d**), indicating that suppressor strains produce ppGpp that is rapidly turned over under basal conditions. Notably, the hydrolase-inactive variants of supA and sup17 could not be obtained, likely due to the toxicity associated with excessive ppGpp accumulation.

Because C-terminal domains of RelA/SpoT homologs are known to regulate the catalytic activities of the N-terminal enzymatic domains, we next systematically truncated these regulatory regions to assess how suppressor mutations alter intramolecular control of SpoT activity. Consistent with this model, truncation of individual C-terminal domains revealed that distinct suppressor mutations differentially modify the contribution of these domains to growth control (**Extended Data Fig. 8**), without abolishing the requirement for basal ppGpp synthesis. These findings indicate that SpoT H414 functions within a broader intramolecular regulatory network that fine-tunes basal ppGpp production, rather than acting as an isolated molecular switch.

277 Together, these data demonstrate that a sub-basal but non-zero ppGpp pool - close to the detection
278 limit of conventional assays - is sufficient to support growth in minimal medium, and that SpoT
279 activity is finely tuned to maintain this pool within a narrow physiological range.

280 **Negative feedback between ppGpp and SpoT abundance stabilizes basal ppGpp**

281 To investigate how SpoT activity is regulated, we examined SpoT protein levels using an antiserum
282 raised against the C-terminal regulatory domains of SpoT. Immunoblot analysis revealed that SpoT
283 protein levels were markedly elevated in *spoT^{H414A}* cells compared to *spoT^{wt}* and suppressor strains
284 (**Fig. 6e**).

285 Because *spoT^{H414A}* cells produce sub-basal ppGpp levels, whereas suppressor strains restore
286 physiological function with slightly elevated but tightly controlled ppGpp, these results suggest the
287 existence of a negative feedback mechanism in which basal ppGpp limits SpoT abundance. Such
288 feedback would stabilize intracellular ppGpp levels by adjusting SpoT protein concentration in
289 response to alarmone availability. This regulatory relationship provides a parsimonious mechanistic
290 framework for how SpoT may maintain a narrow basal ppGpp range sufficient for metabolic
291 homeostasis without eliciting an acute stringent response.

292 **Graded restoration of transcriptional programs reflects quantitative tuning of basal SpoT** 293 **activity**

294 To understand how suppressor mutations restore growth and acid resistance, we performed RNA
295 sequencing on the four suppressor strains. Parallel coordinates and multidimensional scaling
296 analyses revealed that strong suppressors (supA and sup41) clustered closely with *spoT^{wt}* cells,
297 whereas weaker suppressors (supB and sup17) occupied intermediate positions between *spoT^{H414A}*
298 and *spoT^{wt}* (**Extended Data Fig. 9a,9b**).

299 Across all suppressors, a core set of genes involved in amino acid metabolism and stress adaptation
 300 was consistently restored (**Extended Data Fig. 9c**), whereas genes aberrantly induced in *spoT^{H414A}*
 301 cells were broadly repressed (**Extended Data Fig. 9d**). Moreover, strong suppressors (supA and
 302 sup41) largely restored expression of acid resistance genes and concomitantly repressed phage
 303 shock and SOS response pathways; in contrast, weaker suppressors (supB and sup17) exhibited
 304 only partial correction of these transcriptional defects (**Extended Data Fig. 10**), consistent with
 305 their physiological phenotypes (**Fig. 5c**).

306 Together, these results indicate that suppressor mutations restore SpoT-dependent regulation in a
 307 graded fashion, with transcriptional recovery closely correlating with physiological rescue.

308 **The regulatory function of SpoT H414 is conserved across enteric pathogens**

309 Finally, we asked whether the regulatory role of SpoT H414 is conserved in pathogenic
 310 enterobacteria. We introduced the corresponding H414A substitution into SpoT homologs from
 311 *Salmonella enterica* serovar Typhimurium and *Shigella flexneri* and expressed these proteins in an
 312 *E. coli* ppGpp⁰ background.

313 Wild-type SpoT homologs from both species largely restored growth in M9Glc and acid resistance,
 314 whereas the corresponding H414A variants failed to support either phenotype (**Fig. 7a-d**). Notably,
 315 glutamate supplementation rescued both growth and acid resistance defects in the *Shigella* SpoT_{sf}
 316 H414A strain but not in the *Salmonella* SpoT_{st} H414A variant. This differential rescue correlated
 317 with the extent of sequence divergence within the C-terminal regulatory regions of SpoT. *S. flexneri*
 318 SpoT_{sf} differs from *E. coli* SpoT_{ec} at only a single conserved position (A614G) within the CC
 319 domain, whereas *S. Typhimurium* SpoT_{st} shares 98.15% overall identity but contains multiple amino
 320 acid substitutions distributed across the C-terminal domains, including a cluster of nine

substitutions within linker-2 between TGS and CC domains (**Extended Data Fig. 11**). These sequence differences are likely to modulate intramolecular regulatory interactions within SpoT, thereby influencing the ability of the H414A variants to respond to glutamate supplementation, akin to the differential behaviors of the intragenetic suppressor strains (**Fig. 5d**).

Altogether, these results demonstrate that SpoT H414 plays a conserved and functionally important role in coordinating growth and acid resistance across enteric pathogens, highlighting the physiological and evolutionary relevance of basal SpoT regulation.

Discussion

Basal ppGpp defines a distinct regulatory regime coordinating metabolism and stress resistance

The stringent response has classically been viewed as an acute adaptation to amino acid starvation, driven by RelA-dependent bursts of ppGpp that globally reprogram transcription and metabolism (**Fig. 7e**, top). In this canonical framework, ppGpp functions as an emergency alarmone that rapidly suppresses growth-associated biosynthetic programs and redirects cellular resources upon severe nutrient limitation.

Our findings reveal a complementary and previously underappreciated mode of ppGpp signalling that operates outside this acute starvation paradigm. By dissecting the function of a conserved regulatory residue in SpoT, H414, we identify a basal ppGpp state that is actively maintained during nutrient-limited growth and is essential for coordinating metabolic homeostasis with stress preparedness (**Fig. 7e**, middle). Perturbation of this state uncovers a sharp physiological threshold below which metabolic balance and stress resistance collapse (**Fig. 7e**, bottom).

Importantly, the phenotypes observed in *spoT^{H414A}* cells are not readily explained by defects in canonical RelA-mediated stringent response activation. All experiments were conducted in a *ΔrelA* background and under conditions that do not elicit acute amino acid starvation. Moreover, the transcriptional profile of *spoT^{H414A}* cells is fundamentally distinct from those reported in classical stringent response studies driven by RelA-dependent ppGpp bursts^{15,16}. Loss of SpoT H414 does not recapitulate either RelA-activated or ppGpp⁰ transcriptional states. Instead, *spoT^{H414A}* cells exhibit a selective and imbalanced response marked by overcommitment to arginine and nucleotide biosynthesis, depletion of central metabolites such as glutamate, and failure to sustain specific stress-response programs, most notably the glutamate-dependent acid resistance system.

Together, these observations support the existence of a regulatory regime operating at ppGpp levels above zero yet below the basal state normally maintained by wild-type SpoT. Within this low-amplitude range, ppGpp does not function as a global transcriptional on-off switch, but instead acts as a quantitative tuning signal that constrains metabolic flux allocation and preserves stress preparedness. Loss of this basal control therefore exposes physiological vulnerabilities that are not captured by classical models of the stringent response. In this framework, SpoT functions not merely as a modulator of RelA activity, but as a primary regulator of a stabilized physiological state. This low but non-zero ppGpp levels act not as an emergency brake on growth, but as a buffering signal that integrates metabolism with stress preparedness during nutrient-limited growth.

SpoT-mediated control of glutamate homeostasis underlies stress preparedness without compromising basal viability

A central mechanistic insight from this study is that SpoT-dependent basal ppGpp constrains metabolic flux allocation to preserve glutamate homeostasis. Transcriptomic profiling of *spoT^{H414A}* cells revealed coordinated induction of arginine biosynthesis (**Fig. 3a,b**), which directly consumes

365 glutamate, together with upregulation of *de novo* purine nucleotide biosynthesis (**Extended Data**
 366 **Fig. 3a,b**), which indirectly increases glutamate demand through enhanced glutamine utilization.
 367 This pattern reflects a loss of quantitative control over anabolic commitment rather than selective
 368 activation of a single biosynthetic pathway.

369 Genetic and physiological analyses identify arginine biosynthesis as the primary driver of glutamate
 370 depletion in this context. Blocking the first committed step of arginine synthesis prevents glutamate
 371 starvation and restores growth (**Fig. 3d**), whereas forced arginine overproduction phenocopies the
 372 *spoT^{H414A}* defect (**Fig. 3e**).

373 Glutamate occupies a uniquely central position in bacterial physiology, serving both as a metabolic
 374 hub and as the essential substrate for the glutamate-dependent Gad acid resistance system.
 375 Accordingly, glutamate depletion provides a parsimonious explanation for the coupled phenotypes
 376 observed in *spoT^{H414A}* cells, including impaired growth in minimal medium and failure of acid
 377 resistance. Glutamate supplementation and GadE-dependent activation of the acid resistance
 378 program rescue survival under low-pH challenge (**Fig. 4a; Extended Data Fig. 4b**), indicating that
 379 SpoT-dependent basal ppGpp enforces a metabolic prioritization strategy that preserves metabolites
 380 required for effective stress defence.

381 Importantly, disruption of this basal regulatory state does not cause immediate loss of viability
 382 under neutral, well-buffered conditions (**Extended Data Fig. 1g**), despite severe repression of acid
 383 resistance genes and extreme sensitivity to low pH (**Fig. 2c**). This separation between basal growth
 384 capacity and stress survival indicates that SpoT-derived basal ppGpp primarily maintains
 385 physiological preparedness rather than minimal viability. This distinction helps explain why the
 386 most pronounced phenotypes of *spoT^{H414A}* cells emerge only upon environmental challenge.

387 Notably, RelA is dispensable for acid resistance under the conditions examined (**Fig. 2c**),
 388 highlighting a functional division of labour between the two stringent response enzymes. Whereas
 389 RelA mediates acute, high-amplitude responses to amino acid starvation, SpoT maintains a low-
 390 level regulatory state that buffers metabolic allocation and supports long-term stress readiness.

391 **SpoT maintains basal ppGpp through coupled activity tuning and abundance feedback**

392 The identification of intragenic suppressors of *spoT*^{H414A} reveals that SpoT encodes an intrinsic
 393 regulatory system capable of quantitatively tuning basal ppGpp levels. Suppressor mutations
 394 distributed across both the catalytic and regulatory regions of SpoT restore growth (**Fig. 5c**) and
 395 acid resistance (**Fig. 5d**) without producing large, readily detectable increases in ppGpp (**Fig. 6a**).
 396 These observations indicate that physiological rescue does not require restoration of wild-type
 397 ppGpp abundance, but instead depends on re-establishing a narrowly defined sub-basal ppGpp
 398 state, consistent with the graded restoration of transcriptional programs observed across suppressor
 399 strains (**Extended Data Fig. 9,10**).

400 Direct perturbation of SpoT enzymatic activities supports this interpretation. Inactivation of ppGpp
 401 synthesis abolishes suppressor-mediated growth recovery (**Fig. 6b**), whereas inactivation of SpoT
 402 hydrolase activity leads to substantial ppGpp accumulation (**Fig. 6c**) and accelerated growth
 403 resumption (**Fig. 6d**). These results indicate that suppressor mutations rebalance SpoT synthetic and
 404 hydrolytic activities rather than bypassing the requirement for ppGpp. The inability to construct
 405 synthetase-only variants in specific suppressor backgrounds further underscores the importance of
 406 quantitative control. In these contexts, elevated ppGpp levels are likely deleterious, consistent with
 407 the well-established growth-inhibitory effects of excessive ppGpp. Thus, both insufficient and
 408 excessive ppGpp are incompatible with optimal growth, defining a sharply constrained
 409 physiological window for basal ppGpp function.

410 However, tuning of catalytic activity alone is unlikely to be sufficient to stabilize a low-amplitude
411 signalling state over extended periods of growth. Consistent with this, SpoT protein levels are
412 markedly elevated in *spoT^{H414A}* cells but reduced in suppressor backgrounds (**Fig. 6e**), revealing an
413 additional layer of regulation mediated by negative feedback on SpoT abundance. This inverse
414 relationship suggests that basal ppGpp limits SpoT accumulation, thereby constraining total
415 enzymatic capacity for ppGpp turnover.

416 Together, quantitative tuning of SpoT catalytic activity and feedback control of SpoT abundance
417 may enable precise maintenance of ppGpp within a narrow functional window. This coupled
418 regulatory architecture provides a mechanistic model for how bacteria stabilize a low-level ppGpp
419 signalling state sufficient for metabolic coordination and stress preparedness, yet below the
420 threshold that triggers growth arrest and the canonical stringent response.

421 **Conservation and implications of basal SpoT regulation**

422 The functional conservation of H414 across *Escherichia coli*, *Salmonella*, and *Shigella* SpoT
423 proteins demonstrates that this residue is broadly required for growth in minimal medium and
424 survival under extreme acid stress (**Fig. 7a-d**). At the same time, the differential ability of
425 *Salmonella* and *Shigella* SpoT H414A variants to respond to glutamate supplementation, akin to the
426 intragenetic suppressors (**Fig. 5d**), suggests that sequence divergence within C-terminal regulatory
427 regions modulates how basal SpoT regulation is implemented across species.

428 These observations indicate that, while the requirement for SpoT-mediated basal ppGpp control is
429 conserved, individual bacteria may tune SpoT regulatory domains to optimize metabolic and stress-
430 response coordination according to their metabolic capacity, ecological niches and life cycles. In
431 this framework, basal ppGpp does not represent a rigid or uniform signalling state, but rather a

432 conserved regulatory principle flexibly adapted through species-specific intramolecular tuning of
433 SpoT.

434 Several important questions remain, including the nature of the signal sensed by SpoT H414 and the
435 absolute intracellular concentrations and temporal dynamics of basal ppGpp. Addressing these
436 questions will require new approaches capable of resolving low-abundance alarmone pools with
437 high sensitivity, ideally at single-cell resolution, and across diverse growth conditions and bacterial
438 species. Elucidating how basal ppGpp selectively modulates distinct metabolic and stress-response
439 pathways will further clarify how this regulatory regime integrates diverse physiological demands.

440 Given the central role of acid resistance in enteric pathogenesis, SpoT-mediated basal ppGpp
441 regulation may represent a previously underappreciated vulnerability that could be exploited to
442 compromise bacterial survival under host-associated stresses.

