Mutations in respiratory complex I promote antibiotic persistence through alterations in intracellular acidity and protein synthesis

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Antibiotic persistence describes the presence of phenotypic variants within an isogenic bacterial population that are transiently tolerant to antibiotic treatment. Perturbations of metabolic homeostasis can promote antibiotic persistence, but the precise mechanisms are not well understood. Here, we use laboratory evolution, population-wide sequencing and biochemical characterizations to identify mutations in respiratory complex I and discover how they promote persistence in Escherichia coli. We show that persistence-inducing perturbations of metabolic homeostasis are associated with cytoplasmic acidiﬁcation. Such cytoplasmic acidiﬁcation is further strengthened by compromised proton pumping in the complex I mutants. While RpoS regulon activation induces persistence in the wild type, the aggravated cytoplasmic acidiﬁcation in the complex I mutants leads to increased persistence via global shutdown of protein synthesis. Thus, we propose that cytoplasmic acidiﬁcation, ampliﬁed by a compromised complex I, can act as a signaling hub for perturbed metabolic homeostasis in antibiotic persisters.
Bacterial persistence describes the presence of phenotypic variants within an isogenic population that are transiently tolerant to otherwise lethal antibiotic therapy1–4. Such antibiotic-tolerant cells can endanger human health as they prolong therapy and can ultimately lead to relapsing infections5,6,7. In addition, tolerance levels are shown to increase during evolution under periodic antibiotic treatment8–10 and such increased tolerance levels were shown to proceed and accelerate the development of genetic resistance11,18–21. Recent advances towards understanding bacterial persistence have been made over the last years. First, toxin-antitoxin systems were found to be involved in persistence induction and awakening22–24. Second, the stringent response, a stress response that coordinates adaptation to stresses through altered levels of messenger nucleotides ppGpp and pppGpp (i.e. (p)ppGpp), has been linked to persistence via either increased levels of (p)ppGpp25,26 or a plethora of pathways including the induction of the RpoS regulon27–29, the inhibition of major processes such as DNA replication, transcription, and translation30–32 and the activation of toxin-antitoxin systems33,34,35. Third, the phenomenon of persistence in the context of metabolic homeostasis lead to persister cell formation. We propose that cytoplasmic acidic pH as a key mutational target for increased persister formation upon perturbations of metabolic homeostasis such as very fast growth36, enzyme saturation37, or metabolic network such as respiratory complex I38 as well as other imbalances of the metabolic network such as very fast growth39,40, enzyme saturation41, or a perturbed TCA cycle and/or respiration42,43. Given the multitude of various metabolic perturbations leading to persistence, we hypothesize that one generic signal exists that triggers persistence upon perturbed metabolic homeostasis. Yet, it is unknown what this trigger is and how it initiates persister cell formation.

Here, we describe a mechanism for how different perturbations of metabolic homeostasis lead to persister cell formation. Through laboratory evolution of persistence and whole-genome population-wide sequencing, we identified respiratory complex I as a key mutational target for increased persister cell formation upon entry into stationary phase, in model and pathogenic E. coli strains. Complex I mutants with increased persistence display compromised proton translocation leading to significant cytoplasmic acidification upon perturbations of metabolic homeostasis. We propose that acidification induces the persister state in two ways: through activation of the RpoS regulon and, upon more severe cytoplasmic acidification, through shutdown of protein synthesis. Our findings show how cytoplasmic acidification, amplified by a compromised complex I, acts as a central signaling hub to connect a perturbed metabolic homeostasis with persister cell formation.

Results
Evolution towards increased persistence leads to mutations in nuo. To discover genetic determinants of persister cell formation upon perturbations of metabolic homeostasis, we exploited laboratory evolution selecting for increased persistence in an environment that cycles between dilution and regrowth to stationary phase and antibiotic treatments (Figs 1a, 2a, b). In this work, we investigated the genomic changes that occurred in 37 populations of an E. coli lab strain that were previously evolved under such cycling environment with daily exposure to amikacin (ten populations), kanamycin, tobramycin, or gentamicin (nine populations each)18. For comparison and generalization, we also included three populations of the uropathogen UTI89 that we evolved under daily exposure to amikacin in the current work, where we found 1000 to 10,000-fold increases in persistence, similar to the previously evolved populations of the lab strain (Supplementary Fig. 1a).

To detect mutations in these 40 E. coli populations, we applied deep, whole-genome sequencing on population samples at the endpoint of evolution after 5–10 days. Initially focusing on mutations present at, or above, a frequency of 5% (see "Methods"), we identified on average 3.15 mutations per population amounting to a total of 128 mutations across all populations (Supplementary Data 1; Supplementary Fig. 1b, c). Statistical analyses indicate that the evolved populations consist entirely of mutants (Supplementary Fig. 1d, e). Confirming the low mutational complexity as inferred from population-wide sequencing, genome sequences of randomly picked clones at endpoints only contained one or two mutations (Supplementary Table I). Mutations are significantly enriched in coding regions of 29 genes, especially targeting genes coding for inner-membrane proteins (Supplementary Fig. 1f–h). Several targets are repeatedly hit by identical mutations in different populations, and/or emerge as multiple alleles (Supplementary Fig. 1j, k).

Mutations in nuo predominantly hit regions encoding transmembrane domains involved in proton translocation and are causal to high persistence. The E. coli nuo operon consists of 13 genes coding for NADH/ubiquinone oxidoreductase (respiratory complex I). The complex serves as an entry point for electrons from NADH into the electron transport chain (ETC) (Fig. 2a). 87 out of the 93 identified mutations and 41 unique alleles are present in the genes nuoAHJKLMN of complex I. Notably, the structurally and functionally distinct part of the complex, encoded by nuboCDDEFGI, contains significantly fewer mutations (only six) (Supplementary Fig. 2a, b). NuboCDDEFGI forms the cytoplasmic arm that catalyzes NADH oxidation and transports the electrons via a series of iron-sulfur clusters to the ubiquinone reduction site, 100 Å apart39. NuoAHJKLMN builds the membrane arm that translocates protons across the membrane, thus contributing to the electrochemical membrane potential, i.e. proton motive force (PMF)31,32 (Fig. 2a). The mutations we found are primarily in the proton translocating units of the membrane arm and specifically hit codons of amino acids enriched in hydrophobic residues (83 times) and of those residues located in transmembrane helices (64 times) (Fig. 2c, Supplementary Fig. 2a, b). While many of the altered amino acids are in close proximity to regions involved in proton translocation (Fig. 2a inset, Supplementary Fig. 2c, d), the mutations do not directly affect amino acids reported to be crucial for proton translocation, such as amino acids involved in long-range proton translocation (Fig. 2a inset, Supplementary Fig. 2c, d).
structural changes along a central axis in the membrane arm, or
in outlining the proton half-channels, or in the actual binding
and export of protons (Fig. 2a). Based on these findings, we expected
that the mutations do not abolish the overall complex I function,
but rather induce smaller functional changes in complex I, likely
connected with proton translocation, leading to the observed
increase in persistence.

To confirm causality of the mutations in noo for persistence,
we selected clones with single mutations in each one of the
subunits L, M and N (called noo further on)—which are
subunits that are all proposed to contain one distinct proton
translocation path—and restored the chromosomal wild-type
alleles in these mutants by site-specific mutagenesis. Here we
found that the strains again became susceptible towards amikacin,
i.e. the antibiotic that imposed selective pressure during evolution
(Fig. 2d). In addition, the minimal inhibitory concentration of
amikacin remained unchanged in the noo mutants, excluding an
increase in resistance as an explanation for their increased survival (Supplementary Fig. 2e). In fact, the noo mutants show
clear cross-tolerance to other antibiotics in the stationary phase
(Supplementary Fig. 2f–h), which is a known hallmark of
persistence.3 As already expected from the above-described characteristics of the mutations (e.g., that they are specific to
the proton translocation arm and are not targeting crucial amino
acids), the effect of the point mutations cannot simply be
mimicked by destroying complex I functionality entirely, either
by knocking out the individual subunits or the entire complex
(Fig. 2e, Supplementary Fig. 2f, g). Restoring the wild-type alleles
abrogates not only the amikacin tolerance as mentioned above,
but also the multidrug tolerance of the point mutants for the
related aminoglycoside kanamycin and for the unrelated
fluoroquinolone ofloxacin (Supplementary Fig. 2i), demonstrating
that noo mutations are causal to the high-persistence,
multidrug-tolerant phenotype.

