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Non-lethal oxidative stress boosts bacterial survival and evolvability under lethal exposure

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Abstract

When oxygen began to accumulate by photosynthesis and to be utilized for respiration, reactive oxygen species such as hydrogen peroxide, a toxic by-product of aerobic respiration, became a challenge. Later on, multicellular organisms began to employ reactive oxygen species as potent weapons against microbes. Although bacterial defences against lethal and sub-lethal oxidative stress have been studied in model bacteria, the role of prevalent fluctuating concentrations remain unexplored. It is known that sub-lethal exposure of *E. coli* to hydrogen peroxide results in enhanced survival upon further exposure. Here we show how pre-treated or 'primed' cells survive better when they later face are subsequently exposed to otherwise lethal concentrations concentrations. The basis and the duration of the response (memory) was also determined by time-lapse quantitative proteomics. A low level of peroxide induces several scavenging enzymes that are quite stable,

protecting cells from future exposure. We then asked if the phenotypic resistance against H_2O_2 alters resistance evolution against oxygen stress. Experimental evolution for H_2O_2 resistance revealed faster evolution and higher levels of resistance in primed cells. Several mutations were found to be associated with resistance in evolved populations affecting different loci but counterintuitively, none of them were implicated in scavenging systems. Our results have important implications for host colonisation and infections where microbes often encounter reactive oxygen species in gradients.

Author summary

Throughout evolutionary time bacteria were exposed to reactive oxygen species and evolved the ability to scavenge toxic oxygen radicals. Multicellular organisms evolved also the ability to produce such oxygen species against pathogens. Recent studies also suggest that reactive oxygen species such as hydrogen peroxide play an important role during host gut colonisation by its microbiota. Traditionally, experiments with different antimicrobials are carried out using fixed concentrations while in nature, including intra-host environment, microbes experience this type of stress in steps or gradients. Here we show that bacteria treated with sub-lethal concentrations of hydrogen peroxide (priming) survive far better than non-treated cells when they later encounter a lethal concentration. We also found that 'priming' response has a protective role from lethal mutagenesis. This protection is provided by long-living proteins that, upon priming, remains at a high level for some generations as we could determine by time-lapse LC-Mass spectrometry. Bacteria that were primed evolved resistance faster and to a higher level. Moreover, mutations that increase resistance to hydrogen peroxide, as determined by whole genome sequencing, do not occur in the scavenger codifying genes but in other different gene functions, at least in *E. coli*.

Introduction

The ability to solicit an improved stress response when encountering repeated stress relies on 'remembering' a similar event from the past (memory) and it is a common trait to many biological entities [1]. Bacteria encounter increasing and repeated stress imposed by the host immune system, during the course of an infection or the colonization of a host [2,3]. Obata et al, for example, recently demonstrated that low levels of hydrogen peroxide (H_2O_2) in the gut of *Drosophila melanogaster* shape the composition of the gut microbiota resulting in differential survival of the flies [3]. Here, we report how bacteria respond to low levels of ROS exposure and how this impacts bacterial fitness. We then investigate if the phenotypic response to a sub-lethal dose of H_2O_2 facilitates resistance evolution, hence providing a test of the phenotype-first hypothesis, which proposes that environmentally initiated phenotypic change precedes or even facilitates evolutionary adaptation [4].

In *Escherichia coli*, the defences against oxidative stress depend on transcriptional regulators such as OxyR or SoxR that detect different changes in the redox balance and induce the production of detoxifying enzymes, DNA repair and protection system and other proteins [5]. Oxidation of OxyR by hydrogen peroxide (H_2O_2) leads to the formation of an intra-molecular disulfide bond between cysteine residues 199 and 208 and OxyR is deactivated by enzymatic reduction with glutaredoxin I (Grx) or thioredoxin (Trx). OxyR positively regulates catalases and peroxidases. Because also the Grx/GorA system is transcriptionally regulated by OxyR, the whole response is autoregulated [6].

The OxyR-mediated oxidative stress response results in scavenging of H_2O_2 and hence mitigates the toxicity of this by-product of aerobic metabolism. It includes the induction of the Suf proteins that form a complex to supply apo-enzymes with iron-sulfur clusters. This system replaces the Isc system, which is disrupted by H_2O_2 stress [7,8]. The iron sulphur clusters of dehydratases are one of the most H_2O_2-sensitive systems. The repair of those clusters by Suf is

necessary to prevent the failure of the TCA cycle. The iron-sulfur clusters are enzymes that employ ferrous iron as a co-factor which can increase the risk of fuelling a Fenton reaction. The nucleoid-binding protein Dps binds intracellular free iron to limit the generation of hydroxyl radical close to the vulnerable DNA [9].