Figures and legends

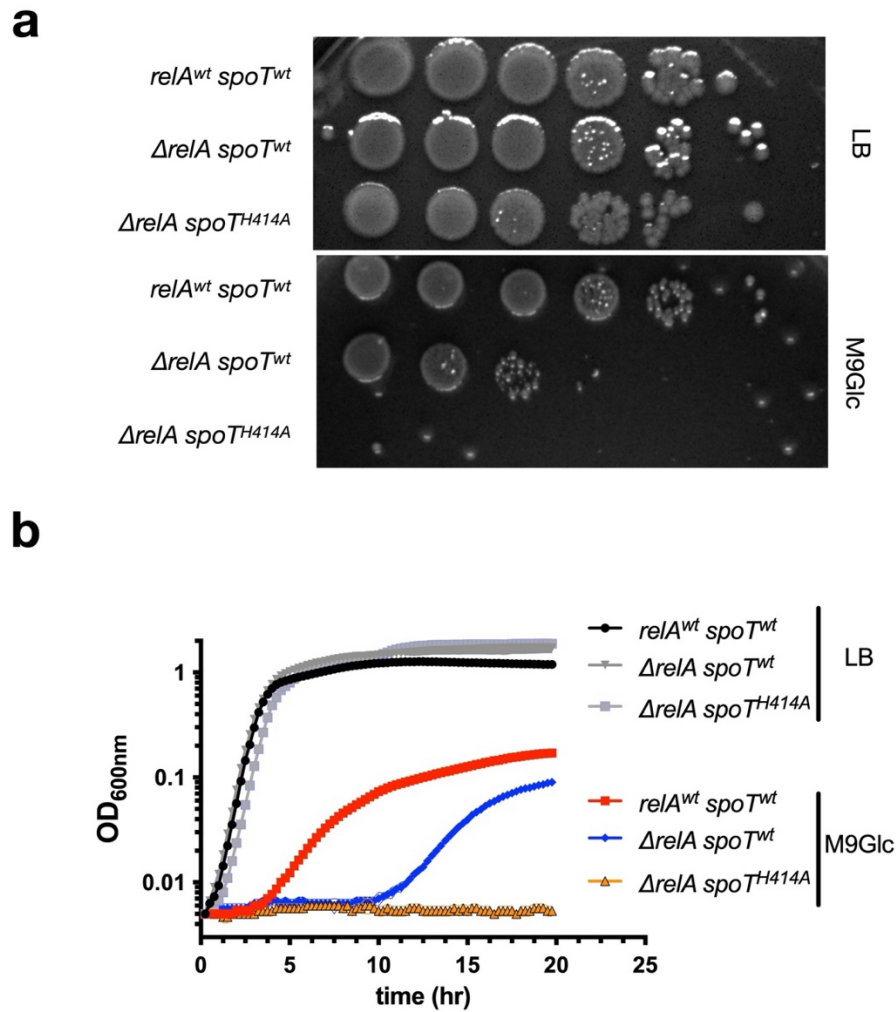


Figure 1. A conserved SpoT H414A mutation reveals defective growth on M9Glc medium. (a) Growth of wild type *E. coli* MG1655 (*relA^{wt} spoT^{wt}*), and *ΔrelA spoT^{wt}*, *ΔrelA spoT^{H414A}* strains on LB and M9Glc solid media. **(b)** Growth dynamics of the same strains in liquid LB and M9Glc media.

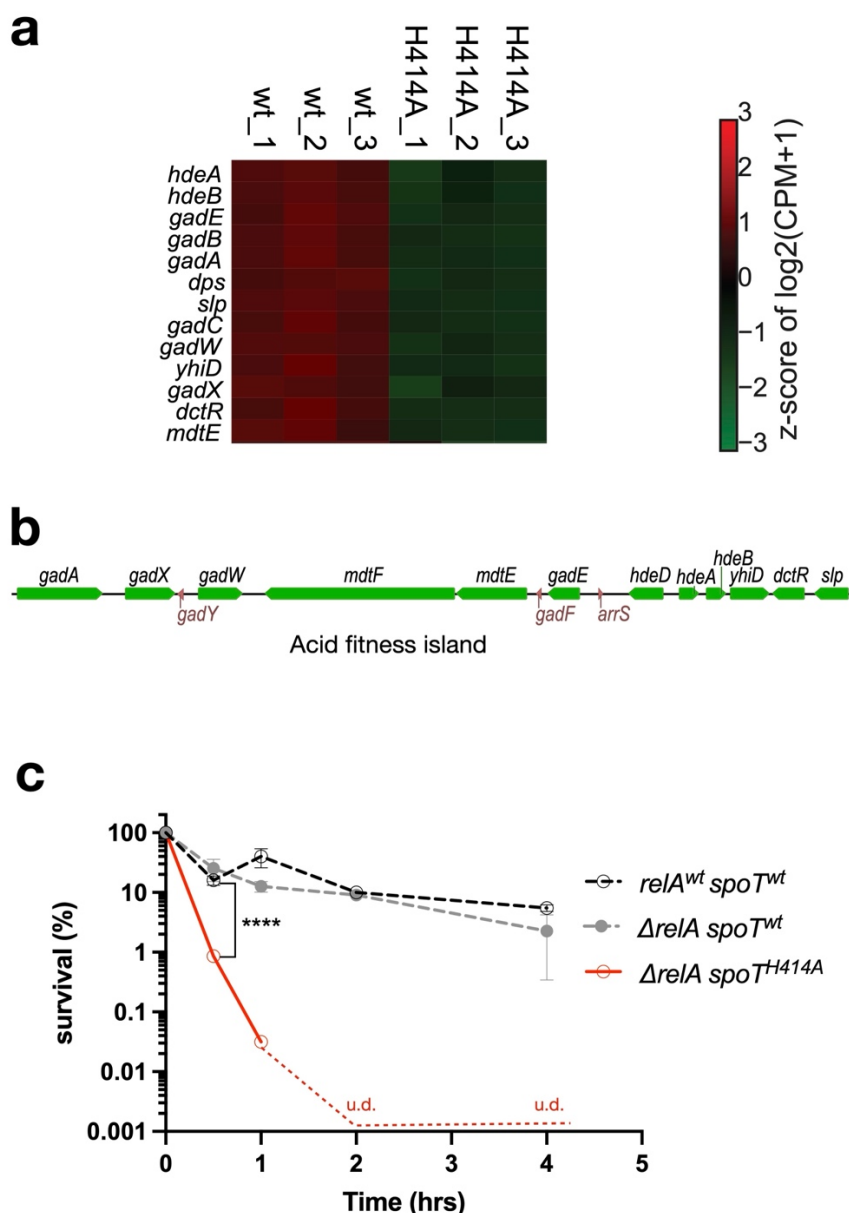
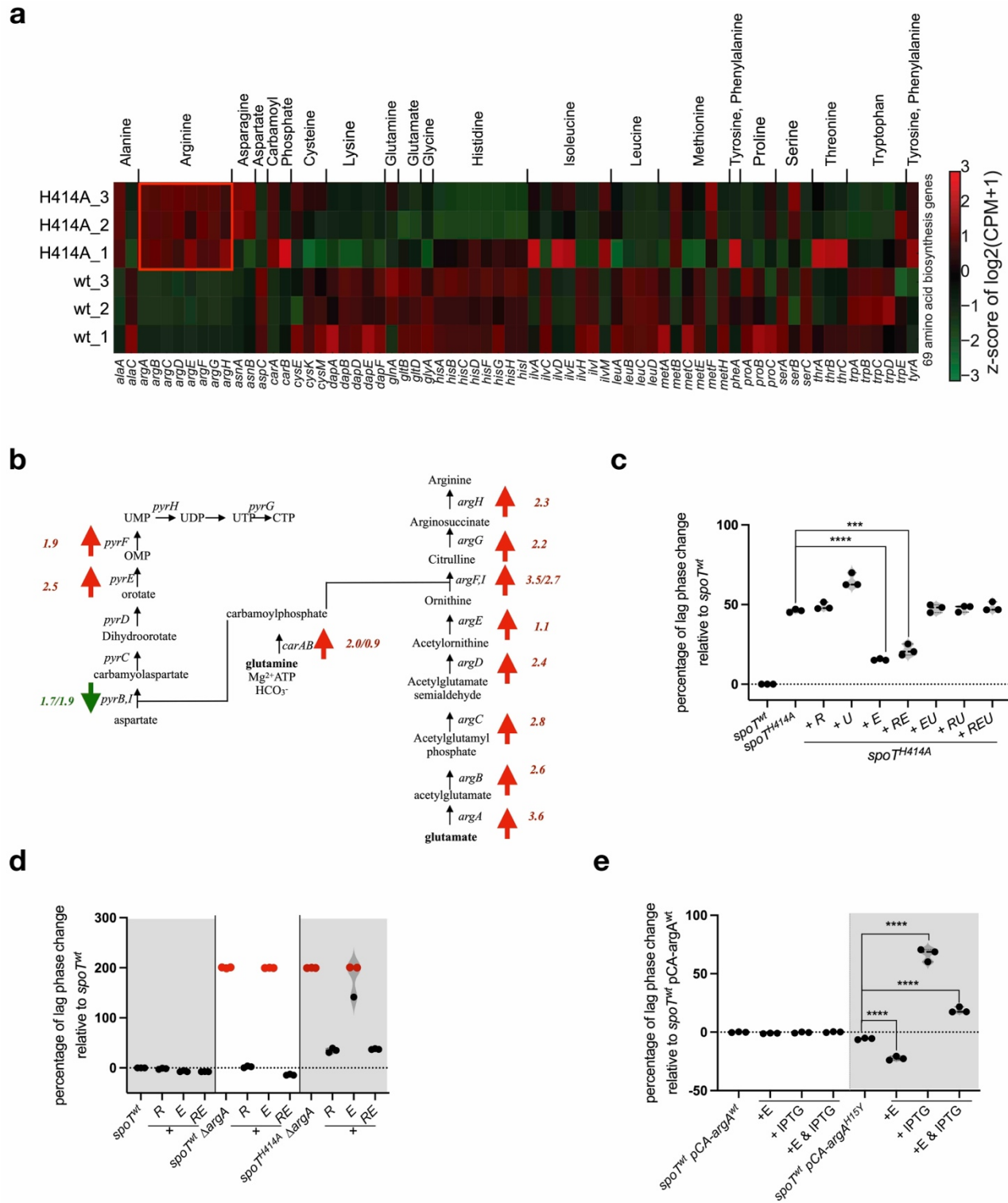


Figure 2. Transcriptomic analysis reveals collapse of acid resistance programs. (a) Heatmap of genes encoded in the *E. coli* acid fitness island in *spoT*^{H414A} relative to *spoT*^{wt}. **(b)** Genetic organization of the *E. coli* acid fitness island. **(c)** Acid resistance assay of wild type MG1655 (*relA*^{wt} *spoT*^{wt}), Δ *relA* *spoT*^{H414A} and Δ *relA* *spoT*^{wt}. Survival of indicated strains following challenge at pH 2.5, expressed as the percentage of survival (i.e., colony-forming units (CFUs) remaining relative to starting CFUs) for each strain. Data are means $\pm$ s.d. from biological replicates. u.d., undetectable.

Statistical significance was assessed using unpaired two-tailed Student's *t*-tests. **** denote $P < 0.0001$.



458 **Figure 3. Arginine metabolic overflow drives glutamate depletion in *spoT^{H414A}*.** (a) Heatmap of
 459 genes involved in amino acid biosynthesis, comparing *spoT^{H414A}* and *spoT^{wt}* strains. The arginine
 460 biosynthesis genes are highlighted. (b) Schematic of the arginine biosynthesis pathway in *E. coli*.
 461 Genes upregulated in *spoT^{H414A}* cells are indicated by red upward arrows, accompanied by average
 462 log₂ fold-change values relative to *spoT^{wt}*. (c) Normalized lag phase duration of *spoT^{H414A}* cells
 463 grown in M9Glc medium with supplementation of arginine (5.2 mM, R), uracil (0.2 mM, U),
 464 glutamate (0.6 mM, E), or the indicated combinations, expressed relative to the *spoT^{wt}* strain. (d)
 465 Normalized lag phase duration of indicated strains grown in M9Glc medium with supplementation
 466 of arginine (R), glutamate (E), or both, relative to *spoT^{wt}*. Red dots indicate failure to resume
 467 growth within the 72-h observation window. (e) Normalized lag phase duration of *spoT^{wt}* strains
 468 harboring pCA24N-*argA^{wt}* or the feedback-resistant pCA24N- *argA^{H15Y}* allele, grown with or
 469 without IPTG induction and/or glutamate supplementation (0.6 mM), relative to the uninduced
 470 *argA^{wt}* control. Data are means ± s.d. from biological replicates. Statistical significance was
 471 assessed using unpaired two-tailed Student's *t*-tests. ****, *** denote *P* < 0.0001 and 0.001,
 472 respectively.

473

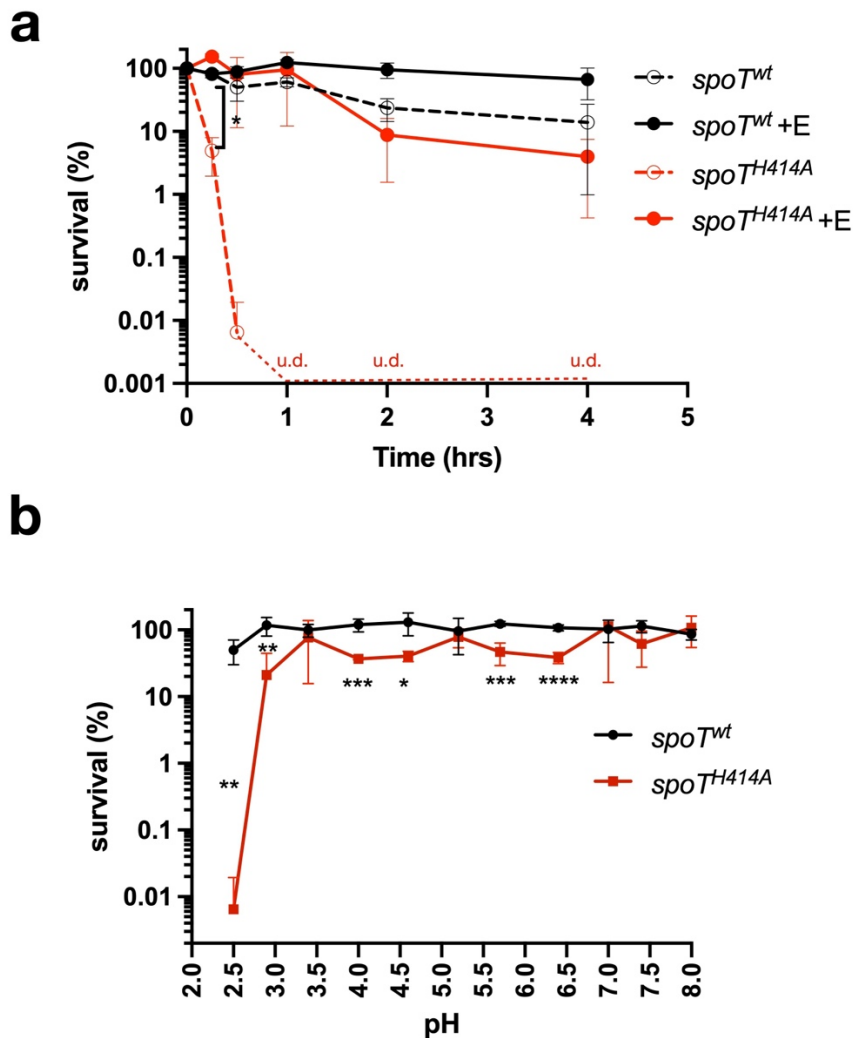


Figure 4. SpoT H414 is required for survival under extreme acid stress.