High-persistence-conferring variants of complex I oxidize
NADH and transfer electrons but are impaired in proton
translocation. To test whether the identified noo mutations
indeed alter proton translocation, we produced and purified wild-type
complex I and three variants, carrying a variation in either
subunit L, M, or N. Despite its huge size (±550 kDa) and its
multitude of cofactors, we were able to purify wild-type complex I
and its variants with retained activity from E. coli membranes
through several chromatographic steps (Supplementary Fig. 3a, b;
see “Methods”). Yields of the preparations were similar between
the samples (±0.2 mg protein g
−1 cells) as were the banding
patterns after SDS PAGE, both in line with previously obtained
results35,36 (Supplementary Fig. 3c). Native gel electrophoresis
confirmed stability and purity of the preparations, and in situ
NADH oxidation demonstrated activity of the complexes
(Supplementary Fig. 3d). The mutations did not introduce instability
to the variants as judged from their “melting points”37 (Supplemen-
tary Fig. 3e, f). Thus, the purification yielded pure and active
samples of the wild-type complex I and the three variants.

To profile the biochemical activities of the purified complexes,
we made use 1) of proteoliposomes, i.e. vesicles made from E. coli
polar lipids reconstituted with the individual preparations of
complexes, of membrane fractions containing the wild-type
complex I or the variants, and 2) of inside-out vesicles (ISOVs)
prepared from \textit{E. coli} membrane fractions (see “Methods”). Like proteoliposomes, the latter are closed compartments but additionally retain the full complexity of the in vivo membrane composition. NADH oxidation (and subsequent reduction of decyl ubiquinon in the membrane) was measured spectro-potentialistically as a decrease in NADH absorption at 340 nm, where we found that the liposomes reconstituted with wild-type complex I and the variants oxidized NADH at a similar rate (Fig. 3a and inset). Electrons from NADH ultimately flow down the ETC via the quinone/quinol couple to the terminal oxidases, where in well-aerated conditions oxygen is reduced to water. Respirometry on membranes that contain wild-type complex I or its variants as sole NADH dehydrogenase (see “Methods”) revealed highly similar O$_2$ reduction rates upon NADH addition (Fig. 3b and inset). Thus, NADH oxidation and electron transfer are both not affected by the \textit{nuo\textsuperscript{*}} mutations.

To examine proton translation as third functionality of complex I, we made use of the fluorescence of 9-amino-6-chloro-2-methoxyacridine (ACMA) that can be quenched upon alkalization induced by proton translocation (Supplementary Fig. 3g, h). Compared to wild-type complex I, all variants showed, to varying degrees (50–75%), impaired proton translocation upon addition of NADH to the proteoliposomes (Fig. 3c). We confirmed this result by testing the activity of the variants in ISOVs (Fig. 3d). The ISOVs results furthermore quantitatively agreed with their more complex composition. As ISOVs also contain the terminal quinol oxidases, proton gradients measured with ACMA reflect the combined action of the quinol oxidases (1H$^+$/e$^-$ for \textit{bd}-type and 2H$^+$/e$^-$ for \textit{bo3}-type oxidase) and complex I (2H$^+$/e$^-$)\textsuperscript{58}. ISOVs with complex I variants mainly translocate protons by the quinol oxidases and lack contribution

\textbf{Fig. 2} High persistence-conferring \textit{nuo\textsuperscript{*}} mutations target hydrophobic amino acids in transmembrane subunits and do not cause a complete loss of function of the complex. \textit{a} Modeled structure of \textit{E. coli} complex I (see Methods). The cytoplasmic subunits NuoBCDEFGI (gray surfaces) catalyze NADH oxidation and transfer two electrons over a series of iron-sulfur clusters (not shown) to the quinone reduction site. The quinol flows further down the electron transport chain in the membrane (gray background) to be re-oxidized by terminal oxidases (not shown). The hydrophobic subunits NuoAHJKLMN (in colored surfaces) translocate four H$^+$ ions per molecule of NADH that is oxidized by the cytoplasmic subunits. The membrane part is magnified in the inset and is annotated with the positions of amino acid variants found in high-persistence mutants (red spheres) and with residues that are crucial for proton translocation (blue sticks, based on refs. \textsuperscript{51–53}). \textit{b}, \textit{c} High-persistence mutations are significantly enriched in \textit{b} the membrane units and \textit{c} predominantly target hydrophobic amino acids (Chi$^2$ comparison to random mutations, see Methods). \textit{d} The \textit{nuo\textsuperscript{*}} mutations are causal for high persistence. Mutants with single mutations in each one of subunits L, M and N lose their high tolerance for amikacin (5h with 400 \textmu gm l$^{-1}$) when \textit{nuo\textsuperscript{*}} mutations are repaired (mean ±stdevs, n = 3; *p < 0.0001 for a within-strain comparison from a two-way ANOVA with Sidák’s posttest). \textit{e} Killing dynamics with amikacin (400 \textmu gm l$^{-1}$) confirm the high persistence of \textit{nuo\textsuperscript{*}} point mutants in stationary phase and show that their effect cannot be mimicked by a single gene or operon knockout (in red). A model describing biphasic killing dynamics (lines ±95% CIs) was fitted to the data (means ±stdevs, n = 3; * fits are different based on AIC criterion). See also Supplementary Fig. 2. Source data are provided as a Source Data file.
from complex I (so 1–2 H⁺/e⁻), while complex I significantly contributes to proton translocation in wild-type ISOVs (so 3–4 H⁺/e⁻), which concurs with the 50–70% lower ACMA quenching in ISOVs with complex I variants compared to wild-type ISOVs (Fig. 3d). Overall, these results demonstrate that the identified nuo* mutations in complex I target proton translocation and uncouple translocation from electron transfer.

Neither impaired antibiotic uptake nor decreased energy levels fully explains complex I-dependent increased persistence. Given their compromised proton translocation, we hypothesized that the nuo* mutants could have a decreased proton motive force (PMF). Previously, persisters were shown to tolerate aminoglycosides by means of a decreased PMF leading to lower antibiotic uptake. To test this hypothesis, we assessed the electrical gradient, i.e. the part of the PMF driving the uptake of charged aminoglycosides, using bis-(1,3-dibarbituric acid)-trimethine oxonol (DiBAC₄(3)), a fluorescent dye whose uptake inversely correlates with the electrical potential across the membrane. Using flow cytometry, we assessed the level of DiBAC₄(3) of single cells to quantify their electrical gradient. Treatment with the dissipator carbonyl cyanide m-chlorophenyl hydrazone (CCCP) served as a depolarized control. The nuo* mutants showed a decreased electrical potential compared to the wild type (higher curves in Fig. 4a), resembling an expected consequence from the impaired proton translocation in complex I. However, the decrease in electrical gradient is minor—only about 50% when compared to the wild-type control. The second red vertical line indicates addition of pollenidin, which indicates that O₂ reduction indeed originates from NADH oxidation by complex I and that variants and wild-type complex I are equally sensitive to this inhibitor. c, d Proton reduction is impaired in all variants as estimated from the difference in fluorescence quenching of the pH-sensitive fluorophore ACMA in c proteoliposomes containing reconstituted complex I and in d ISOVs generated from membranes (means ±sems; n = 3). Relative fluorescent values were obtained by comparing the fluorescence to the start and through rescaling between 0 and 100% where 100% is the maximum value of each sample and 0% is the lowest value of the run (i.e. wild-type complex I which showed ±50% quenching, similar to values found in literature). Proteoliposomes are leaky and therefore revert the ACMA quench over time. ISOVs, while additionally showing the activity of the terminal oxidases, are much tighter and show no reversion. In a, d, reactions were started by adding NADH (first red vertical line) and individual graphs are nudged horizontally and, in a, b also vertically to allow comparison. See also Supplementary Fig. 3. Source data are provided as a Source Data file.
treatment. More importantly, under the assumption that depolarization fully explains the tolerant state, the differences in electrical gradient do not match with the differences in persister levels. For example, the 0.1% persister level of the wild type agrees with an electrical potential that is only present in 1% or less of the cells in the nuo* mutants (cyan lines in Fig. 4a) while these mutants have a persister level of ±50% (Fig. 2d, e, Supplementary Fig. 2f-i). Furthermore, and consistent with the small decrease in electrical gradient, nuo* mutants did not display decreased antibiotic uptake, as indicated by similar or even higher intracellular amikacin concentrations compared to the wild type (Fig. 4b). In addition, increased tolerance by a decreased antibiotic uptake as a result of a decreased electrical gradient would also not explain why the nuo* mutations also offer protection against oxolinic (Supplementary Fig. 2g), a member of the fluoroquinolone antibiotics whose uptake does not depend on an electrical gradient61–63 and which is also readily taken up by the nuo* mutants (Supplementary Fig. 4a). Altogether, these experiments show that a difference in antibiotic uptake does not explain the increased persister levels in the nuo* mutants.