In a classic paper it has been shown that the pre-treatment with a low dose (60 µM) of hydrogen peroxide can increase the survival of *E. coli* upon subsequent exposure to a lethal dose (30 mM) [10]. However, neither the duration of such priming responses nor the molecular mechanisms of its maintenance, i.e. the memory, have been studied. Our study has two main aims. First, we investigated the main factors in the H_2O_2 priming response and for how long the response is sustained. Second, we used this system to test the hypothesis that inducible phenotypes accelerate adaptive evolution [4]. Therefore, we experimentally evolved *Escherichia coli* under a growing level of Hydrogen peroxide with and without priming . For H_2O_2-resistant populations evolved after priming and non-priming regimes, genome re-sequencing analyses were performed to identify mutations. We focus our study on growing bacteria (exponential phase), as this better reflects the situations during an infection or colonisation of host surfaces including the gut. Moreover, this allows us to focus on low H_2O_2-primed resistance in the absence of the stationary phase-induced RpoS-dependent general stress response, which also generates pronounced phenotypic H_2O_2 resistance [11,12].

Results and Discussion

Priming by H_2O_2 results in higher bacterial survival

We find that the minimal inhibitory concentration for hydrogen peroxide is 1 mM and find a clear dose-effect in survival rate (figure 1a, ranging from 50 µM to 1 mM, figure 1a, $p=2.1x10^{-16}$, DRC model fitted based on maximum likelihood [13]). Based on these results we primed cells with H_2O_2 0.1 mM, the maximum concentration that shows no difference in growth rate compared to

non-treated cells, and monitored survival at minimal inhibitory concentration, 1 mM, starting 30 min after removal of the treatment. Primed (pre-treated) populations of *E. coli* showed higher survival than naïve cells by more than one order of magnitude (Cox proportional hazard model, $p<0.05$, figure 1b).

These results are in agreement with data previously reported by Imlay et al [10], showing survival protection even to doses as high as 30 mM, a concentration close to hydrogen peroxide usage as a disinfectant. Our concentrations are also in the range of some *in vivo* situations. For example, tailfin transection on zebrafish larvae induces a rapid increase in H_2O_2 levels ranging from 100–200 µM in the wound margins [2]. In some cases, more than 100 µM of H_2O_2 have been reported in human and other animal aqueous and vitreous humors of the eye [14].

Duration of the priming response

As the priming response protects the cells effectively from lethal exposure to H_2O_2, an important question is for how long this response remains effective? To address this question, we pre-treated *E. coli* again with H_2O_2 0.1 mM during 30 minutes, but we applied the trigger concentration (1 mM) at different times-points (30, 60, 90, 120 and 150 minutes after the 30 min priming period, also keeping cell density constant. We observed a significant decay of the priming response from 120 minutes after H_2O_2 pre-treatment removal (figure 2), roughly four divisions, suggesting that the priming effect is also trans-generational. After 150 minutes, the survival rate of primed populations no longer differed from naïve populations. Another study has shown long-term memory based on an epigenetic switch able to control a bimodal virulence alternation also in the scale of hours in *E. coli* [15].

How is the state of priming maintained for up to five generations? Bacteria can store information about recent stress via stable transcripts or proteins [15,16]. We investigated the memory at the protein level and initially studied the impact of the two concentrations used in most of our experiments (0.1 and 1

mM) on the proteome of *E. coli* by quantitative LC-mass spectrometry. From the quantitative proteomic dataset, we detected many of the known enzymes that are induced by hydrogen peroxide just five minutes after the addition of H_2O_2 (figures 3, and tables S1 and S2). For both concentrations, we detected and quantified many proteins belonging to OxyR regulon but many other relevant genes such as *ahpC*/*F*, *xthA* and *suf* operon showed a weak difference or no response at all. We should keep in mind that the samples in this experiment were taken after only five minutes of treatment. The reason for short exposure is that viability after the 1 mM treatment would be too at later timepoints. For the dose of 0.1 mM it is possible to collect viable cells after a longer period of exposure and these results are presented in the figure 4.

To explore the temporal dynamics of the proteome after a 30-minute stimulus with H_2O_2 (0.1 mM), we followed the changes over almost three hours (five time points: 30, 60, 90, 120 and 150 minutes). The decline in anti-H_2O_2 protein levels correlates well with the decrease of the response (figure 4). Some important proteins such as KatG, AhpCF and YaaA declined slowly after removal of the H_2O_2, corresponding probably to a sustained production with a minor contribution of dilution due to cell division and poor degradation rate. These results indicate that many of these proteins are stable and show a significantly higher abundance than in the control even at 150 minutes after H_2O_2 treatment. Other proteins such as GrxA, YaaA and XthA, SufA, SufS, AcrA-AcrB completely declined at this point indicating that these proteins may be subject to proteolysis and have shorter half-lives (figure 4) but also that the stressful situation is alleviated. The overall results of this proteomic experiment strongly suggest that the memory of priming response in *E. coli* is mediated by the scavenging proteins such as KatG and AhpCF.