(a) Acid resistance assay of $\Delta relA$ *spoT^{H414A}* and $\Delta relA$ *spoT^{wt}* as in Figure 2c. Where indicated (+E), glutamate (0.6 mM) was included in the acid challenge medium. u.d., undetectable. **(b)**

Survival of *spoT^{H414A}* and *spoT^{wt}* strains following 30 min challenge in buffers adjusted to the

indicated pH values, normalized to starting CFUs. Data are means $\pm$ s.d. from biological replicates.

Statistical significance was assessed using unpaired two-tailed Student's *t*-tests. ****, ***, **, * denote $P < 0.0001$, 0.001, 0.01, and 0.05, respectively.

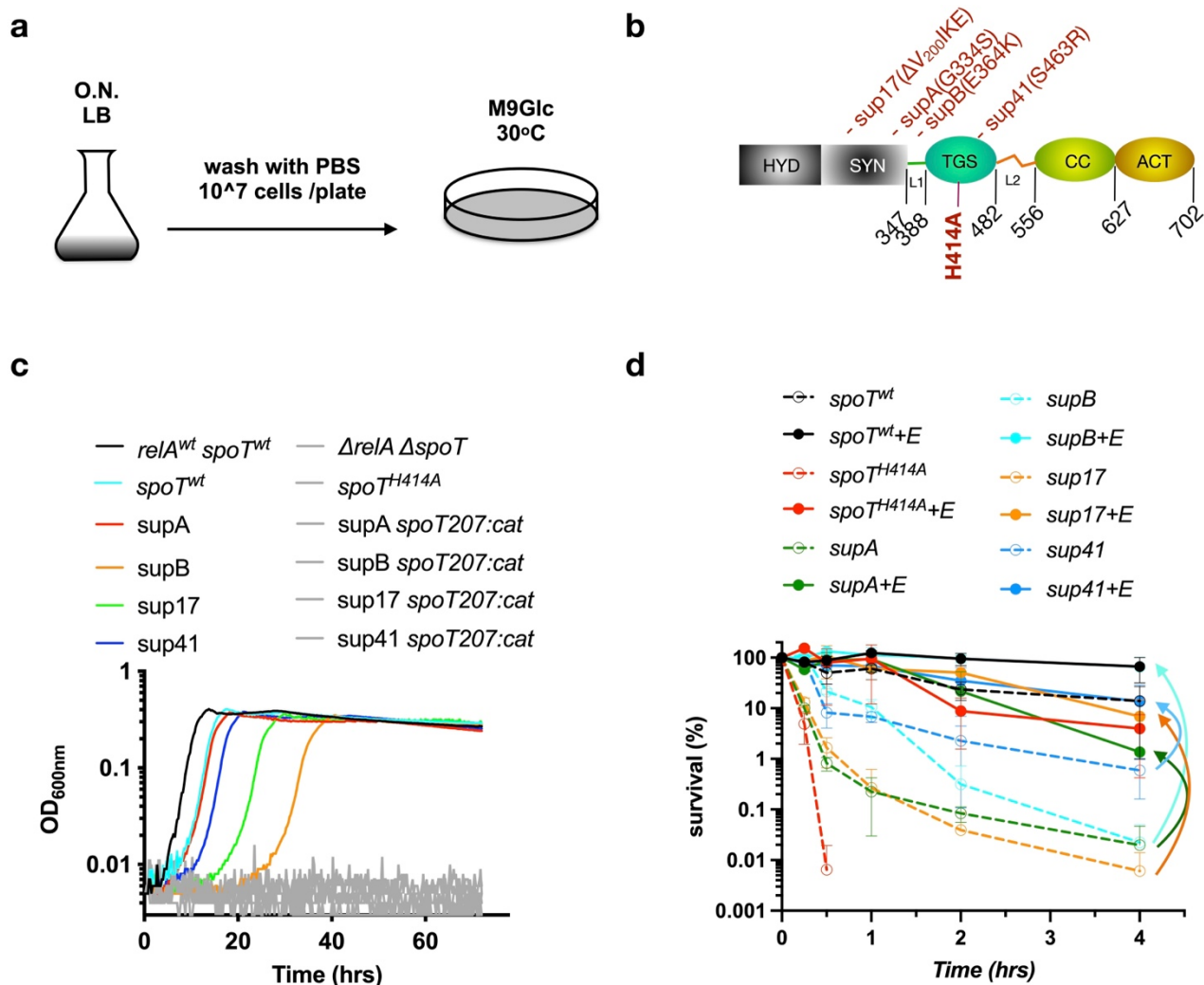


Figure 5. Intragenic SpoT suppressor mutations restore growth and acid resistance. (a)

Schematic illustrating the isolation of spontaneous suppressor mutations that enable growth of

spoT^{H414A} cells on M9Glc medium. **(b)** Domain architecture of the SpoT protein, indicating the

locations of suppressor mutations. Amino-acid positions are shown below. L1/L2, linker-1/linker-2;

HYD, hydrolase domain; SYN, synthetase domain; TGS, ThrRS-GTPase-SpoT domain; CC,

conserved cysteine domain; ACT, aspartate kinase-chorismate mutase-TyrA domain. **(c)** Growth

dynamics of four suppressor strains (supA, supB, sup17, sup41) compared with *spoT*^{H414A}, *spoT*^{wt},

ppGpp⁰ (Δ *relA* Δ *spoT*), and wild-type MG1655 (*relA*^{wt} *spoT*^{wt}). Also shown are suppressor strains

in which *spoT* was deleted (*spoT207::cat*). **(d)** Survival of indicated strains following challenge at

pH 2.5. Where indicated (+E), glutamate (0.6 mM) was included. Data are means $\pm$ s.d. Statistical significance was assessed using unpaired two-tailed Student's *t*-tests. ***, **, * denote $P < 0.001$, 0.01, and 0.05, respectively.

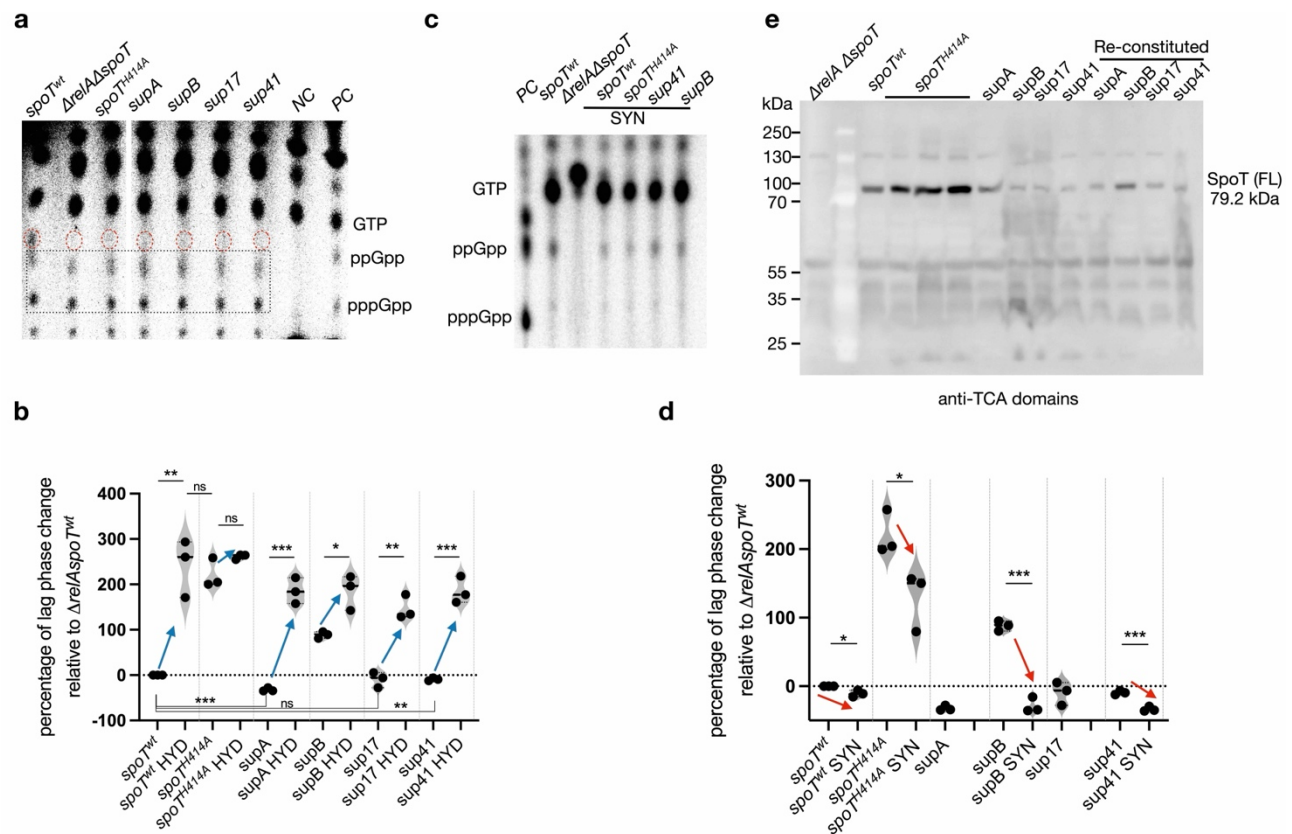


Figure 6. A sub-basal ppGpp pool is sufficient for physiological function. (a) Thin-layer chromatography (TLC) analysis of 32 P-labeled nucleotides from indicated strains grown in low-phosphate MOPS minimal medium supplemented with 0.2% glucose. NC and PC denote valine-treated Δ *relA* Δ *spoT* and wild-type MG1655 controls, respectively. Red circles mark ppGpp; boxed regions indicate nonspecific spots also present in Δ *relA* Δ *spoT* samples. **(b)** Effect of inactivating SpoT ppGpp synthetase activity (E319Q; HYD) on growth recovery in M9Glc. Lag phase duration is normalized to *spoT^{wt}*. **(c)** TLC analysis of 32 P-labeled nucleotides from strains carrying SpoT hydrolase-inactive mutations (H72A D73A; SYN). **(d)** Growth recovery of hydrolase-inactive strains (SYN), quantified as normalized lag phase duration relative to *spoT^{wt}*. Note that the

hydrolase-inactive (SYN) variants of supA and sup17 could not be obtained, thereby not tested. (e) Immunoblot analysis of SpoT protein levels using an antiserum raised against the SpoT C-terminal (TGS-CC-ACT; anti-TCA) region. Cells were grown in M9Glc to early exponential phase ($OD_{600nm} \approx 0.2$) prior to sampling. *spoT^{H414A}* samples were collected after prolonged incubation, as in Extended Data Fig. 1c. *ΔrelA ΔspoT* cells grown in LB served as a negative control.