Another consequence of the decreased proton translocation in the mutants could be a lowered ATP generation by the PMF-driven ATP synthase. Recently, a lower energy status has been linked to persistence35,21,41–43, while another study did not find decreased ATP levels in persisters26. We used a genetically encoded fluorescent reporter, Perceval, to measure ATP:ADP ratios in stationary phase populations. The nuo* mutations led to a decreased ATP:ADP ratio compared to the wild type (Fig. 4c), which would be in line with the hypothesis of a de-energized cell status as cause of tolerance. However, the ATP:ADP ratios in the nuo* mutants are also decreased in the exponential phase (Supplementary Fig. 4e), a condition in which none of the mutants show increased tolerance (Supplementary Fig. 2h). While lower energy status might still contribute to the increased tolerance as an important prerequisite, we conclude that neither decreased antibiotic uptake nor a lower energy status by altered PMF can single-handedly explain the increased persister levels of the nuo* mutants, a result which we also confirmed for the evolved mutants of the uropathogenic strain (Supplementary Fig. 4c–g).

Increased cytoplasmic acidification in nuo* mutants is causal for increased persistence. Next, we hypothesized that the impaired proton translocation of the complex I variants might influence pH homeostasis during metabolic perturbations, which in turn could lead to increased persistence. To test this hypothesis, we used the ratiometric fluorescent pH sensor pHluorin (Supplementary Fig. 4h) compared the cytoplasmic pH between the wild type and the high-persistence nuo* mutants. In exponential phase, a condition where we do not observe increased persistence in the nuo* mutants (Supplementary Fig. 2h), the pH in the nuo* mutants did not significantly differ from the one in the wild type exhibiting an average pH value of 7.73 ±0.01 (Supplementary Fig. 4i). However, in stationary phase, all high-persistence nuo* mutants showed similar decreases in ATP:ADP ratios in exponential phase when they do not display increased persistence (Supplementary Figs. 4b, 2h).

See also Supplementary Fig. 4. Source data are provided as a Source Data file.
again found a highly similar intracellular pH of 7.97 ± 0.02 in all strains (Fig. 5b), which dropped to 7.35 ± 0.03 directly after the shift to fumarate in all strains. While in the wild type, the pH stabilized around this level, the cytoplasm of the high-persistence nuo* mutants continued to acidify to pH values around 7.07 ± 0.13 (at 220 min after the nutrient shift) (Fig. 5b). Thus, also with a completely different way of perturbing metabolic homeostasis and inducing persistence, we find that the cytoplasm acidifies, and that this acidification is stronger in the nuo* mutants. Remarkably, also here, the decreased pH of the nuo* mutants correlated with an increased tolerance (Fig. 5c, Supplementary Fig. 5b). Thus, the decreased cytoplasmic pH, which seems to be a general feature associated with persistence-inducing conditions involving metabolic perturbations, is further aggravated in the nuo* mutants, likely due to their compromised proton translocation, being causal for their increased tolerance.

### RpoS contributes to increased persistence

Next, we asked how the lowered intracellular pH in the nuo* mutants could lead to increased tolerance. In our previous study, we found that the persisters generated through sudden nutrient shifts have an increased RpoS response with higher RpoS levels. The stationary-phase specific nature of the increased persistence in the nuo* mutants also points to a role for RpoS, which is the sigma factor. As RpoS is posttranscriptionally and posttranslationally upregulated at low pH, we hypothesized that the increased tolerance could be due to an increased pH-induced RpoS response.

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**Fig. 5 Increased cytoplasmic acidification in nuo* mutants is causal for increased persistence.**

a) Cytoplasmic pH measured in cell populations using the ratiometric pHluorin sensor, is significantly decreased in the high-persistence nuo* mutants in comparison to the wild type in stationary phase, where the external pH is 8.1 ± 0.06 (means ±stdevs, n ≥ 17; *** p < 0.001 for a phenotype-level comparison from a linear mixed model). b) Cytoplasmic pH of cells growing in exponential phase on M9 glucose medium decreases in all strains upon a shift to M9 fumarate medium (dotted line; pH ≥ 7), known to induce persister formation. Dynamic measurement of cytoplasmic pH after a nutrient shift shows continued decreases over time only in the high-persistence nuo* mutants (means ±sems, n ≥ 6; * slopes are different based on AIC criterion with the wild-type slope which is non-significantly different from 0 based on an F test). c) Survival of amikacin treatment (for 4 h with 400 µg ml⁻¹) assessed 30 min after the nutrient shift (in the same experiment as shown in b) reveals a significant negative correlation between survival and cytoplasmic pH (means ±sems, n = 4). The linear regression line (±95% confidence interval) along with Pearson r and p values (top right corner) are shown in red. d) Cytoplasmic acidification is causal for increased persister levels. Resuspending stationary phase cells in sterile spent medium buffered at different pH values increased the survival of amikacin treatment (for 5 h with 400 µg ml⁻¹) from a pH of 6.5 and below in the wild type only when benzoate and methylamine are simultaneously added (ΔpH dissipators at 40 mM; boxed bars) to equilibrate cytoplasmic to extracellular pH. Adding this weak acid-base pair does not influence tolerance levels in unbuffered spent medium that has a pH of 8.1 ± 0.06 (means ±stdevs, n ≥ 2; ** p < 0.001 and ns = non-significant for within-PH comparison from a linear mixed model with Šidák’s posttest). See also Supplementary Figs. 4-5. Source data are provided as a Source Data file.
Indeed, supporting a role for RpoS, we found that deleting rpoS reduces persistence of the nuo* mutants to some extent in stationary phase conditions (Supplementary Fig. 6; see “Methods”). Surprisingly, however, high-persistence nuo* mutants lacking both RelA and SpoT, which are incapable of producing (p)ppGpp, remain highly antibiotic-tolerant in stationary phase conditions (Supplementary Fig. 6). Thus, alternative and/or redundant routes need to exist that connect pH to RpoS induction. Moreover, since the nuo*
mutants lacking RpoS are still more antibiotic tolerant than the wild type (Fig. 6a, b) and since the correlation between cytoplasmic pH (Supplementary Fig. 5d, e) and tolerance remains significant in those strains (Supplementary Figs. 4k, l, 5a), a route additional to RpoS needs to connect the stronger decrease in cytoplasmic pH with the increased antibiotic tolerance.

**Decreased pH inhibits translation and causes increased persistence.** To investigate how the strong pH decrease in the nuo* mutants could cause the increased tolerance, we subjected the wild type and the nuo* mutant to proteome analyses at different time points during the controlled shift from glucose to fumarate, where we had found that the nuo* mutants acidify more strongly than the wild type (Fig. 5b). We mapped the high-dimensional proteome data onto a two-dimensional space created by a previously performed principal component analysis of a similar proteome dataset (Fig. 6c, Supplementary Fig. 5c; see "Methods")\(^26,69\). One dimension reflects a "growth axis" along which proteomes from cultures growing at steady-state in different growth conditions cluster. The other dimension reflects a "stress axis". It was previously found that the proteome of the wild type after the nutrient shift leading to persister formation, moved down the "stress" dimension, in a process that continued over >8 h\(^26\).