An interesting approach to visualise the global impact of H_2O_2 on bacterial physiology is a network analysis based on protein-protein and function [17]. If we examine, for example, the proteome response to priming concentration (H_2O_2 0.1 mM during 30 minutes, we observe a high degree of connectivity in network of protein-protein interaction and functional relation of both, up- and

downregulated proteins (figure 5). If we compare our network with a large-scale protein-protein interaction network of *E. coli* [18], we find a wide perturbation including the most important nodes. This analysis also suggests that there is a proteome-wide readjustment to cope with hydrogen peroxide stress that does not only involve scavenging systems and shows the profound impact of oxidative stress across the entire proteome in a dose that does not change the growth rate in a rich medium.

Another contributing factor to memory could be based on long-life transcripts. Thus, we sequenced the full transcriptome after exposure to H_2O_2. RNAseq captured both, sRNA and mRNA, and we sampled just before the decline of the response (120 minutes after the stimulus). The highest expressed transcript was OxyS, a small regulatory RNA (sRNA) induced by active OxyR (tables S3 and S4). OxyS regulates several genes, and although several targets have been identified, its function is not fully understood [19]. We did not detect other transcripts related to the OxyR regulon.

OxyS is a potential candidate to explain the duration of priming because it is relatively stable with a half-life between 18 to 20 minutes during the exponential phase [20–22]. To explore this, we used an *oxyS*-deficient mutant to test its susceptibility to H_2O_2 over 30 minutes. We did not find any significant difference in susceptibility (figure 6) consistent with prior reports [21,23,24]. Although OxyS did not provide us with a mechanism to explain the duration of the memory, its stability could play some role in alleviating DNA damage as recently suggested [25]. However, such protection does not seem to have a significant impact on survival, according to our results.

Based on the comparison of proteomic and transcriptomic data we can say that the capacity of the cells ‘to remember’ the stimulus is mainly based on the stability of scavenging proteins as documented in the proteomic dataset, that after induction remain present at higher concentrations than in the control samples as long as 120 minutes after removal of peroxide. This indicates that these enzymes are not degraded but probably their production go on later after

the stimulus with a minor impact of proteolysis and dilution during cell divisions which could explain the pattern that we observe in the decline of the response.

Priming response is compromised by disrupting important H_2O_2-scavenging genes

The expression of important genes for H_2O_2 stress survival was also confirmed by qPCR 30 minutes after the addition of H_2O_2 (0.1 mM). H_2O_2 induced a significant up-regulation of representative genes such as *katG*, *ahpF*/*ahpC*, *dps*, *mntH* and *sufA* (figure S1 and table S5). As previously described in the literature, *oxyR* and *fur* genes do not change in expression level when cells are treated with H_2O_2, since their activation relies on swapping between active or inactive forms of the proteins depending on the intracellular level of H_2O_2 or iron respectably [6,7].

To understand the priming response to H_2O_2 at molecular level, we constructed a set of mutants for the coding gene of key proteins that we found in our proteomic dataset. In proliferating *E. coli*, OxyR is the major regulator controlling cellular response to H_2O_2 [26]. We explored the involvement of OxyR in the priming response because many of the upregulated detected proteins are under this regulator. We found that by disrupting OxyR, there was a dramatic change in sensitivity with full loss of viability after 30 minutes (figure 6). The priming response is completely abolished (Figure 7), indicating that the enhanced survival, due to pre-exposure to H_2O_2, depends on OxyR. OxyR is a fundamental transcription factor, probably the most important one, that protects *E. coli* against H_2O_2 during the exponential phase [26,27]. The active form of OxyR positively regulates dozens of genes.

Next, we selected, from the most highly expressed proteins and taking into account previous reports [7,9], many from the OxyR regulon, to determine which ones contribute more significantly to survival (figure 6) and priming (figure 7). Removal of KatG (catalase), AhpF (one subunit of the alkyl peroxidase AhpCF) or RecA (DNA repair) significantly decreases survival in the presence of hydrogen peroxide (1 mM). The double mutant for KatG/AhpF