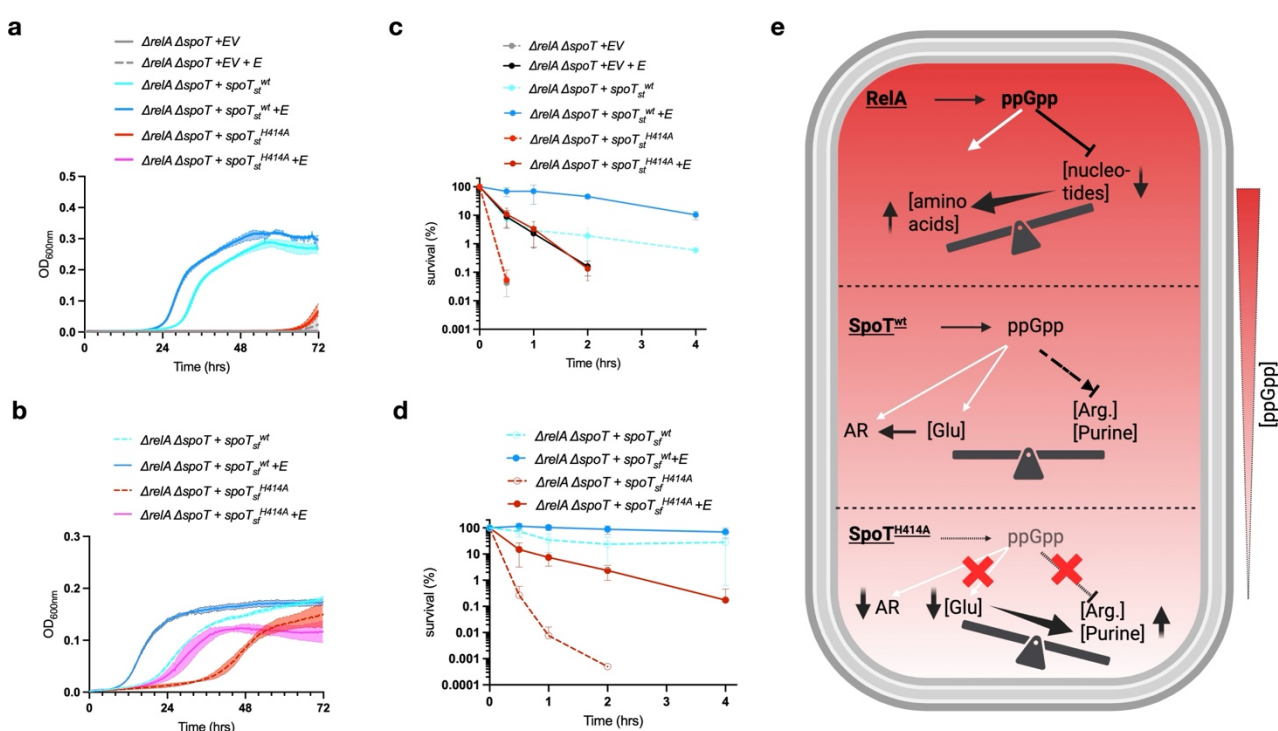


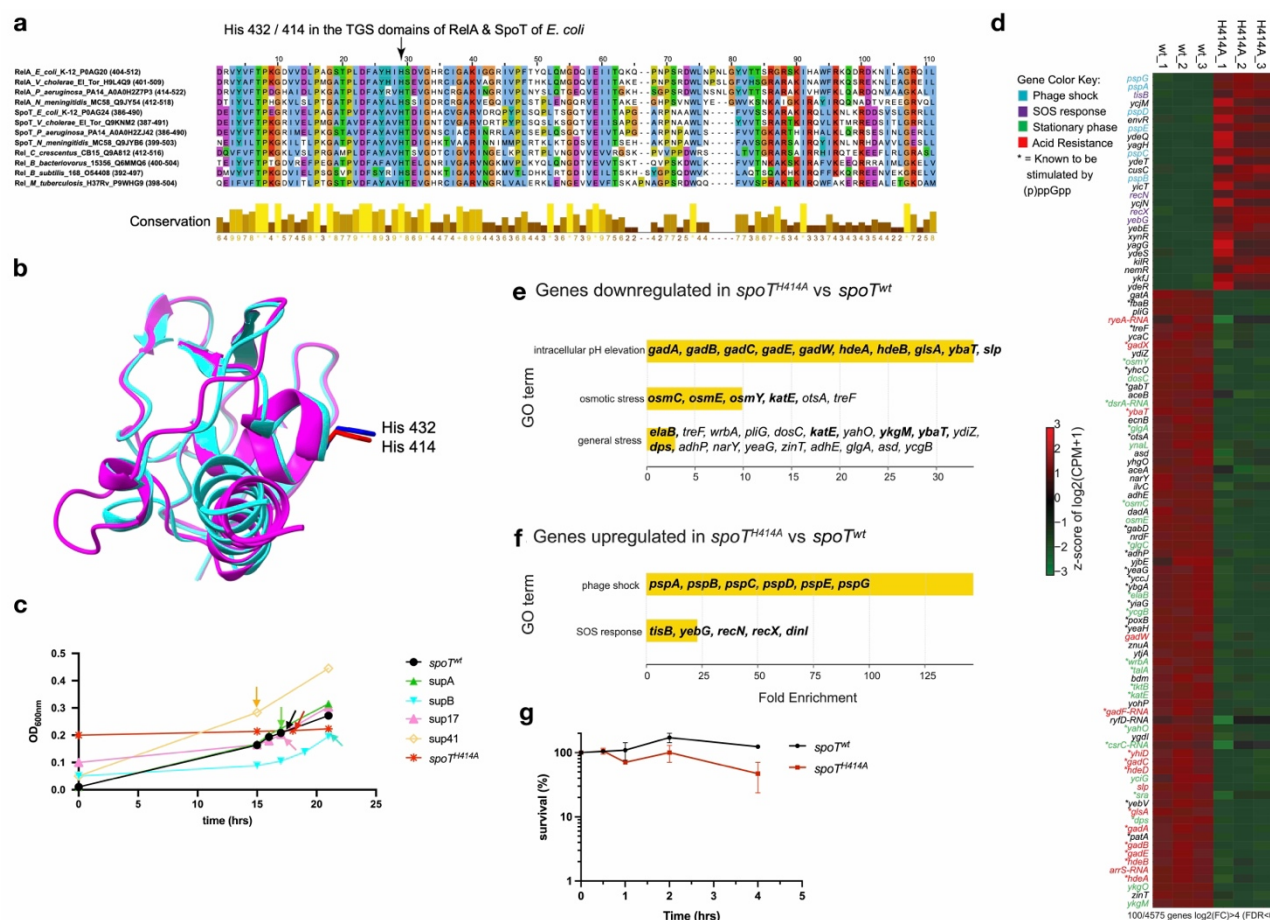
Figure 7. Conservation of SpoT H414 function and model for basal ppGpp regulation. (a,b)

Growth of *E. coli* $\Delta relA \Delta spoT$ strains complemented with SpoT homologs from *Salmonella enterica* serovar Typhimurium (*SpoT_{st}*) (a) or *Shigella flexneri* (*SpoT_{sf}*) (b). E, glutamate (0.6 mM); EV, empty vector. Data represent at least three biological replicates. (c,d) Survival of complemented $\Delta relA \Delta spoT$ strains expressing *SpoT_{st}* (c) or *SpoT_{sf}* (d) following challenge at pH 2.5, normalized to starting CFUs. Where indicated (+E), glutamate (0.6 mM) was included. (e) Top: Under acute amino-acid starvation, RelA is rapidly activated by ribosome-associated signals to generate a high-amplitude ppGpp surge, globally reprogramming transcription and metabolism to suppress

520 nucleotide pathways and promote amino-acid availability. Middle: SpoT maintains a low but
 521 physiologically essential basal ppGpp pool that is distinct from and independent of RelA. This basal
 522 ppGpp state coordinates metabolic homeostasis by restraining excessive arginine and purine
 523 biosynthesis, preserving intracellular glutamate levels, and priming multiple acid resistance
 524 programs, including the glutamate-dependent Gad system (AR). Bottom: In *spoT^{H414A}* cells, a sub-
 525 basal level of ppGpp remains below the threshold required for effective metabolic and stress
 526 control. Therefore, arginine and purine biosynthetic pathways become overactivated, intracellular
 527 glutamate is depleted, and acid resistance programs are inadequately induced, resulting in growth
 528 failure in minimal medium and extreme sensitivity to acid stress.

529

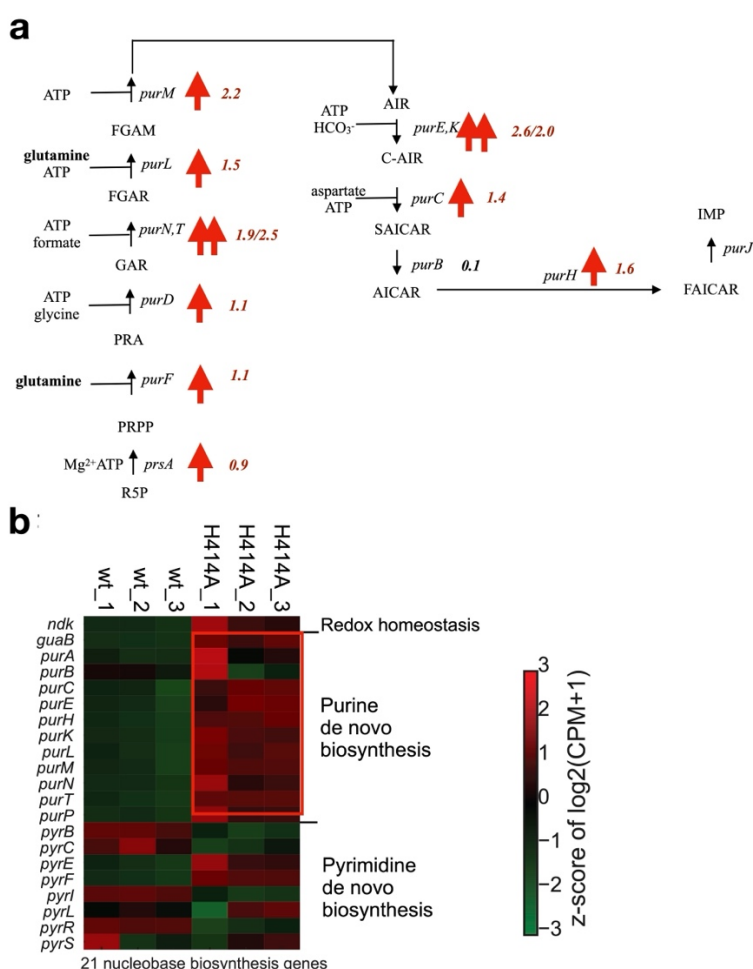
530 EXTENDED DATA FIGURE LEGENDS



531

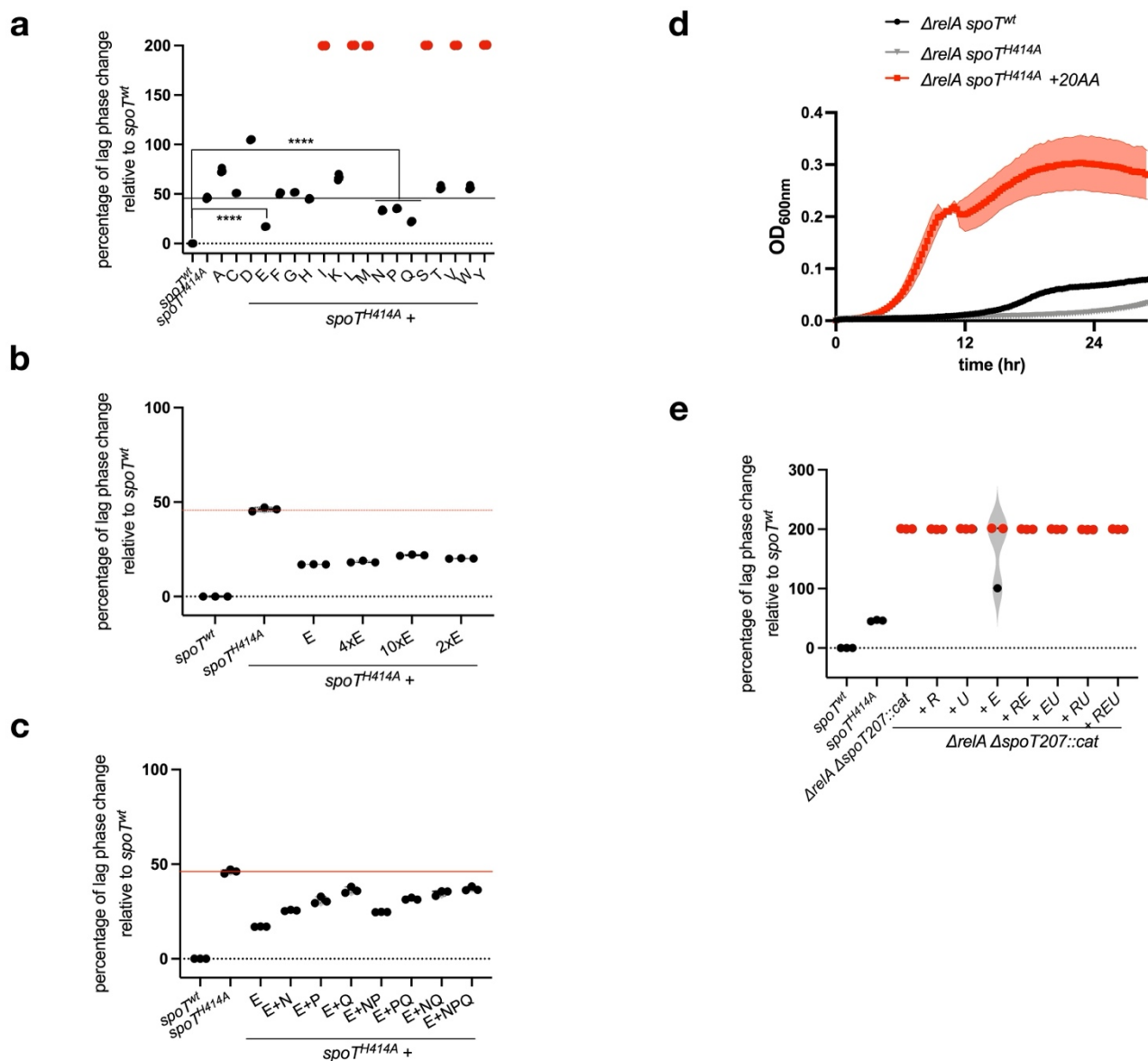
532 **Extended Data Fig. 1 (a)** Sequence alignment of 12 TGS domains of RelA, SpoT and their
533 homologous proteins from α , β , γ and δ proteobacteria, Firmicute and Actinomycete. The highly
534 conserved position corresponding to His 432 of RelA and 414 of SpoT of *E. coli* is indicated with
535 an arrow. **(b)** Superpositioning of the TGS domains of RelA and SpoT from *E. coli* (rmsd over 91
536 of 98 atom pairs is 0.762 Å) generated by AF3. **(c)** Representative growth curves of $\Delta relA$
537 *spoTH414A*, $\Delta relA spoTH414A$ and the four suppressor strains in M9Glc media for collecting RNA
538 sequencing samples. Cells were collected when their OD_{600nm} approach ca. 0.2, except for *spoTH414A*
539 cells, which could not grow in this medium, thus these cells were collected after incubated for 18
540 hrs in the same medium. Coloured arrows indicate the sampling points for each strain. **(d)** Heatmap

541 of genes with greater than 16-fold changes between $\Delta relA$ $spoT^{H414A}$ and $\Delta relA$ $spoT^{wt}$ cells growing
 542 in M9Glc medium. **(e)** Gene ontology groups of genes downregulated in the $spoT^{H414A}$ strain.
 543 Genes downregulated at least 16-fold were extracted using DEGUST ($|\log_2(FC)| > 4$; FDR < 0.05)
 544 and analysed by AmiGO and Panther. The 58 genes downregulated more than 16-fold ($\log_2(FC) <$
 545 -4) are listed in **Table S3**. **(f)** Gene ontology groups of genes upregulated at least 16-fold in the
 546 $spoT^{H414A}$ strain. The 28 genes upregulated more than 16-fold ($\log_2(FC) > 4$) are listed in **Table**
 547 **S3**. Note the differences in the Fold Enrichment scales in **(e)** and **(f)**. Gene names in bold in **(e, f)**
 548 are strongly up or downregulated. **(g)** Normalized percentage of surviving viable cells of the $spoT^{wt}$
 549 and $spoT^{H414A}$ strains growing in M9Glc medium. Two replicates were performed and presented.