Here, agreeing with these previous results, we find that wild-type cells, when shifted from glucose to fumarate, display proteome dynamics moving down the stress dimension (closed, gray symbols in Fig. 6c), characterized by induction of the global stress response\(^26\). Secondly, we found that the proteome of the wild-type strain lacking rpoS (open, gray symbols in Fig. 6c) changes steadily over time towards a proteome of cells with normal steady-state growth on fumarate (red dot in Fig. 6c). This is in line with the previous finding that, in an ΔrpoS mutant, almost all cells resume growth after a rapid shift to fumarate\(^26\). Furthermore, this observation supports our earlier conjectured hypothesis\(^32\) that an ΔrpoS mutant strain invests resources in restoring metabolic homeostasis, and not in massive activation of stress responses, which is also corroborated by a somewhat restored pH homeostasis (i.e., reversal of the initial acidification) in the absence of RpoS in the wild type after a shift to fumarate (Supplementary Fig. 5d).

The proteome of nuo* mutants carrying a functional rpoS gene (closed, blue symbols in Fig. 6c) showed changes in the direction of an induced stress response for the first 30 min after the nutrient shift, similar to the changes of the wild-type proteome. Surprisingly, however, after 30 min the proteome stops changing abruptly (Fig. 6c), suggesting that the proteome might be locked in a frozen state with no further net turnover of proteins (i.e., either no or a balanced translation and degradation). Notably, it was also around 30 min that the cytoplasmic pH started to deviate between the wild type and the nuo* mutants (Fig. 5b) and that a correlation emerged between cytoplasmic pH and antibiotic tolerance (Fig. 5c, Supplementary Fig. 5a). Additionally, nuo* mutants lacking rpoS show a similar halt in proteome dynamics (Fig. 6c), which is in agreement with the only minor decreases in tolerance upon deletion of rpoS in these backgrounds. Thus, these data suggest that after about 30 min, which is when the pH in the nuo* mutants has dropped to pH values below 7.2, net protein turnover comes to a halt.

To test whether protein turnover is also halted in the stationary phase conditions on complex medium, we performed radioactive labeling experiments to follow protein synthesis. Here, we found that translation is indeed impaired in all nuo* mutants under the conditions that they were originally selected for (Fig. 6d), excluding the possibility that any net changes in the proteome were masked by a translation activity that balances out protein degradation. Thus, we conclude that the strong cytoplasmic acidification in the nuo* mutants halts changes in proteome turnover. We conjectured that halted protein translation, because of inactivity of amikacin’s antibiotic targets, could render the nuo* mutants more tolerant than the wild type.

To test whether the halted translation in the nuo* mutants is indeed responsible for increased persistence, we artificially blocked translation after shifting cells to fumarate by the addition of a sublethal dose of chloramphenicol and checked for tolerance against amikacin after 30 min. Here, we found that such a pretreatment, similar to what was found previously\(^70\), results in a strong induction of persistence in the wild type, comparable to the levels in the nuo* mutants without pretreatment (Fig. 6e). Additionally, such an artificial inhibition of translation also abolishes the need for rpoS both in the wild type and nuo* mutants. Thus, we conclude that the pH-induced halted translation in the nuo* mutants is indeed the main factor responsible for the observed increased persistence against amikacin, bypassing the need for a full-blown activation of stress responses by RpoS as mechanism for tolerance as is the case for the wild type.

**Discussion**

Here, we present a mechanistic model, which explains how persister cells are formed upon perturbations of metabolic homeostasis (Fig. 7). In this model, strong metabolic perturbations, such as the shift into stationary phase or abrupt nutrient shifts, lead to cytoplasmic acidification, likely through protons released through continued ATP consumption without ATP regeneration, which has been linked to acidification in other systems\(^71–75\). Mutations in complex I that compromise proton translocation result in a further decreased cytoplasmic pH upon metabolic perturbations. Part of the following induction of persistence, especially in the wild type, is conferred by the RpoS-dependent global stress response. In the nuo* mutants with increased cytoplasmic acidification, persistence is additionally conferred by a global halting of protein synthesis. At the system level, additional feedback and interaction mechanisms exist: an RpoS response inhibits restoration of metabolic homeostasis\(^26\) and the pH-induced halted protein synthesis further prevents restoration of homeostasis, while also halting a further activation of the RpoS response. Our newly identified mechanism also agrees with a growing body of recent research that suggest that bacterial metabolism, chemiosmotic homeostasis and membrane bioenergetics— in addition to drug uptake and activation of primary targets—dictates the lethality of bactericidal antibiotics\(^76–78\).

Our work identifies complex I as an important persistence factor. Loss-of-function mutations in complex I (e.g., transposon or frameshift mutations) are long known to abrogate the growth advantage in long-term stationary-phase conditions of rpoS ‘GASP’ mutants in absence of antibiotics\(^79\). More recently, such mutations were shown to increase gentamicin tolerance in *E. coli*\(^80\) and aminoglycoside resistance in *E. coli*\(^81–83\) and *Pseudomonas aeruginosa*\(^84\) through a reduced PMF and antibiotic uptake. Such an effect could also be mimicked by artificially decreasing complex I activity\(^85–88\) and was recently proposed to underly the antibiotic tolerance of a point mutant in *nuoN* in *P. aeruginosa*\(^89\). Here, we describe a new way in which complex I alters antibiotic susceptibility, i.e. by changes that solely impair proton translocation activity of the complex. Mutations identified in this work specifically lie in the proximity of the three previously confirmed proton pathways in Nuol, M and N and were found at the interface of the NuoAHJK subunits, thereby corroborating a previously hypothesized fourth channel at this site\(^51,53,90\). Additionally, the nuo* mutants still take up antibiotics
and show cross-tolerance towards other antibiotics, in contrast to the collateral hypersensitivity towards other antibiotics of (loss-of-function) mutations in nuo (and other genes) described in the context of aminoglycoside resistance due to reduced drug uptake\(^\text{83,91}\). Therefore, the nuo\(^*\) mutations identified in the current study constitute a new mechanism in which complex I influences antibiotic survival in bacteria, i.e. by inducing persister formation through increased cytoplasmic acidification upon strong perturbations of metabolic homeostasis.

In this work, we uncovered that cytoplasmic acidification—induced by perturbations of metabolic homeostasis, i.e. upon nutrient shifts or entry into stationary phase—acts as a signal for persister formation in E. coli. In fact, a drop in cytoplasmic pH was observed in Salmonella upon macrophage engulfment and this acidification was furthermore important for the injection of modulating effector proteins that allow Salmonella to survive inside the vacuole\(^\text{92}\). At the same time, but without a connection to cytoplasmic acidification, Helaine and colleagues showed that macrophage internalization induced intracellular persister formation\(^\text{93}\). Indole, the inter-kingdom signal molecule that was previously connected to the persister state\(^\text{94–96}\), was shown to regulate intracellular acidification upon entry in stationary phase\(^\text{97}\) although very recently, the tryptophanase that catalyzes this acidification after ±30 min of perturbation of metabolic homeostasis, ultimately causing cells to enter dormancy\(^\text{25}\), similar to the glassy cytoplasm connected with dormancy in other species\(^\text{108–111}\). Additional evidence for the significance of cytoplasm fluidity is the recently identified correlation of protein aggregate formation with persistence\(^\text{41,112–113}\), while, at the same time, widespread and pH-dependent aggregation of native-like proteins underlies the transition of the cytoplasm to a solid-like state\(^\text{75}\). Recently, mutations in translation-related genes were selected during laboratory evolution towards increased persistence and shown to affect translation more specifically\(^\text{116}\). Similarly, changes in cytoplasmic pH might also modulate ribosome activity more directly than via the induction of a global glass-like dormant cytoplasm, for example by unbalancing the ionic fluxes of other cations like K\(^+\), Ca\(^{2+}\), and Mg\(^2\) that were recently associated with ribosome functioning and/or antibiotic killing\(^\text{6,7,8,117}\). Therefore, a decrease in cytoplasmic pH could lead via a myriad of ways (e.g., cytoplasmic rigidity, ionic imbalances) to halted protein turnover and thus to increased tolerance.