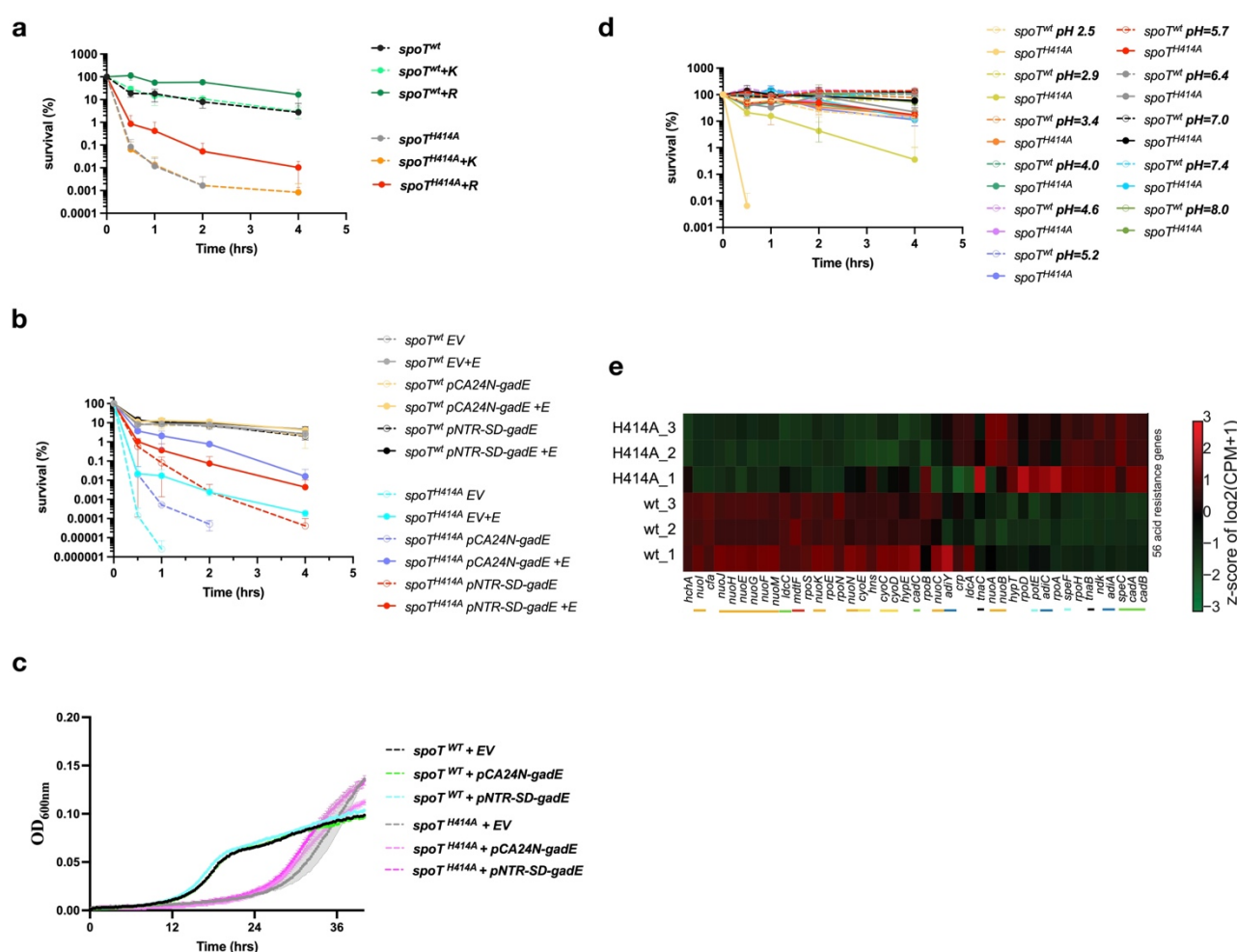
550

551 **Extended Data Fig. 2 (a)** Purine nucleotide de novo biosynthesis pathway in *E. coli*. The
552 upregulated genes are indicated by a red upward arrow alongside average log₂(FC) values of
553 *spoT^{H414A}* versus *spoT^{wt}*. R5P, ribose-5-phosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate; PRA,
554 5-phosphoribosylamine; GAR, 5'-phosphoribosylglycinamide; FGAR, 5'-phosphoribosyl-N-
555 formylglycineamide; FGAM, 5'-phosphoribosylformylglycinamidine; AIR, 5-aminoimidazole
556 ribonucleotide; C-AIR, 5-carboxyamino-1-(5-phospho-D-ribosyl)imidazole; SAICAR, 1-(5-
557 phosphoribosyl)-4-(N-succino-carboxamide)-5-aminoimidazole; AICAR, 5-amino-1-(5-phospho-D-
558 ribosyl)imidazole-4-carboxamide; FAICAR, 5-formamido-1-(5-phospho-D-ribosyl)imidazole-4-
559 carboxamide; IMP, inosine 5'-monophosphate. **(b)** Heatmap of genes involved in purine and
560 pyrimidine nucleotide de novo biosynthesis. The red box indicates purine de novo biosynthesis
561 genes.



Extended Data Fig. 3 (a) Normalized lag phase length of the $spoT^{H414A}$ strain without or with supplemented each of the 20 amino acids (in single letters; A, alanine 0.8mM; C, cysteine 0.1mM; D, aspartic acid 0.4mM; E, glutamate 0.6mM; F, phenylalanine 0.4mM; G, glycine 0.8mM; H, histidine 0.2mM; I, isoleucine 0.4mM; K, lysine 0.4mM; L, leucine 0.8mM; M, methionine 0.2mM; N, asparagine 0.4mM; P, proline 0.4mM; Q, glutamine 0.6mM; R, arginine 5.2mM; S, serine 10mM; T, threonine 0.4mM; V, valine 0.6mM; W, tryptophan 0.1mM; Y, tyrosine 0.2mM), relative to the $spoT^{wt}$ strain in M9Glc medium. **(b)** Normalized lag phase length of the $spoT^{H414A}$ strain without or

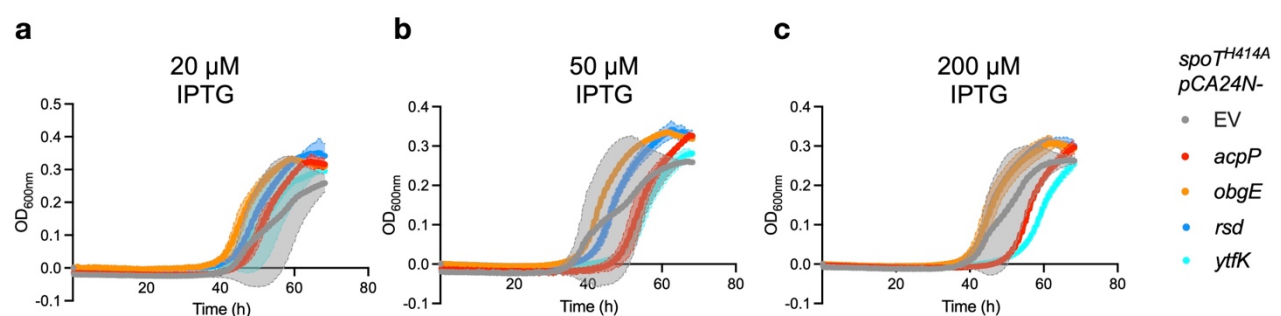
570 with supplemented 1x, 2x, 4x, 10x glutamate (E, 1x = 0.6 mM), relative to the *spoT^{wt}* strain in M9Glc
 571 medium. (c) Normalized lag phase length of the *spoT^{H414A}* strain without or with supplemented
 572 glutamate (0.6 mM) together with glutamine (Q, 0.6 mM), proline (P, 0.4 mM), asparagine (N, 0.4
 573 mM), relative to the *spoT^{wt}* strain in M9Glc medium. (d) Representative growth curves of $\Delta relA$
 574 *spoT^{H414A}*, $\Delta relA$ *spoT^{wt}* strains without or with all 20 amino acids (+20AA) (see method for details)
 575 in M9Glc medium. Two biological replicates each with two technical replicates were performed and
 576 presented. (e) Normalized lag phase length of the $\Delta relA \Delta spoT207::cat$ (ppGpp⁰) strain without or
 577 with supplemented arginine (5.2 mM, R), uracil (0.2 mM, U), glutamate (0.6 mM, E) or their varied
 578 combinations, relative to the *spoT^{wt}* strain in M9Glc medium. Red dots indicate fails of regrowth
 579 within the tested time range of 72 hrs.



580

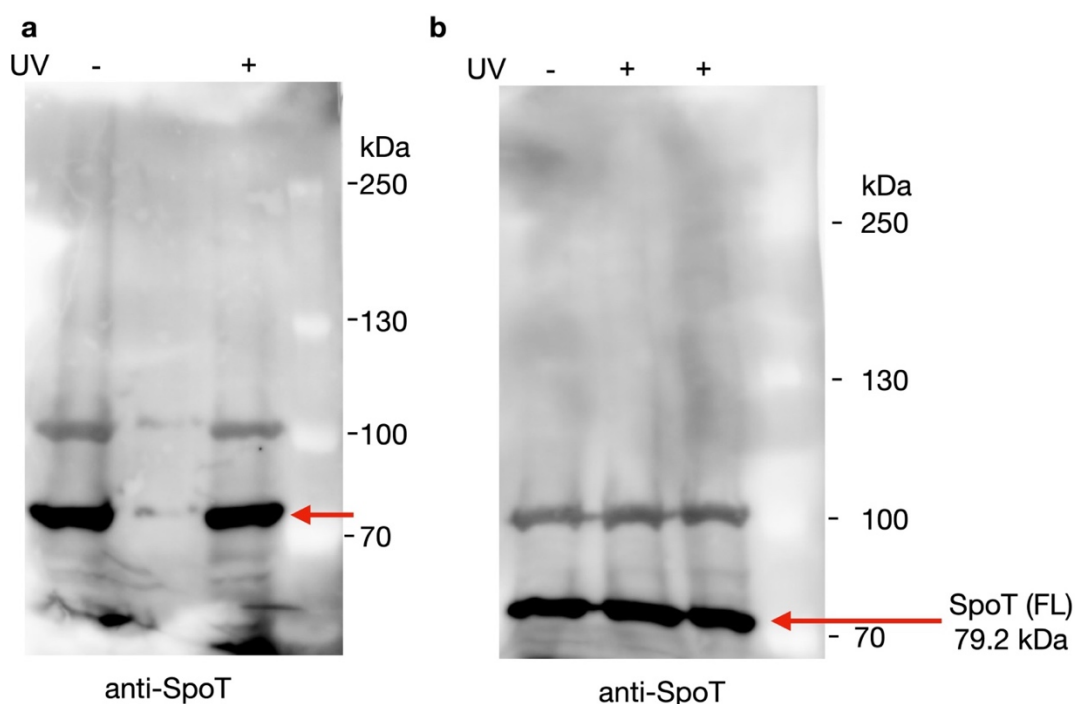
Extended Data Fig. 4 (a) Normalized percentage of surviving cells of various strains after challenged with pH=2.5. The data were normalized to the starting CFUs of each respective tested strain. +K, +R, lysine (0.6 mM), arginine (0.6 mM) included in the pH=2.5 medium, respectively.

(b) Normalized percentage of surviving cells of *spoT*^{wt} or *spoT*^{H414A} strains containing various plasmid vectors (pCA24N⁴², pNTR-SD⁴³) after challenged with pH=2.5. The data were normalized to the starting CFUs of each respective tested strain. EV, empty vector pCA24N. +E, glutamate (0.6 mM) included in the pH=2.5 medium. **(c)** Representative growth curves of *spoT*^{H414A} and *spoT*^{wt} strains containing either the empty vector (EV) or a *gadE* gene on pCA24N or pNTR-SD vectors in M9Glc medium. Two biological replicates each with two technical replicates were performed and presented. **(d)** Normalized percentage of surviving cells of *spoT*^{wt} and *spoT*^{H414A} strains after challenged with media of various pH values. The data were normalized to the starting CFUs of each respective tested strain. **(e)** Heatmap of genes involved in acid resistance. Colored bars indicate genes belonging to the same acid resistance systems: orange, *nuo* genes encoding NADH dehydrogenase I; yellow, *cyo* genes encoding cytochrome *bo* oxidase; green, lysine dependent acid resistance systems; cyan, ornithine dependent acid resistance system; black, alkaline stress; dark blue, arginine dependent acid resistance system.



Extended Data Fig. 5. Growth dynamics of *spoT*^{H414A} strains expressing candidate SpoT regulators.

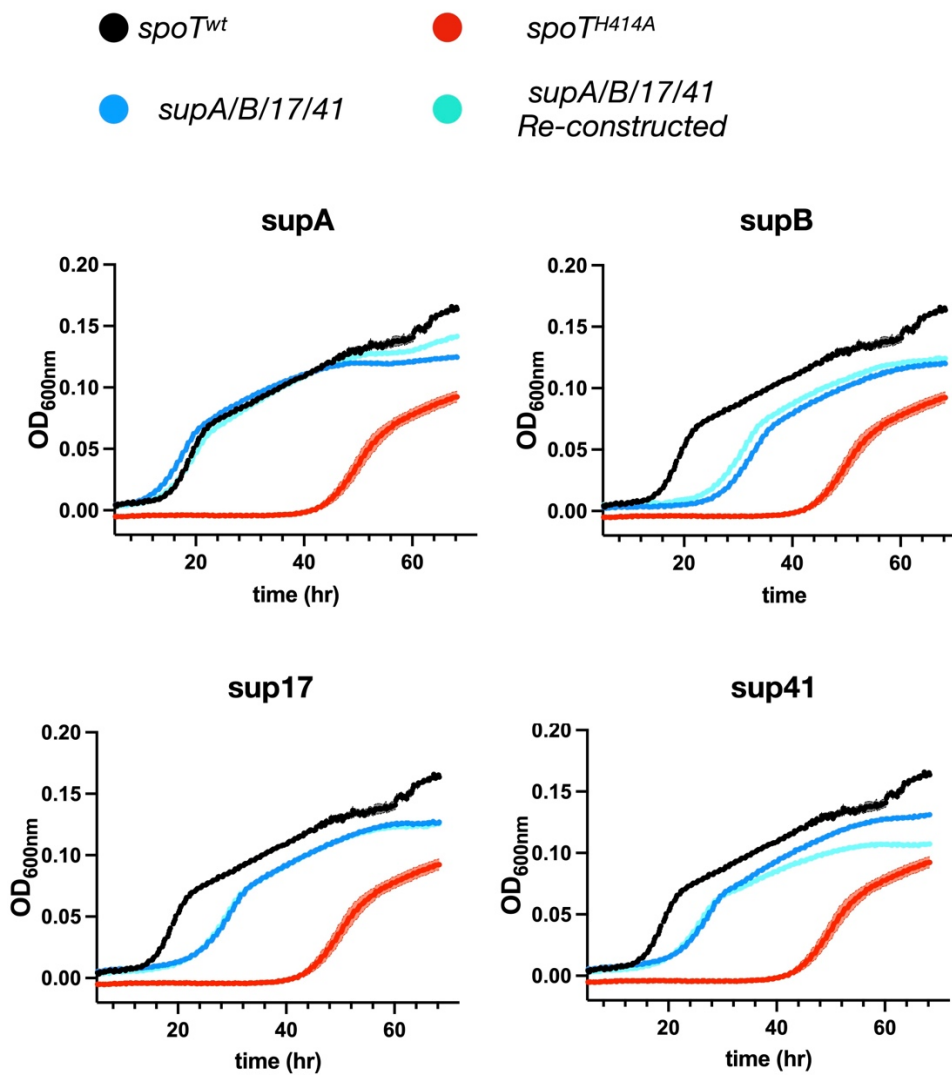
600 Growth curves of *spoT*^{H414A} strains carrying either the empty vector (EV) pCA24N or pCA24N
 601 derivatives expressing *acpP*, *obgE*, *rsd*, or *ytfK*. Cells were grown in M9Glc medium supplemented
 602 with the indicated concentrations of IPTG to induce expression of the respective genes. Data
 603 represent the mean $\pm$ s.d. from three independent biological replicates.



605 **Extended Data Fig. 6. Western blot analysis of SpoT following unnatural amino acid-**
 606 **mediated UV crosslinking.**

607 SpoT carrying a photo-crosslinkable unnatural amino acid at position 414 was expressed in a $\Delta relA$
 608 $\Delta spoT$ background and subjected to UV irradiation (365 nm) to induce covalent crosslinking to
 609 proximal proteins. Samples collected in the absence (-) or presence (+) of UV irradiation were
 610 analyzed by SDS-PAGE and immunoblotting using anti-SpoT antiserum. Two independent
 611 representative experiments are shown (**a**, **b**). The positions of full-length SpoT are indicated by the
 612 red arrows.

613

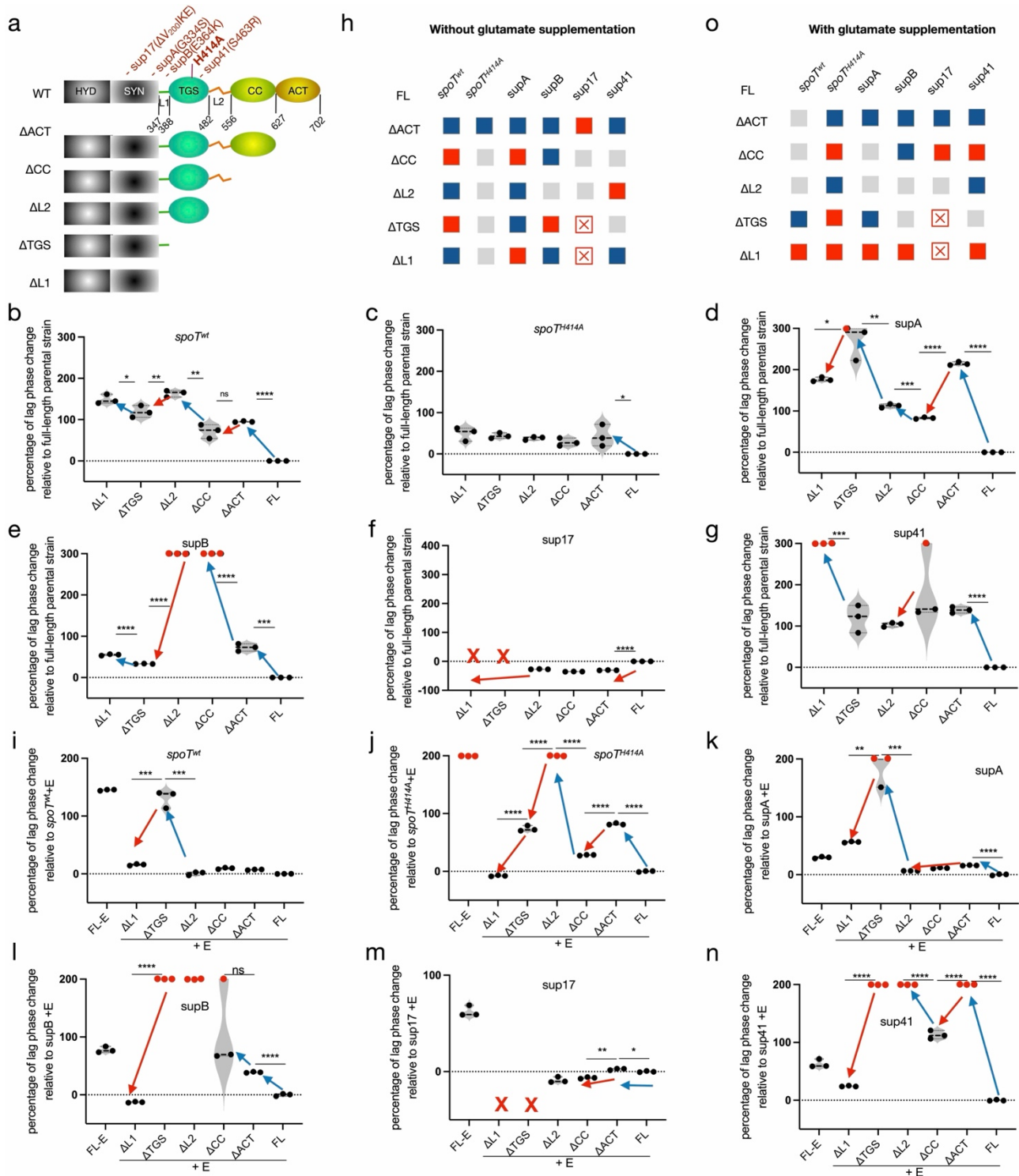


614

615 **Extended Data Fig. 7** Growth dynamics of re-constructed four suppressors (cyan)

616 (*supA/supB/sup17/sup41*) in comparison to *spoT^{H414A}*, *spoT^{wt}*, and the four random suppressors

617 (blue).

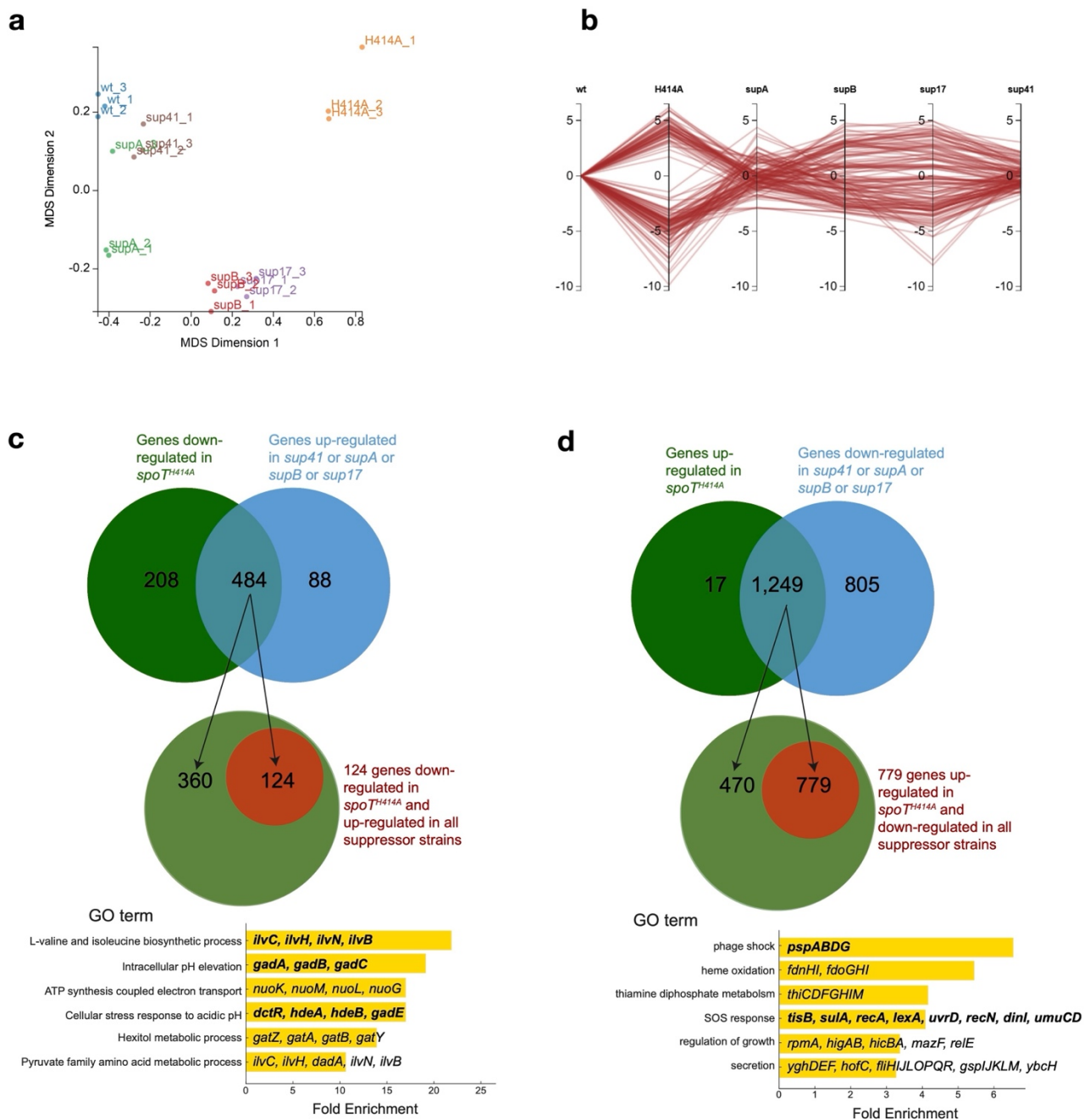


Extended Data Fig. 8 Distributed C-terminal domain regulation tunes SpoT-dependent

growth under basal conditions. (a) Schematic representation of sequential truncations of SpoT C-

terminal regulatory domains introduced into different genetic backgrounds. (b-g) Normalized lag

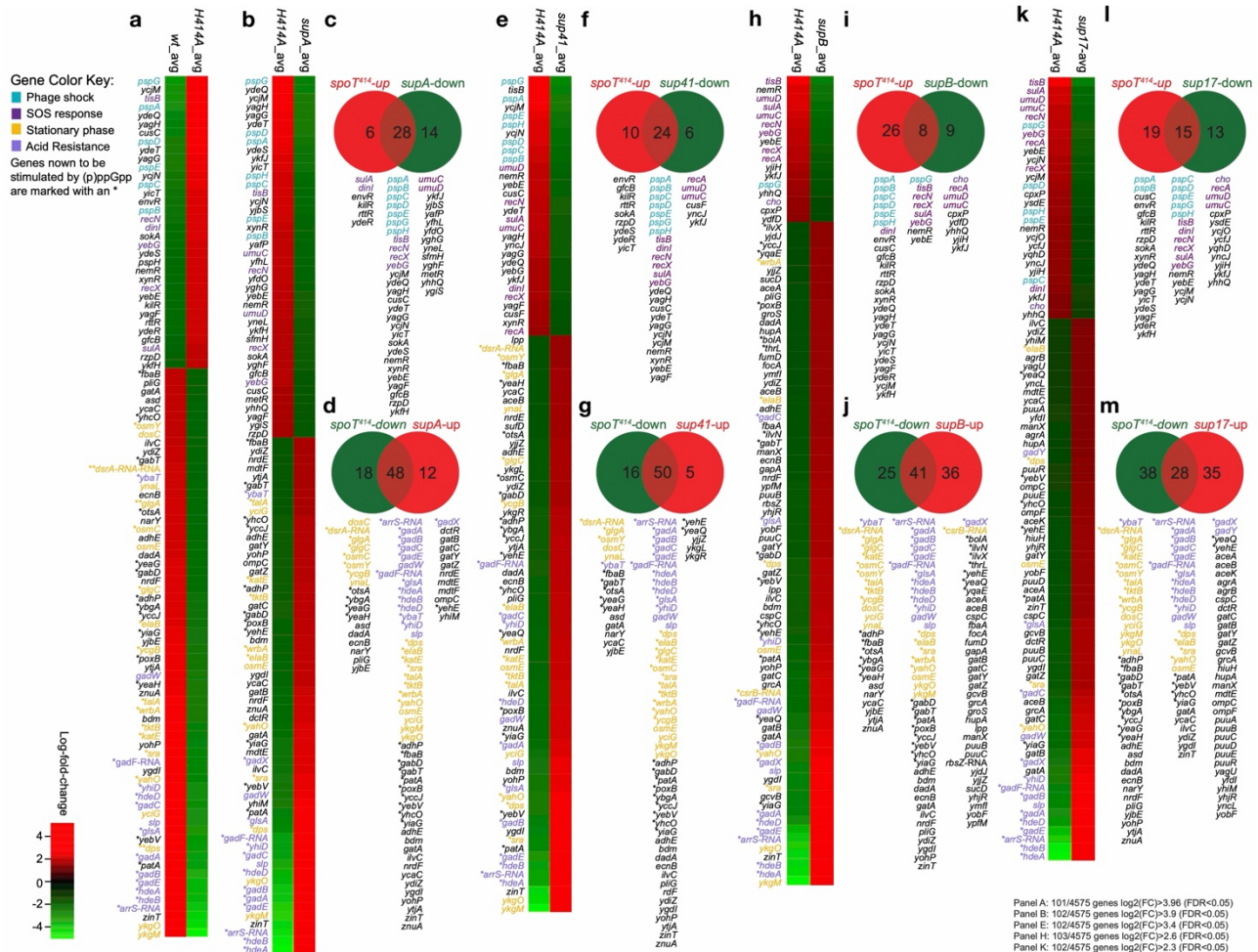
622 phase duration during growth in M9Glc minimal medium, expressed as a percentage relative to the
 623 corresponding full-length (FL) SpoT parental strain. Red dots indicate strains that failed to resume
 624 growth within the 72-h observation period. Red crosses indicate truncation variants that could not
 625 be obtained. **(h)** Summary of the effects of individual domain deletions on growth resumption in
 626 M9Glc, based on data in panels **b-g**. Blue and red blocks indicate positive or negative effects on
 627 growth, respectively. Red crosses denote non-viable genotypes, and grey blocks indicate conditions
 628 in which no clear effect could be inferred due to indistinguishable growth behavior. **(i-n)**
 629 Normalized lag phase duration during growth in M9Glc supplemented with glutamate (0.6 mM),
 630 expressed relative to the corresponding FL parental strains. **(o)** Summary of domain-specific effects
 631 on growth resumption in the presence of glutamate, based on panels **i-n**. Color coding is as in panel
 632 **h**.



Extended Data Fig. 9 Suppressor mutations partially restore global transcriptional programs disrupted in *spoT^{H414A}* cells.

(a) Parallel coordinates plot showing genes with strong differential expression ($|\log_2 \text{fold change}| > 4$, $\text{FDR} < 0.05$) across *spoT^{wt}*, *spoT^{H414A}* and four intragenic suppressor strains. (b) Multidimensional scaling (MDS) plot of transcriptomic profiles from the same six strains, with

individual biological replicates shown. Growth conditions and sampling strategy are described in the Materials and Methods. Panels **a** and **b** were both generated by DEGUST. **(c)** Cross-suppressor analysis of genes downregulated in *spotT^{H414A}*. Top, Venn diagram showing overlap of genes downregulated in *spotT^{H414A}* and upregulated in one or more suppressor strains. Middle, subset of genes downregulated in *spotT^{H414A}* and consistently upregulated in all four suppressor strains. Bottom, Gene Ontology (GO) enrichment analysis of the 124 genes restored across all suppressors. All genes are listed in **Table S4**. **(d)** Cross-suppressor analysis of genes upregulated in *spotT^{H414A}*. Top, Venn diagram showing overlap of genes upregulated in *spotT^{H414A}* and downregulated in one or more suppressor strains. Middle, subset of genes upregulated in *spotT^{H414A}* and consistently downregulated in all four suppressor strains. Bottom, GO enrichment analysis of the 779 genes consistently repressed across all suppressors. Genes were extracted from DEGUST using thresholds of $|\log_2 \text{fold change}| > 1$ and $\text{FDR} < 0.05$. Gene lists are provided in **Table S4**. Within GO term annotations, gene names shown in bold indicate transcripts with particularly strong differential expression.