With this work, we have unraveled the molecular basis of how metabolically induced persister cells are formed, where the dynamic nature of cytoplasmic pH, as a signal for strong metabolic perturbations, resembles a global connector of metabolism with persister formation. The generic nature of this mechanism, converging at cytoplasmic acidification, is consistent with the fact that numerous effects, including perturbations of many metabolic genes\(^\text{118,119}\), abrupt nutrient shifts, changes in extracellular pH\(^\text{93}\),

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**Fig. 7 Model connecting metabolic homeostasis, cytoplasmic acidification and two paths for persister cell formation.** A strong perturbation of metabolic homeostasis by nutrient shifts or entry into stationary phase acidifies the cytoplasm. Such acidification could occur due to an imbalance between ATP consumption and regeneration. In these conditions, complex I acts as a regulator of cytoplasmic pH: wild-type proton translocation activity counter-balances acidification; impaired proton translocation in the nuo\(^*\) mutants enhances cytoplasmic acidification after ±30 min of perturbation of metabolic homeostasis. Mild cytoplasmic acidification leads to increased persistence by an increased RpoS response, possibly mediated by inhibition of SpoT mediated hydrolysis of (p)ppGpp (dominant path in black arrows in the wild type). Stronger cytoplasmic acidification halts protein synthesis and renders antibiotic targets inactive leading to a second persistence mechanism (dominant path in red arrows in nuo\(^*\) mutants with impaired complex I function.) At various points in this model, feedback loops are in place that affect persister formation. For instance, RpoS inhibits adaptation to maintain metabolic homeostasis, recovering from the initial pH drop. In turn, strong intracellular acidification via halted protein synthesis inhibits full RpoS activation.
sublethal concentrations of antibiotics and oxidative stress have all been observed to induce tolerance and could all converge in intracellular acidification.

**Methods**

**Bacterial strains, media, and cultivation.** All bacterial strains used in this study are listed in Supplementary Data 3, with the most important strains being SX43 or SX513 (the ancestral lab strains used in most of our experiments) or WT, which are close relatives to BW25113, and UTI89 (a uropathogenic E. coli strain). Genomic mutants were constructed with either P1vir phage transduction as described before or through homologous recombineering described before. Where possible, mutants from an ordered gene knockout library in E. coli, the Keio collection, were used as templates to create new mutants and they were cured from their Km cassette by transformation with pCP20 expressing the FLP recombinase. At all steps, validity of mutants was checked by either PCR with gel electrophoresis or targeted Sanger sequencing or both using primers listed in Supplementary Data.

Experiments were performed using M9 minimal medium prepared as previously described, Mueller Hinton Broth (MHB, Becton Dickinson; widely used in antibiotic sensitivity testing), Isosony broth (LB) medium (10 g L\(^{-1}\) NaCl, 10 g L\(^{-1}\) trypton, 5 g L\(^{-1}\) yeast extract with/without 15 g L\(^{-1}\) agar), M63 minimum salts medium as previously described or ‘spent’ MHB medium. The carbon source for MHB and LB is an undefined mixture of peptides with minor traces of sugars. The spent MHB medium was prepared by removing wild-type cells from an overnight culture (37°C, 200 rpm or 1g) by centrifugation and filtration through a 0.2 μm filter. For spent MHB medium buffered at a certain pH, the appropriate Good’s buffering agent was added (50 mM 2-(N-morpholino)ethanesulfonic acid for pH 5.5 and 6 and 50 mM piperezine-N,N′-bis(2-ethanesulfonic acid) for pH 6.5) with/without potassium benzoate (40 mM) and methylamine hydrochloride (40 mM) to dissipate ΔpH. Next, the pH was adjusted using HCl or KOH and the medium was filter sterilized. For minimal media, carbon source stock solutions were prepared in demineralized water, adjusted to pH 7.0 using NaOH or HCl, respectively, and sterilized filtered through a 0.2 μm polyethersulfone (PES) filter. Glucose was added in a final concentration of 5 g L\(^{-1}\) and fumurate in a final concentration of 2 g L\(^{-1}\), respectively. Antibiotics, IPTG and arabinose were all prepared in demineralized water and sterile filtered through a 0.2 μm filter. Bacterial cultures were cultivated either in 50 ml medium in a 150 ml or 10 ml medium in a 100 ml Erlenmeyer flask closed with a 38 mm silicone sponge closure (Belco Glass) at 37°C, 300 rpm and 5 cm throw (5 x g) or in 100 ml medium in a 250 ml Erlenmeyer flask closed using cellulose stoppers (VWR) at 37°C, 200 rpm and 2.5 cm throw (1 x g). Overnight cultures were diluted 1:100 in fresh medium and further diluted 1:100 as soon as OD\(_{600}\) of 0.5 is reached to keep cells in the exponential growth phase.

Assessment of the antibiotic sensitivity. MICs, survival levels, and killing curves were determined according to the methods described previously. We determined an overnight culture was diluted in fresh MHB to an inoculum of 1 × 10^5 colony-forming units per milliliter (CFU ml\(^{-1}\)) and incubated in a range of two-fold antibiotic dilutions for 16–20 h. After incubation, the lowest antibiotic concentration where no growth was observed was determined as the absorbance at 595 nm (BioTek), we defined as the MIC. Alternatively, commercially available MIC test strips were used (Liofilchem). Antibiotic survival level in the stationary phase is obtained by taking the CFU ml\(^{-1}\) (usually for 3h) and total CFU ml\(^{-1}\) (before total antibiotic treatment (generally 1–5 × 10^5 CFU ml\(^{-1}\)). CFU ml\(^{-1}\) were obtained by making serial dilutions, spiking plates, and semi-automated quantification after 2 days of incubation at 37°C (Eddyfer and Flash & Grow). When using spent medium, stationary-phase cells were resuspended in an equal volume of the spent medium and treatment was started immediately.

The antibiotics that were used are all aminoglycosides or fluorquinolones. We used these two classes as they are potent bactericidal drugs that furthermore can kill sensitive stationary phase cells and result in a biphasic killing pattern, indicative for the survival of persister cells. Furthermore, these antibiotics were used in our previous work which forms the basis of the current work.

Assessment of antibiotic tolerance through cytometry upon nutrient shifts in M9 medium. Tolerance experiments upon nutrient shifts were always combined with regular dilutions to maintain growth in the exponential phase. At OD\(_{600}\) of 0.5, pH was measured and persisted were induced by a switch to M9 fumarate medium. At multiple time points after the nutrient switch, cultures were sampled, and intracellular pH was determined as previously described. For determination of antibiotic tolerance, we treated the cells with amikacin (4 h at 400 μg ml\(^{-1}\) at 0.5 h after the switch. As a control we blocked translation using a low dose of chloramphenicol (25 μg ml\(^{-1}\)) added direct after the switch to fumarate. After 4 h, amikacin treated cells were diluted 1:10 in fresh medium to keep their ability to grow tracked using flow cytometry (for 3h, see refs. 26,127). Tolerant cells resumed growth, became bigger and lost their pHluorin fluorescent intensity whereas non-growing cells retained their size and fluorescent intensity. The fraction of cells that did not resumed growth was determined by observing no cell size changes and no loss of fluorescence. Cells were measured every 30 min from 0 to 3 h after the transfer to LB medium. Fractions of non-recovering cells from the period of 2.5 and 3 h after transfer to LB were used to calculate the fractions of tolerant cells by subtracting the nondividing cells from the total cells added to the culture at the start.

**Evolution experiments.** Experimental evolution using the uropathogenic E. coli strain UTI89 was performed as before. Briefly, parallel cultures were grown overnight to stationary phase in MHB (±18 h), treated for 5 h with amikacin (400 μg ml\(^{-1}\); 100–200-fold MIC) to eliminate all non-persisters, washed three times in MgSO\(_4\) (10 mM) to remove antibiotics and diluted 1/100 into fresh MHB to allow another cycle of batch growth. A sigmoidal fit expected for the spread of a mutant in a haploid population was fitted to the data obtained under daily treatment.

**Genome-wide next-generation sequencing.** We detected mutations arising in evolved populations or clones by genome-wide sequencing using Illumina’s HiSeq platform. Genomic DNA was extracted by using the DNeasy Blood and Tissue Kit (Qiagen). Purify and concentrate were assessed by Nanodrop, gel electrophoresis and Qubit. Average insert size of the prepared libraries was ±300 bp and sequencing was conducted at EMBL’s GeneCore in Germany, the VIB Nucleomics core in Belgium, Eurofins, or the Genomics core of UZ Gasthuisberg in Belgium. Raw data files have been deposited in the NCBI SRA database with following accession IDs: PRJNA498891 (populations of the lab strain), PRJNA270307 (clonal data of the lab strain), PRJNA498771 (urogenotypic populations), PRJNA498708 (urogenotypic clones) and PRJNA687774 (to check the alleleAaspg deletion strains). The 100–150 pair-end input data was analyzed using Qiagen’s CLC Genomics Workbench version 11.0. Full details on the used workflow and parameters are available upon request. Briefly, reads were mapped to reference sequences after quality control, read trimming and filtering (NC_000913.3 for lab strain, NC_007946.1 and NC_007941.1 for the uropathogen UTI89). Mutation lists were obtained with CLC’s low frequency variant detection tool. Further filtering of these lists was based on several quality features and prior experiences (scripts available upon request). Importantly, for clones, cutoff frequencies of 75% were used, while for population analysis, an initial cutoff frequency of 5% was used while later on, this cutoff was dropped for the regions of the target genes specifically and data were filtered on minimum coverage only (coverage >30, forward & reverse read count >0; script available upon request). Cignal results were furthermore confirmed by sequencing targeted Sanger clones with primers in Supplementary Data 4. While we did not specifically remove intergenic or synonymous mutations, only 3 and 0, respectively, were identified in the entire dataset at or above the cutoff frequency of 5%. The per-base software package Circos was used (http://circos.ca/) to visualize data. Scripts and configuration files are available upon request.

Mutations from the UTI background were added to figures, tables and data based on a pairwise alignment to the lab strain background. For all the Chi2 tests to examine for significant enrichment, we used 200,000 replicates in Monte Carlo simulations to compute p-values and computed the null hypothesis as the condition in which mutations would be hitting randomly in the genome (Supplementary Fig. 10), in genes (Supplementary Fig. 12), in genes coding for membrane proteins (Supplementary Fig. 1c), in genes coding for inner membrane proteins (Supplementary Fig. 1f), in genes of the mnu operon (Fig. 2b), in mnu genes coding for the membrane-spanning subunit (Fig. 2c, Supplementary Fig. 3a, b). To do so, we used the genome-wide annotations in NC_000913:201186353 bp in total, 4089853 bp of which are part of core genomes (400000 tries of which code for membrane proteins, 822021 bp of which code for inner-membrane proteins, 146736 bp of which code for complex I of which 7110bp are for genes of the membrane subunit. For the membrane subunits, amino acids were scored to be part of either transmembrane helices or non-membrane-spanning loops based on predictions by Protter (wlab.ethz.ch/protter) and scored to be either hydrophobic or hydrophilic based on a scale computed before and a cutoff value of 0.6.

**Genomically repairing nuo mutation.** P1vir phage transduction was used to genomically revert the identified mutations in mnuL, M and N by using the yfp::Km \(^{\text{Keio}}\) mutant as donor strain as described elsewhere.

**Generating 3D structure of complex I from E. coli.** No full protein structure exists for complex I of E. coli. To this end, we modeled the protein structure of the cytoplasmic domain (nuoBCDFEGI; uniprot IDs: P0AFC7, P33599, P0AFD1, P31979, P33602, P0AFD6) and membrane arm (nuoAHKLMMN; uniprot IDs:
Purification of complex I and its variants. Complex I transcribed from the pBADnuo nuoFHis6 was expressed in, and purified from an E. coli strain lacking complex I and the alternative NADH dehydrogenase as performed before by the Friedrich group. On this construct using primers nuoL_FW and nuoL_RV, nuoM_FW and nuoM_RV and nuoN_FW and nuoN_RV (Supplementary Data 4) generated the variants carrying the point mutations of interest. Specifically, after 1:100 dilution in buffered flasks containing autodissociation medium, BW25113 nuoADnuoDHHis6 with pBADnuo nuoFHIs6 was grown aerobically until late exponential phase (OD600 = 3–4). From that point onwards, purification steps were carried out at 4 °C. Cells were harvested through centrifugation at 3000g for 15 min. Cell pellets were suspended with a Teflon-in-glass homogenizer in a 5-fold volume of buffer A (50 mM NaCl, 50 mM MES/NaOH, pH 6.0) with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and gentle pipetting, extruded with 31 passes through a 0.1 μm polycarbonate membrane and used on the same day. The NADH/ferricyanide oxidoreductase activity before and after addition of 0.5% DDM showed no significant differences between variants and wild type. The ratio of both activities was on average 0.75, indicating that 75% of the complex was oriented in the membrane and the NADH binding site was accessible from the buffer. Comparing to the NADH/FeCN oxidoreductase activity of purified proteins, the average protein reconstitution efficiency was 21%. Preparation of ISOV vesicles. BW25113 nuoADnuoDHHis6 cells with pBADnuo nuoFHIs6 were induced and harvested as described above. Inside-out vesicles (ISOVs) containing the complex I variants and endogenous lipids and other proteins; were prepared at 4 °C from frozen cells following a slightly modified, previously described procedure.58 Cell pellets were suspended in equal amount of washing buffer (50 mM KH2PO4/KOH, 5 mM MgSO4, pH 7.5), sedimented (10 min, 5000 g), suspended in 1 μM of the membrane permeable carbonyl cyanide m-chlorophenyl hydrazone (CCP). Experiments were carried out at 1 h with 500 μM of the protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCP) before monitoring the decreasing reaction of 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) upon reaction with thiol groups that are released upon heating and unfolding. 300 nM of complex I cytochrome c (complex I in buffer A) was mixed with a 5-fold excess of CPM, overlaid with silicone oil and assessed in a Perkin Elmer LS-55 fluorescence spectrophotometer (ex. 384 nm, em. 470 nm). A dose-response equation was fitted to the data in GraphPad Prism 8 using the least squares method: Y = Bottom + (Top – Bottom)/(1 + (X/IC50)β) + HLBlop where β = 1

O2 reduction assay. Reduction of O2 by electrons released from NADH by complex I was determined in buffer A* on 5 μl of membranes with a Clark-type electrode (Hansatech). The reaction was started by adding 1.25 mM NADH while 10 μM piericidin A was used to inhibit the reaction.

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Determination of purity and stability of complex I preparations Initial purification and stability of complex I were verified using polyacrylamide gel electrophoresis (PAGE). Under denaturing conditions, a sodium dodecyl sulfate (SDS) PAGE was run as described previously59 with a 3.9% stacking gel and a 10% separating gel. In addition, colorless native (CN) PAGE was performed as described previously135 with a 3.5% stacking gel and a 4–13% gradient separating gel (pH 6.0). For nitroblue tetrazolium (NBT) staining the gel was incubated for 5 min with 1 mg ml−1 NBT in 100 mM MOPS, pH 8 and the reaction was started by an addition of 100 μM NADH.

In addition, PAGE stability of purified complex I was verified with thermal shift assays. ThermofAD measures the intrinsic fluorescence of flavin, a cofactor of complex I, to determine the unfolding temperature of the complex.136 Briefly, 25 μl of complex I (in buffer A, 1 μg ml−1) was heated in a CFX96 qPCR thermocycler (Bio-Rad) with fluorescence measurements at regular interval (ex. 470–500, em. 506–536 nm). After subtracting blank data, a first derivative was calculated to obtain melting points as inflection points/maxima peaks. In addition, a Thermofluor-adapted assay was made use of the increased fluorescent yield

Reconstitution of complex I into liposomes. Purified complex I was reconstituted in liposomes at 4 °C138. Briefly, complex I (2 mg ml−1) was mixed with a 4–fold excess of E. coli polar lipids (extract from Avantium) which were dissolved in lipid-buffer (5 mM MES/NaOH, pH 6.0) and DDM (100 μg ml−1) by sonication. The mixture was stirred gently for 10 min before BioBeads SM-2 (Bio-Rad) were added in an 8-fold excess, accounted for their binding capacity of and the presence of DDM in the sample and stirred gently for another 3 h. The proteoliposomes were sedimented by centrifugation for 45 min at 150,000g, resuspended in protein-lipid-buffer (5 mM MES/NaOH, pH 6.0) and 50 mM MgCl2 by gentle pipetting, extruded with 31 passes through a 0.1 μm polycarbonate membrane and used on the same day. The NADH/ferricyanide oxidoreductase activity before and after addition of 0.5% DDM showed no significant differences between variants and wild type. The ratio of both activities was on average 0.75, indicating that 75% of the complex was oriented in the membrane and the NADH binding site was accessible from the buffer. Comparing to the NADH/FeCN oxidoreductase activity of purified proteins, the average protein reconstitution efficiency was 21%.
sediments were suspended in 750 μl of PBS and lysed by incubation at 100 °C for 7 min. After centrifugation (10 min, 14,000 rpm or 16,873 g) 100 μl supernatants were pipetted in 8 mm diameter holes on MHB agar plates inoculated with Bacillus subtilis ATCC 6051 as the indicator organism. Plates were incubated for 24 h at 37 °C after which the diameter of the inhibition zone was measured. Amikacin concentrations were deduced from a standard curve spanning a two-fold concentration range from 2 to 64 μg ml−1.