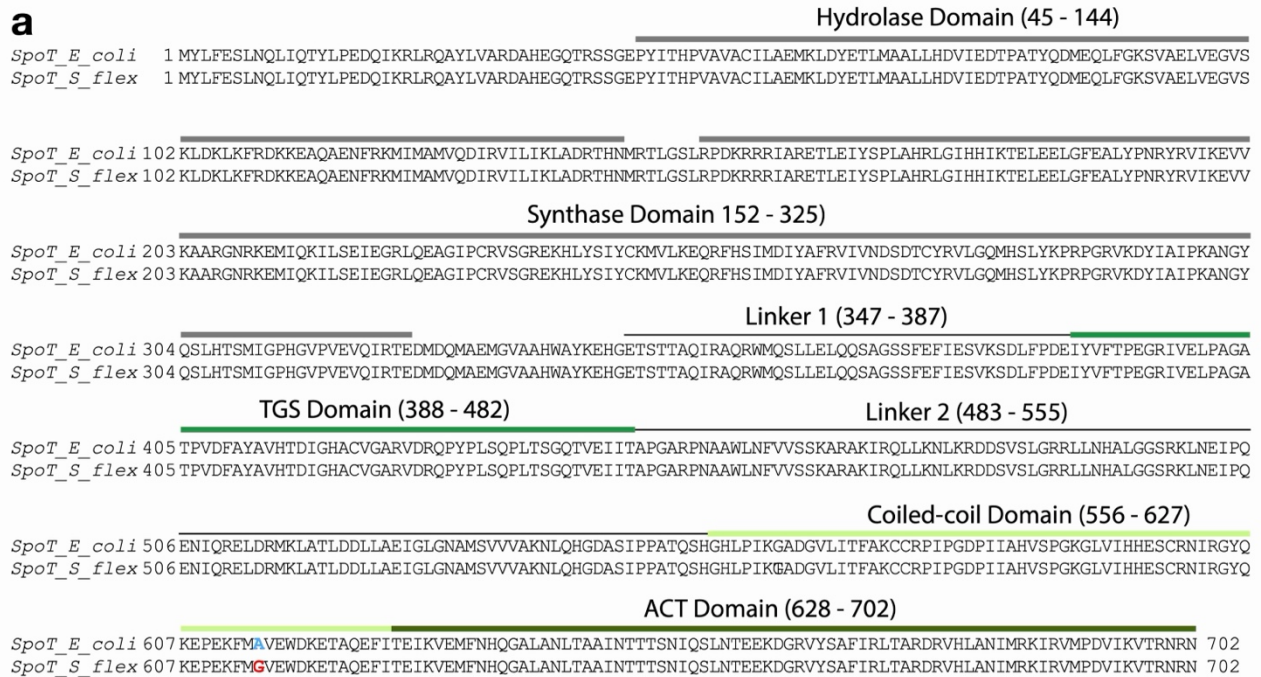


Extended Data Fig. 10 Suppressor mutations differentially correct transcriptional dysregulation in *spoTH414A* cells.

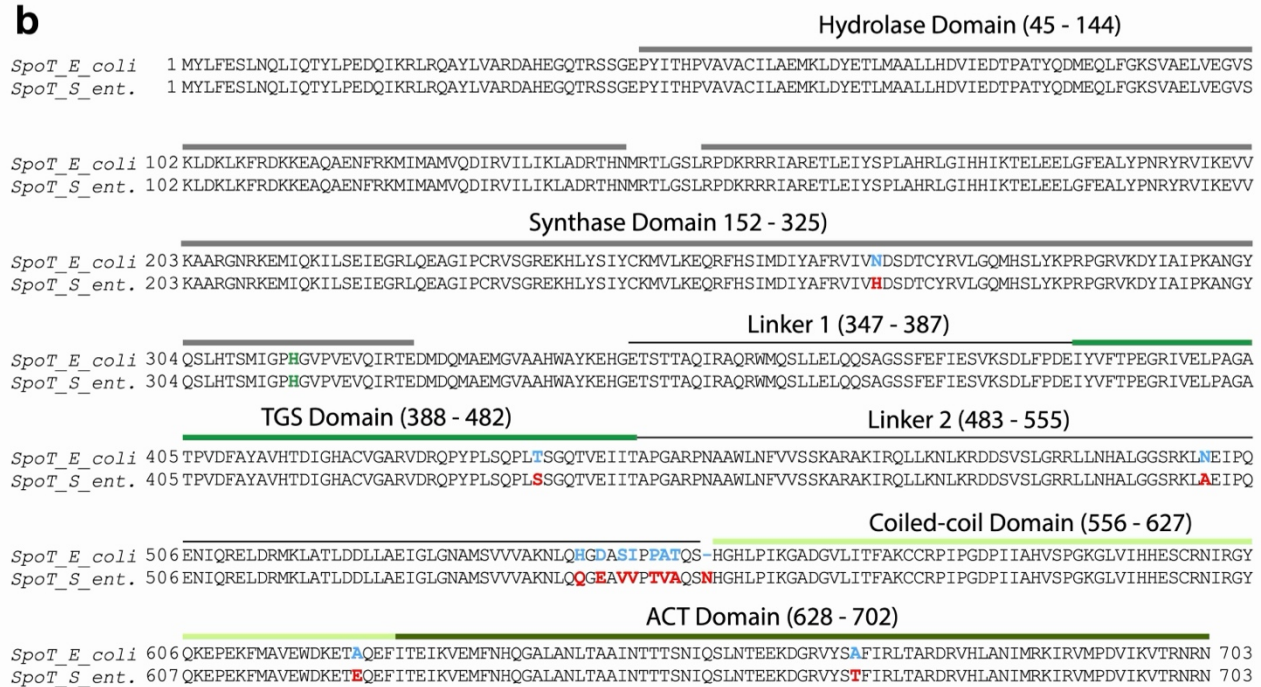
(a) Heatmap of genes differentially expressed in *spoTH414A* relative to *spoT^{wt}*. DEGUST analysis using thresholds of $|\log_2 \text{fold change}| > 3.96$ and $\text{FDR} < 0.05$ identified 100 genes from a total of 4,575 genes analyzed. Highlighted gene classes include phage shock response genes (*psp*, blue), SOS response genes (magenta), acid stress response genes (deep purple), and stationary-phase-induced genes (gold). Genes marked with an asterisk (*) denote transcripts previously reported to be ppGpp-inducible. (b, e, h, k) Heatmaps comparing *spoTH414A* with each suppressor strain. FDR thresholds were identical to those used in panel (a), while $|\log_2 \text{fold change}|$ cutoffs were adjusted to return approximately 100 genes per comparison. DEGUST identified 102 genes for *supA* ($|\log_2 \text{fold}$

664 change| > 3.9), 97 genes for sup41 (|log₂ fold change| > 3.4), 94 genes for supB (|log₂ fold change| >
665 2.6), and 91 genes for sup17 (|log₂ fold change| > 2.3). Gene lists for each comparison are provided
666 in **Table S5. (c, d, f, g, i, j, l, m)** Venn diagram analyses showing overlap of genes dysregulated in
667 *spoT^{H414A}* and in each suppressor strain. Genes differentially expressed in *spoT^{H414A}* relative to
668 *spoT^{wt}* (from panel **a**) were used as the reference gene set for all Venn analyses, with upregulated
669 and downregulated genes analyzed separately.

a



b



670

671 **Extended Data Fig. 11 Sequence conservation and divergence of SpoT in enteric bacteria.**

672 Pairwise sequence comparison of *Escherichia coli* SpoT_{ec} with SpoT homologs from *Salmonella*

673 *enterica* serovar Typhimurium SL1334 (SpoT_{st}) and *Shigella flexneri* (SpoT_{sf}). SpoT_{sf} is nearly

674 identical to SpoT_{ec}, differing by a single conserved substitution (A614G) within the CC domain.

675 SpoT_{st} (702 amino acids) shares 98.15% overall sequence identity with SpoT_{ec} (701 amino acids),
676 but contains several amino acid substitutions distributed across multiple domains, including the
677 hydrolase (HYD; N267H), TGS (T437S), linker-2 (L2; nine substitutions: N501A, H542Q, D544E,
678 S546V, I547V, P549T, A550V, T551A, and an insertion of N between residues 553-554), CC
679 (A621E), and ACT (A668T) domains.
680

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687

688 AUTHOR CONTRIBUTIONS

689 Y.E.Z designed this study; Y.Y.L, M.L.S and Y.E.Z acquired fundings; Y.Y.L, M.L.S, Y.E.Z
690 acquired experimental data; X.C.Z, L.D, K.G, Y.E.Z performed the bioinformatics analyses; Y.E.Z,
691 K.G, wrote the draft manuscript; and all authors analysed the data and edited the manuscript.

692

693 DECLARATIONS OF INTERESTS

694 The authors declare that they have no conflicts of interest.

695

696 EXPERIMENTAL MODEL AND SUBJECT DETAILS

697 *E. coli* DH5 α (Stratagene) was used for cloning and *E. coli* K-12 MG1655 for experiments requiring
698 an *E. coli* wt strain background (see **Table S1** for further details). For cloning, nutrient broth (Oxoid),
699 with agar and antibiotics (chloramphenicol (25 μ g/ml), ampicillin (100 μ g/ml), and/or kanamycin (25
700 μ g/ml)) when appropriate, was used. The different MG1655 strains were cultivated in Lysogeny
701 Broth (LB) as well as M9Glc media (1x M9 salt, pH 7.4 1 mM MgSO₄, 0.1 mM CaCl₂, Thiamine
702 (0.001 mg/1 ml), 0.2 % (g/ml) glucose) at 37 °C with agitation (160 rpm).

703 METHOD DETAILS

704 Isolation of suppressor mutations rescuing the growth of *spoT*^{H414A} in M9Glc

705 The *spoT*^{H414A} (YZ527) strain was grown overnight in LB media and then washed twice with PBS
706 buffer. Approx. 10⁷ cells were plated on one M9Glc plate and incubated at 30°C. Colonies of different
707 sizes were selected between 24-72 hours of incubation, and the restored growth was confirmed in
708 solid and liquid M9Glc medium.

709

710 Growth assay in liquid minimal media

711 To measure the growth curves of various strains in M9Glc media, the OD_{600nm} was measured of
712 overnight cultures grown in LB of each strain, washed twice with 1x Phosphate Buffered Saline (PBS)
713 and normalised in PBS and inoculated into fresh M9Glc media with a starting OD_{600nm} = 0.05 unless
714 otherwise stated. The growth was measured every 15 min for 72 hrs in a plate reader (BioTek)
715 presetted at 30°C with double orbital agitation at 548 cpm. For strains carrying ASKA plasmids (*acpP*,
716 *rsd*, *ytfK*, or *obgE*), protein expression was induced by addition of IPTG at final concentrations of 20,
717 50, or 200 µM, as indicated.

718

719 Anti-serum against SpoT C-terminal domains

720 Because full-length SpoT proved difficult to obtain in a soluble form, we instead generated a
721 truncated SpoT construct encompassing the C-terminal regulatory domains. A DNA fragment
722 corresponding to the *spoT* gene was first amplified using primers pYZ12 and pYZ13 and cloned into
723 the NcoI and BamHI restriction sites of the pET28b expression vector. Subsequently, primers
724 pYZ341 and pYZ342 were used for quick-change mutagenesis to delete the N-terminal catalytic
725 domains, yielding a construct encoding the TGS, CC, and ACT domains of SpoT fused to an N-
726 terminal His₆-TEV tag (pET28b-his.tev.spoT(TGS-CC-ACT)). The resulting plasmid was
727 transformed into *Escherichia coli* BL21(DE3) cells for protein expression. His₆-TEV-SpoT(TGS-
728 CC-ACT) was purified under denaturing conditions using nickel-affinity chromatography. Cell

pellets were resuspended in lysis buffer containing 6 M urea (pH 8.0), and clarified lysates were applied to Ni-NTA resin. Bound protein was eluted with buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5% (v/v) glycerol, 0.5 M imidazole, and 6 M urea. Purified His₆-TEV-SpoT(TGS-CC-ACT) protein was used as antigen to generate polyclonal antiserum in rabbits by Covalab (France), according to standard immunization protocols.

Unnatural amino acid-mediated UV crosslinking

Principle of unnatural amino acid-mediated UV crosslinking

Site-specific incorporation of a photo-crosslinkable unnatural amino acid was used to identify potential protein interaction partners of SpoT. A diazirine-containing lysine analog (DiZPK) was incorporated at position H414 of SpoT via amber stop codon suppression, enabling UV-induced covalent crosslinking to proximal proteins under native growth conditions.

Plasmid construction

To generate an amber codon substitution at position H414 of SpoT, the *spoT* gene was amplified from *Escherichia coli* MG1655 genomic DNA using primer pairs pYZ347/348 and pYZ349/350. Overlap PCR was performed to replace the codon encoding H414 with a TAG stop codon. The resulting PCR product was digested with BamHI and EcoRI and cloned into the corresponding sites of the pNDM220 expression vector, generating pNDM220-RBS-His₆.spoT(H414TAG).His₆. To remove the native N-terminal histidine tag sequence, primers pYZ700 and pYZ701 were used to amplify the N-terminal region of *spoT* from MG1655 genomic DNA. This fragment was digested with BamHI and PmlI and cloned into the BamHI/PmlI sites of the intermediate pNDM220-RBS-His₆.spoT(H414TAG).His₆ plasmid, yielding the final construct pNDM220-RBS-spoT(H414TAG).His₆.

Cell growth and unnatural amino acid incorporation

753 The *E. coli* $\Delta relA \Delta spoT$ strain harboring pNDM220-RBS-spoT(H414TAG).His₆ and pSupAR-
754 MbPylRS(DiZPK) (YZ1350) was grown overnight in LB medium and subcultured 1:50 into fresh
755 LB supplemented with 1% (w/v) glucose. Cultures were grown at 30 °C with shaking until OD_{600nm}
756 $\approx$ 0.2, at which point DiZPK (MedChemExpress; CAS 1337883-32-5) was added to a final
757 concentration of 100 μ M. Cells were incubated for an additional 30 min with shaking at 160 rpm,
758 followed by induction of SpoT expression with 1 mM IPTG for 1 h. Cells were then harvested by
759 centrifugation, washed twice with phosphate-buffered saline (PBS), and resuspended in M9Glc
760 medium. Cultures were incubated for an additional 30 min to allow recovery under minimal medium
761 conditions prior to UV crosslinking.

762 UV crosslinking

763 Cell density was measured, and an aliquot (1 ml) was collected prior to UV exposure as a non-
764 crosslinked control. Samples were pelleted by centrifugation (13,000 rpm, 2 min), supernatants were
765 removed, and cell pellets were flash-frozen. For crosslinking, cultures were exposed to UV light at
766 365 nm for 30 min using an F8T5BL UV lamp. Following irradiation, cells were harvested by
767 centrifugation, washed once with PBS, pelleted, and stored at -20 °C until analysis.

768 Protein extraction and immunoblotting

769 Cell pellets were resuspended in Laemmli sample buffer and normalized to equivalent OD_{600nm}
770 values. Proteins were separated by SDS-PAGE using a 5% resolving gel and transferred to PVDF
771 membranes. Membranes were probed with anti-SpoT antiserum (1:2,000 dilution) for 1 h at room
772 temperature, followed by incubation with HRP-conjugated anti-rabbit secondary antibodies.
773 Immunoreactive bands were detected by chemiluminescence using Pierce® ECL Western Blotting
774 Substrate (Thermo Scientific, #32209) and imaged with an ImageQuant LAS4000 system.

775

776 **Measurements of (p)ppGpp by autoradiography**

(p)ppGpp is a highly labile molecule not amenable to HPLC or mass spectrometry analysis. Therefore, thin layer chromatography (TLC) was used to quantify both pppGpp and ppGpp. The excess phosphates in M9Glc affect efficient labelling of nucleotides by $H_3^{32}PO_4$, therefore MOPS-M9Glc hybrid media was prepared wherein the PBS buffer in M9Glc was replaced by the MOPS buffer of the MOPS media⁴⁴ (i.e., containing MOPS-tricine 40 mM pH 7.2, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, Thiamine (0.001 mg/1 ml), 0.2 % (g/ml) glucose, NH_4Cl 0.001 g/ml, 8.5 mM NaCl, 0.2 mM $KHPO_4$). The strains were grown for ca. 18 hrs in MOPS-M9Glc media at 37 °C with agitation. Afterwards, the strains were adjusted to the OD_{600nm} of 0.05 in MOPS-M9Glc and grown at 37 °C with agitation until OD_{600nm} of 0.2-0.3 was reached. Then, 100 μ l cells were transferred to a 2-ml Eppendorf tube together with 5 μ l of 0.005 mCi $H_3^{32}PO_4$ (100 μ Ci/ml, PerkinElmer). After 1h at 37 °C with 600 rpm agitation, 90 μ l were transferred to a new tube with 20 μ l 2N formic acid, and the sample was stored at -20°C until analysed by TLC as described in¹⁸.

Sample preparation for RNA-sequencing

The strains were grown overnight in LB media before washed twice in PBS and inoculated into M9Glc media ($OD_{600nm} = 0.05$). The strains were grown in M9Glc media until the early exponential phase (approximately $OD_{600nm} = 0.2$) before the cells were collected, and snap frozen with liquid nitrogen and stored at -80 °C. As the strain *spo^{TH414A}* is unable to grow in M9Glc media, the starting OD_{600nm} was set to 0.2, and the cells were collected when the other strains are ready to harvest, i.e. ca. 18 hrs.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen; 74104). Ribosomal RNA (rRNA) was depleted from purified total RNA with Ribo-Zero Plus rRNA Depletion kit (Illumina; 20040892). First and second strand complementary DNA (cDNA) were synthesized by cDNA Synthesis kit (Illumina; 20040895). RNA Index Anchors (Illumina; 20040899) was used for ligation of pre-index

anchors to the ends of the double-stranded cDNA fragments to enable dual indexing. Barcoded cDNA libraries were constructed from anchor-ligated DNA fragments and sequenced on Illumina NovaSeq 6000 Sequencing System from Genewiz (Azenta Life Sciences), yielding a median depth of 39,619,482 paired-end 150 bp reads per sample.

Strain construction

All strains and oligonucleotides used in this study are listed in **Tables S1** and **S2**, respectively. All chromosomal *in situ* mutations of *spoT* alleles were made by using the I-SceI-based λ -Red recombineering method⁴⁵.

The *spoT* E319Q and H72A D73A mutant strains

Briefly, the pWRG99 plasmid was transformed into the target strains (YZ38, YZ359, YZ543, YZ544, YZ545, YZ546) creating the target strains YZ313, YZ345, YZ826, YZ827, YZ828, and YZ829. The SceI site of the pWRG100 vector was PCR amplified using the primers pYZ611 and pYZ612 for the E319Q mutation and the primers pYZ617 and pYZ618 for the H72A D73A mutation. These PCR DNAs were then electroporated into the above strains (YZ38, YZ359, YZ543, YZ544, YZ545, YZ546). Then, the oligos pYZ613 and pYZ616 were used to introduce the E319Q and H72A D73A mutations into the specific *spoT* alleles *in situ*, generating the E319Q mutant strains (YZ872, YZ873, YZ874, YZ875, YZ876) and H72A D73A mutant strains (YZ900, YZ902, YZ904, YZ905).

The C-terminal serial truncated mutant strains of *spoT*

pYZ796 and pYZ797 were used to amplify the SceI from pWRG100 and introduced into YZ313, YZ345, YZ826, YZ827, YZ828, and YZ829. The serial truncations were performed using the repairing oligos pYZ798, pYZ799, pYZ901, pYZ801, pYZ902, deleting the according domains

(ACT, ACT-CC, ACT-CC-Linker2, ACT-CC-Linker2-TGS, ACT-CC-Linker2-TGS-Linker1, respectively).

Reconstruction of the *spoT*^{H414A} suppressor mutations

Primers pYZ619 and pYZ620 were used to amplify the *SceI* DNA from pWRG100 vector before it was introduced into the strain *spoT*^{wt} (YZ313). Then, the primers pYZ328 and pYZ70 were used to amplify the corresponding *spoT* alleles using the chromosomal DNA of the strains *supA* (YZ543), *supB* (YZ544), *sup17* (YZ545), and *sup41* (YZ546) respectively as the templates. These DNAs were used to introduce the specific *spoT* suppressing mutations.

P1 phage transduction

The *spoT* genes of the suppressor strains YZ543, YZ544, YZ545, and YZ546 were inactivated via P1 phage transduction. The donor strain for making the P1 phage lysate was the *spoT*^{207::cat} strain (YZ47)¹³, which contains a chloramphenicol resistance cassette together with *spoT*²⁰⁷ deletion allele for selection. The phage lysate was used to infect strains YZ543, YZ544, YZ545, and YZ546 and the chloramphenicol resistant transductants were selected and confirmed via diagnostic PCR.

Plasmids constructions

To construct *E. coli* (*Ec*), *Salmonella typhimurium*(*st*) or *Shigella flexneri*(*sf*) *spoT* wild type (wt) and H414A mutant plasmids, standard molecular cloning procedures were used. For *E. coli*, the *spoT* coding sequence was PCR-amplified from MG1655 *spoT*^{wt} (YZ38) or *spoT*^{H414A} (YZ527) using primers pYZ1201 and pYZ1202. The pBAD33 vector was isolated from *E. coli* DH5α (YZ593). Both the amplified *spoT* fragments and pBAD33 were digested with *KpnI* and *HindIII* and ligated using T4 DNA ligase to generate pBAD33- *spoT*^{wt} and pBAD33- *spoT*^{H414A}. For *S. Typhimurium*, the

849 H414A mutation was introduced into pGEN-MCS-*spoT_{st}* (YZ692)⁴⁶ by site-directed mutagenesis
 850 using primers pYZ1312 and pYZ1313, yielding pGEN-MCS-*spoT_{st}* (H414A). For *S. flexneri*, an
 851 A614G substitution was introduced by overlap-extension PCR using pBAD33-*spoT_{Ec}*(wt) or
 852 pBAD33- *spoT_{Ec}*(H414A) as templates. Upstream and downstream fragments were amplified with
 853 primer pairs pYZ1201/pYZ1346 and pYZ1202/pYZ1345, respectively, fused by overlap PCR using
 854 primers pYZ1201 and pYZ1202, and cloned into pBAD33 following digestion with *KpnI* and *HindIII*
 855 to generate pBAD33-*spoT_{st}*(WT) and pBAD33-*spoT_{st}*(H414A). To construct the plasmid pCA24N-
 856 argA(H15Y), quick-change mutagenesis was carried out using pCA24N-argA(wt) as template and
 857 the primers pYZ1168 and pYZ1169. All plasmids were transformed into *E. coli* DH5α (see **Table**
 858 **S1**), purified using a commercial miniprep kit (NEB-T1110L), and confirmed by sequencing with
 859 primers pYZ404, pYZ405, and pYZ71 (for pBAD33 constructs), pYZ1305, pYZ1306, and pYZ1228
 860 (for pGEN-MCS constructs) or pYZ34 and pYZ35 (for pCA24N constructs; Eurofins Genomics).

861

862 **Acid resistance assay**

863 Overnight (16 hrs) cultures were grown in LB medium at 37°C with shaking (160 rpm). Cells were
 864 harvested by centrifugation, washed twice with PBS, and normalized to an optical density at 600 nm
 865 (OD_{600nm}) of 0.4 in minimal medium supplemented with 0.2% glucose (M9Glc, pH 7.4). Cultures
 866 were incubated at 37°C with shaking (160 rpm) for 30 min to allow adaptation to M9Glc. After
 867 adaptation, 1 mL cells were pelleted and resuspended in the same volume of M9Glc medium pre-
 868 adjusted to various pH values (from 2.5 to 8.0), with or without supplemented 0.6 mM glutamate (or
 869 other amino acids). Acid challenge was then performed and assessed at 37°C with shaking (160 rpm).
 870 Cell viability was determined at time zero (i.e., before shifting to M9Glc with a low pH), and at 0.5,
 871 1, 2 and 4 h after acid challenges. For viable-cell counts, 40 μL cell culture at each time point was

collected and 10-fold serially diluted (10^0 - 10^{-7}) in LB medium, and 5 μ L of each dilution was spotted on LB agar plates, allowing colony formation and enumeration and calculation of CFUs the next day.

Western blot

Western blot analysis of protein levels of various mutant SpoT was performed as reported¹⁸. Briefly, cells of various strains were collected the same manner as preparation for RNA sequencing (see above, and **Fig. S1C**). These cells were normalised to have an equal OD_{600nm} per ml and mixed with 2x SDS-PAGE loading buffer. Samples were heated for 15 min at 95°C and 20 μ L of each were separated in 10% SDS-PAGE gels. The proteins were transferred to a PVDF membrane (Amersham) and the SpoT proteins detected by first incubation with anti-SpoT antiserum (Covalab) and then the secondary HRP conjugated rabbit IgG antibody (SIGMA). The SpoT bands were visualized by using the Pierce ECL chemiluminescence substrate (Thermo) and the signals were detected by an Imagequant LAS4100.

Bioinformatic analysis

Preparing RNA-seq data. RNA-seq data (fastq files) were mapped by the BWA-MEM tool of Galaxy⁴⁷ and then analysed by the featureCounts tool in Galaxy (<https://usegalaxy.eu/>). The tabular format of the featureCounts files were exported from Galaxy and assembled into one single tabular file from which sub-files containing collections of genes of interest, e.g. amino acid biosynthesis, purine de novo synthesis, were extracted.

Analysis of RNA-seq data. To generate heatmaps and fold-of-changes, the tabular files containing the RNA-seq counts were analysed by DEGUST (<https://degust.erc.monash.edu/>). To generate Venn diagrams, gene lists were analysed by the jVenn application at (<https://jvenn.toulouse.inrae.fr/app/index.html>). Gene Ontology terms were generated by AmiGO

(<https://amigo.geneontology.org/amigo>) and Panther
(<https://pantherdb.org/webservices/go/overrep.jsp>). The json-files generated by PANTHER were
analysed by ChatGPT that generated editable SVG files.

Other Bioinformatic Procedures

Protein sequence alignment was accomplished using JalView⁴⁸, protein tertiary structure modelling
and visualization by AlphaFold 3 and ChimeraX, respectively^{49,50}.

QUANTIFICATION AND STATISTICAL ANALYSIS

Signals of (p)ppGpp from autoradiography were quantified using ImageQuant (GE Healthcare). At
least three biological replicates were performed and one representative shown in the **Figures**.
For other physiological growth experiments, at least two biological replicates each with three
technical replicates were done (see **Figure** legends for details). The length (hours) of lag phases was
defined as the period from the growth start to the time point where the slope of growth curves first
reaches the exponential-phase growth rate. These lag phase length values were then normalized to
the parental strains or conditions, and plotted (see each of the legends for details). The student *t*-test
was used for statistic analysis wherever applicable.

DATA AVAILABILITY

The RNA-seq raw data produced in this study are available through the NCBI Sequence Read
Archive under BioProject accession number PRJNA1097790.

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1069 **Legends to Supplementary Tables**

1070 **Table S1. Bacterial strains used in this study**

1071 **Table S2. Oligonucleotides used in this study**

1072 **Table S3. Differential gene expression analysis of RNA-seq data from *spoT^{wt}* and *spoT^{H414A}***

1073 **strains** . In total, the transcription levels of 4575 genes of MG1655 were analysed. At a setting of
1074 $|\text{Log}_2(\text{FC})| > 1$ and $\text{FDR} < 0.05$, 865 and 949 genes were down and up-regulated, respectively. The
1075 change-of-folds $|\text{Log}_2(\text{FC})|$ of each gene are also shown.

1076

1077 **Table S4. Gene ontology analysis of the four suppressor strains.**

1078 **Sheet #1 (related to Extended Data Fig. 7C):** Genes downregulated in the *spoT^{H414A}* strain relative
1079 to the *spoT^{wt}* strain (Column A). Genes upregulated in either of the four suppressor strains *supA*,
1080 *sup41*, *supB* and *supI7* relative to the *spoT^{H414A}* strain (Columns B to E). The list of genes in
1081 Columns B to E were used to generate a list of genes that were upregulated in at least one
1082 suppressor strain (Column F) and a list of genes that were upregulated in all 4 suppressor strains
1083 (Column G).

1084

1085 **Sheet #2 (related to Extended Data Fig. 7D):** Genes upregulated in the *spoT^{H414A}* strain relative
1086 to the *spoT^{wt}* strain (Column A). Genes downregulated in either of the four suppressor strains
1087 relative to the *spoT^{H414A}* strain. The list of genes in Columns B to E were used to generate a list of
1088 genes that were upregulated in at least one suppressor strain (Column F) and a list of genes that
1089 were downregulated in all 4 suppressor strains (Column G). Gene lists in **Table S4** were generated
1090 by DEGUST at a setting of $|\text{Log}_2(\text{FC})| > 1$ and $\text{FRD} < 0.05$.

1091

1092 **Table S5. Comparison of *spoT^{H414A}* with suppressor strains (related to Extended Data Fig. 8).**

1093 The Table yields the folds-of-changes in the suppressor strains relative to *spoT^{H414A}*. The Log2(FC)
 1094 setting in DEGUST was chosen to yield around 100 genes up and downregulated in each heatmap
 1095 Panels a, b, e, h and k of **Extended Data Fig. 8**.