Ratiometric measurement of ATP:ADP ratios and cytoplasmic pH. ATP:ADP ratio was determined using the ratiometric GFP-based biosensor Perceval84. The pHluorin-his7-Perceval plasmid was obtained from Addgene (plasmid #20336) and the perceval gene was extracted by PCR using primer SPI-10577 and SPI-10578 (Supplementary Data 4). Next, Perceval was subcloned in pBAD/Myc-His-A, linearized through PstI and EcoRI digestion, using Gibson assembly (NEB)15. The 490/405 nm excitation fluorescence ratio (em. 530 nm) correlates with the ATP:ADP ratio and was determined using a Synergy MX Microplate reader (BioTek). Perceval’s sensitivity towards intracellular pH was countered by resuspending cells that were growing on MHB supplemented with 2 g L−1 arabinose and 100 mg ml−1 ampicillin, in M63 medium buffered at pH 7 supplemented with potassium benzoate (40 mM) and methylene hydrochloride (40 mM) before measurement.

Intracellular pH was measured with a GFP-based ratiometric sensor called pHluorin. We transformed the strains of interest with the pGFPR01 or pNTR-SD-pHluorin vector in which pHluorin is expressed from the arabinose-inducible or IPTG-inducible promoters, respectively, ParaB and Pm−1, P. They show pH-dependent fluorescence emission at 505 nm excitation with a broad emission maximum near 530 nm. Cells were either grown on MHB supplemented with 2 g L−1 arabinose or 1mM IPTG or on M9 medium supplemented with 5 g L−1 glucose and 20 g L−1 arabinose with ampicillin (100 mg L−1) to select for the maintenance of the plasmid. Note that no ampicillin and arabinose was added after nutrient shift. Ratiometric measurements to determine pH were performed by either a Synergy MX Microplate reader (BioTek; ex. 410 and 470 nm ±20 nm; em. 530 nm ±20 nm) or a Spark plate reader (Tecan; ex. 380 and 470 nm ±20 nm; em. 530 nm ±20 nm). Cells were either resuspended in M63 minimal salts medium or spun through HPLC spin filters (BaseClear) and resuspended in fresh M9 medium before measurement. Intracellular pH was determined by measuring fluorescence ratios of cultures resuspended for at least 5 min in a range of buffers with different pHs supplemented with 40 mM potassium benzoate and 40 mM methylene hydrochloride to dissipate the transmembrane pH gradient (see Martínez et al., 2012). The regulation between the fluorescence ratio and the intracellular pH was described by fitting a Boltzmann sigmoid:

\[
\text{ratio} = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + \exp(\text{V50} - \text{pH}/\text{Slope}))
\]

Image analysis. Analysis of pictures of protein or DNA gels was done using Vilber’s VisionCapt software for quantification and to estimate sizes.

Dynamic proteomics. For the dynamic proteomics analysis, we followed previously published protocols with minimum modification28,142. Briefly, cells were grown in M63 minimal salts medium supplemented with 1% glucose with regular dilution to maintain growth at an exponential phase. At OD600 of 0.4, a proteomics sample was collected, and persisters were generated by a switch to M9 fumarate medium. At multiple time points after the nutrient shift, samples were collected, all containing 3 × 10⁹ cells. They were centrifuged and washed with phosphate buffered saline (2×) and then cell pellets were resuspended in 80% glycerol and stored at −80 °C until further processing. Samples were run on a dual lane LTQ-Orbitrap Velos mass spectrometer connected to an electrospray ion source with peptide separation through an easy LTQ-Nano 1000 system (all Thermo Fisher Scientific) equipped with a RP-HPLC column (75 μm × 45 cm) packed with C18 resin (ReproSil-Pur C18AQ; Dr. Maisch GmbH) using a linear gradient from 95% solvent A (0.15% formic acid, 2% acetonitrile) to 28% solvent B (98% acetonitrile, 0.15% formic acid) over 90 min at 0.2 μl min−1. The acquisition mode operated one high-resolution MS scan in the FT part at a resolution of 120,000 full width at half maximum (at m/z 400) followed by MS/MS scans in the linear ion trap of the 20 most abundant charge state species. We acquired filtered data, that is to say, singly charged ions (dynamic exclusion duration: 20 s; ion accumulation time: 300 ms (MS) and 50 ms (MS/MS)). The raw files were imported into the Progenesis LC-MS software (Nonlinear Dynamics, Version 4.0) and analyzed using the default settings. MS/MS data were extracted in mgf format and searched against a decoy database of the forward and reverse sequences of the predicted proteome from M. lysodeikticus, where the database of the forward and reverse sequences of the predicted proteome from M. lysodeikticus was built before measurement. A calibration curve was generated by measuring 10 ppm for precursor ions and 0.6 Da for fragment ions. Results were imported to Progenesis, and the false discovery rate (FDR) was set to 1%. The final protein lists containing the cumulative peak areas of all identified peptides for each protein, respectively, were exported from Progenesis LC-MS and further statically analyzed using an in-house developed R script (SafeQuant)28. The raw mass spectrometry data is available through the ProteomeXchange Consortium (ID: PXD000996).

For the analysis, we normalized our data relative to a 2-fold difference in protein concentrations between all the analyzed conditions with the WT at start (before nutrient shift) as a reference. Next, we applied unscaled, two-dimensional data analysis procedure28. To control for potential contamination of samples, we used a previously published and analyzed dataset (from Schmidt et al.69 and Fig. 4a in Radzikowski et al.38, ProteomeXchange IDs: PXD000498 and PXD001968, respectively) to generate the two dimensions that constitute the PCA space. We plotted our data on this space to compare with previously found trends.

Radioactive assay of translation activity. To probe protein synthesis, we opted to follow the incorporation of radioactive L-([4,5-3H]-leucine during stationary phase in complex MHB medium (±1 h after 1:100 dilution as done during the evolution experiment) instead of following induction of a fluorescent protein as done before27 as the latter might be prone to biased results. Specifically, L-([4,5-3H]-leucine was added to stationary phase cultures at 2.5 μCi ml−1 and further incubated shaking at 37 °C. As control, chloramphenicol was additionally added at 64 μg ml−1, which fully abrogated protein synthesis (data not shown). Over the course of an experiment, hourly based samples were taken and resuspended in 3 ml water and added to scintillation liquid (Ultima-Flo M, Perking Elmer). Radioactive signal was measured as counts per minute with a Hidex 300SL scintillation counter.

HPLC-UV detection and quantification of ppGpp. Solutions containing ppGpp (Jena Biosciences) and products of its hydrolysis were analyzed using a HPLC-UV method measuring absorbance at 266 nm. A PL-SAX anion exchange column (PL-SAX 100 Å, 8 μm, 50 × 4.6 mm, Agilent) was used for sample separation at 60 °C. The raw data was imported into the Progenesis software (Nonlinear Dynamics, Version 4.0) and analyzed using the default settings. MS/MS-data were exported in mgf format and searched against a decoy database of the forward and reverse sequences of the predicted proteome from M. lysodeikticus, where the database of the forward and reverse sequences of the predicted proteome from M. lysodeikticus was built before measurement. To probe protein synthesis, we opted to follow the incorporation of radioactive L-([4,5-3H]-leucine during stationary phase in complex MHB medium (±1 h after 1:100 dilution as done during the evolution experiment) instead of following induction of a fluorescent protein as done before27 as the latter might be prone to biased results. Specifically, L-([4,5-3H]-leucine was added to stationary phase cultures at 2.5 μCi ml−1 and further incubated shaking at 37 °C. As control, chloramphenicol was additionally added at 64 μg ml−1, which fully abrogated protein synthesis (data not shown). Over the course of an experiment, hourly based samples were taken and resuspended in 3 ml water and added to scintillation liquid (Ultima-Flo M, Perking Elmer). Radioactive signal was measured as counts per minute with a Hidex 300SL scintillation counter.
SpoT was expressed with an N-terminal His-tag using plasmid pETM11-SpoT. pETM11-SpoT was generated by amplification of spoT using primer pair spoT_NcolS and spoT_Ncol_stop as (Supplementary Data 4) using genomic DNA from E. coli BW25113 as template. Subsequently the DNA fragment was cloned into pETM11 (EMBL Heidelberg) using Ncol and NotI restriction sites. Successful insertion was confirmed by restriction and sequencing analyses. The plasmid was transformed into E. coli BL21 (DE3) pLysS and selected on LB plates containing chloramphenicol. E. coli strain BL21 (DE3) pLysS transformed with plasmid pETM11-SpoT was grown aerobically at 37 °C in 100 ml LB medium in a 1000 ml flask supplemented with kanamycin and chloramphenicol (both at 50 µg ml⁻¹). Gene expression was induced by adding 3.5 µl of 1 M isopropyl-β-D-galactoside (IPTG) and cells were grown for 1 h at 37 °C. Cells were harvested, washed with buffer (50 mM Tris/His Cl pH 7.5, 10% glycerol) and disrupted by passage through a Cell Disruptor (Constant Cell Disruption Systems, Northants, UK) at 25 bar and 4°C in 1% discount buffer (50 mM Tris/HCl pH 7.7, 10% (v/v) glycerol, 300 mM NaCl, 50 mM KCl, 1 mM dithiothreit, 0.5 mM PMSF, and 0.03 mg/ml (w/v) DNase). After removal of intact cells and cell debris by centrifugation (3000g, 10 min, 4°C), the cytosol was incubated overnight at 4 °C with pre-equilibrated Ni-Sepharose (with 50 mM Tris/HCl pH 7.7, 10% (v/v) glycerol, 300 mM NaCl, 10 mM MgCl₂, 2 mM β-mercaptoethanol, 10 mM imidazole). Unbound protein was removed by washing with equilibration buffer and His-SpoT was eluted from the column by increasing imidazole concentrations up to 250 mM. Obtained purified protein degraded already at 4°C (Supplementary Fig. 6f).

To obtain more stable protein, purification was attempted with cytosolic buffer. Cells were cultivated as described above and disrupted in cytosolic buffer pH 7.5 (6 mM K₂HPO₄, 14 mM KH₂PO₄, 140 mM KCl, 5.3% glucose, 10 mM NaCl) containing 0.5% Triton X-100 (v/v) and 0.5 mM PMSF. Removal of intact cells and purification of 6His-SpoT was performed as described above, but with use of cytosolic buffers containing imidazole. Note that pH of the buffers was re-adjusted after imidazole addition using phosphoric acid and KOH, respectively. For purification of SpoT at different pHs, cells were disrupted in cytosolic buffer pH 7.5, bound to Ni-Sepharose, washed with buffer pH 7.5, and eluted with buffers adjusted to the corresponding pH and containing increasing imidazole concentrations. Protein content in samples was analyzed by SDs PAGE (12%) and Bradford protein assay. The purity of the expressed SpoT was determined via the ProteomeXchange Consortium via the PRIDE partner repository under the dataset identifier PRJNA498891, PRJNA498708, PRJNA498717. Parameters and conditions that were used are stated elsewhere. Data were analyzed using either GraphPad Prism 8 or R in the RStudio environment. Relevant information regarding the statistical tests was added to each figure caption where appropriate. In Fig. 1a, c, Supplementary Figs. 1f, I and 2a, b, Ch1 goodness-of-fit tests with 200,000 Monte Carlo simulations to compute the observed distribution and the number of mutations counts in various categories to the expected probabilities based on random occurring mutations. More detailed information can be found under the section explaining the genome-wide sequence analyses and Ch1 statistics are reported in Supplementary Data 4. For ANOVAs and linear (mixed) models, assumptions of homoscedasticity and normality of the residuals were checked visually comparing fitted values with residuals and comparing predicted with actual residuals. Where relevant, the Brown-Forsythe test was used to test whether SDs were equal, the Variance Inflation Factor was used to estimate the degree of multicollinearity and a Ch1 test was performed to test the effectiveness of including random effects/ matching. Post hoc tests (posttests) were performed to make and correct for multiple comparisons. All tests for which they were applied were two-tailed. Throughout the manuscript, a significance level cutoff of alpha = 0.05 was used. For visual purposes, asterisks denote significance levels as defined in the figure captions and each exact p value can be found in Supplementary Data 4. In addition, test statistics and degrees of freedom are reported in Supplementary Data 4.

Enzymatic ppGpp hydrolysis assay. We used both the encapsulated SpoT and regularly purified SpoT to characterize the pH dependency of its ppGpp hydrolysis activity. While the product of the spontaneous hydrolysis reaction leads to GTP, experiments with the limited amounts of the regularly purified SpoT indicated that SpoT hydrolyzes ppGpp to GDP, and that manganese ions are necessary for hydrolysis. Consistent with this, as specified in the materials and methods section, we provided requirements of Rel/Spo homologues for hydrolysis. We used both the encapsulated SpoT and regularly purified SpoT to characterize the pH dependency of its ppGpp hydrolysis activity. While the product of the spontaneous hydrolysis reaction leads to GTP, experiments with the limited amounts of the regularly purified SpoT indicated that SpoT hydrolyzes ppGpp to GDP, and that manganese ions are necessary for hydrolysis. Consistent with this, as specified in the materials and methods section, we provided requirements of Rel/Spo homologues for hydrolysis. We used both the encapsulated SpoT and regularly purified SpoT to characterize the pH dependency of its ppGpp hydrolysis activity. While the product of the spontaneous hydrolysis reaction leads to GTP, experiments with the limited amounts of the regularly purified SpoT indicated that SpoT hydrolyzes ppGpp to GDP, and that manganese ions are necessary for hydrolysis. Consistent with this, as specified in the materials and methods section, we provided requirements of Rel/Spo homologues for hydrolysis.
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The authors declare no competing interests.

Acknowledgements

The authors thank Susan Gottesman for the AB037 strain, Joan L. Slonczewski for the pliurin plasmid, Steven B. Vuk for the BA14 strain and Henri de Greve for the UTI89 strain. We further thank Ying Liu for help with cloning the plasmid for encapsulin-SpoT expression, Franziska Number for providing controls for the ACMA quenching, Alexandros Papagiannakis for valuable suggestions as well as for help with microscopy and Bert Poolman for valuable discussions. B.V.D.B. is recipient of fellowships from the Fund for Scientific Research, Flanders (FWO: 1201917N, 1201922N, V42917NT, and 1G618124N), from the Federation of European Microbiological Societies (FEMS; RG–2016–0052), from the Belgian American Educational Foundation (BAEF; 2016–E083) and from the European Molecular Biology Organization (EMBO; ALTF 344–2017). J.M. was engaged by a fellowship through the Agency for Innovation by Science and Technology (IWT). This work was furthermore supported by grants from FWO (1528318N, G0B2515N and G055517N), K.U. Leuven (C16/17/006), the Flanders Institute for Biotechnology (VIB) (to J.M. and B.V.D.B.), the Deutsche Forschungsgemeinschaft (DFG; STO 278/18-1) (to T.F.) and from the Dutch Research Council (NWO; VIDI grant 864.11.001) to M.H.

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Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-28141-x.

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Peer review information Nature Communications thanks Gregory Cook and the other, anonymous, reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

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