showed even stronger sensitivity. However, the absence of other important proteins induced by H_2O_2 such as YaaA (decreases intracellular iron [28]), LipA (lipoate synthase, important for the repair of iron-sulfur clusters [29]), GltD (glutamate synthase subunit), GhxA or GhrA did not result in differential survival to 1 mM concentration. The OxyS sRNA was the transcript with the highest level of expression in the RNAseq data, but the mutant did not display any increased susceptibility to H_2O_2. Assuming that H_2O_2 sensitivity and priming response are closely linked, we included in the additional priming response experiment mostly those genes with increased susceptibility (figure 7). The removal of KatG indicates that catalase importantly contributes to priming response but it does not fully explain the protection observed for the WT strain (compare to Fig. 1B). In the case of AhpF, we also observe differences in priming response with the naïve state but not that pronounced as the case of KatG deficient strain. The double mutant defective in KatG and AhpF showed a dramatic decrease in priming response but still significantly different from the naïve. Another protein that illustrates an important influence on priming response is RecA, with the mutant showing a decreased priming response to H_2O_2. OxyS mutant is not suppressive for priming response, indicating that its presence contributes neither to the survival or priming. Overall, our data indicate that the priming by a low dose of H_2O_2 is multifactorial, with several OxyR-controlled proteins such as KatG, AhpCF or other factors related to DNA as RecA contributing to the priming response.

Priming response enhances the survival of evolving populations

To find out whether the priming response described above has an influence on the rate by which bacterial resistance against hydrogen peroxide evolves was the goal of the following experiments. We evolved bacterial cultures with a treatment protocol described in detail in M&M section. Parallel populations were evolved where one group was periodically exposed to a sub-lethal concentration of hydrogen peroxide, an activating signal that should protect the populations in comparison with naïve ones when later exposed to a higher

dose. We continued daily intermittent exposures to hydrogen peroxide doubling both, priming and triggering concentrations until extinction occured. We observed that the extinction rate was faster for the naïve populations in comparison to primed populations (Log-rank test, $p < 0.05$), figure 8). Naïve cells evolved resistance of up 8 mM H_2O_2, whereas primed cells evolved resistance to at least 16 mM H_2O_2. This first result indicates that priming potentially increases the evolvability of pre-treated populations.

Further analysis of the resistant populations by whole-genome sequencing revealed that all populations harboured different sets of mutations. Surprisingly, we did not find any modification in the enzymatic scavenger systems, such as catalases or any other proteins related to peroxide protection. We found several hundreds of single nucleotide polymorphisms (SNPs) and other type of mutations that were unique to each population for both regimes (figure 9). These mutations probably represent many of the neutral or non-lethal changes that populations accumulated during the exposure to H_2O_2, that is not simply a selective agent, but it also speeds up evolution by increasing the mutation rate. At the moment we cannot be certain about the contribution of each mutation to H_2O_2 stress adaptation and they will be subject of detailed studies in the future. However, it is possible to speculate about the possible role of many of the mutations we found. For example, a set of changes are located in genes coding for iron binding proteins or related with iron transport such as *iceT*, *feoA, yaaX*/*yaaA* or *rsxC*. The control of intracellular iron is crucial to decrease the adverse effects of Fenton chemistry [7].

There was also a set of more frequent changes that were common to both types of population, evolved under priming and non-priming conditions (figure 9). The most frequent mutations were *yodB* (a cytochrome b561 homolog), intergenic mutations between *insA* and *uspC* (universal stress protein C), intergenic mutations between *flhD* and *insB1*. Another frequent mutation was in the gene *yagH*, belonging to the CP4-6 prophage. CP4-6 is a cryptic prophage in *E. coli* that has been suggested could play a role in bacterial survival under adverse environmental conditions [30].

Priming alters the mutational spectrum and H_2O_2-induced mutagenesis in evolving populations

To assess if the evolved populations have a similar mutation spectrum, we analysed the total pool of mutations segregated by the treatments and determined the proportion of every type of found mutation. We used Monte Carlo hypergeometric test implemented by iMARS [31], to assess the overall differences between mutational spectra. Both groups, of the evolving populations under priming and non-priming conditions, differed significantly ($p=0.00021$, figure 6). ROS induces a particular type of mutations, with a characteristic signature in the DNA. The guanine base in genomic DNA is highly susceptible to oxidative stress due to having the lowest oxidation potential. Therefore, G·C→T·A and G·C→C·G transversion mutations frequently occur under oxidative conditions [32,33]. Thus, we investigated the proportion of C→A and C→G substitutions between the two types of evolving regimes, but we did not find significant differences ($p=0.056$ and $p=0.11$ respectively, two-tailed Fisher's exact-test, figure 10).

A possible explanation for these results is that the pre-activation of antioxidant defences helps to decrease the number of non-adaptive mutations and lethality by decreasing the extent to which the Fenton reaction occurs. We observed an increase in the frequency of substitutions G→A, T→C, G→C, T→G and A→G in naïve populations compared to primed populations, suggesting that the primed cells have DNA-damage control. Primed populations showed an increase in frameshift mutations by deletion of 1 bp, and additions of C or G; these last two were only present in primed populations that also showed higher number of A→C substitutions.

It is expected that the priming response would decrease DNA damage and hence also the rate of lethal mutations. To assess this possibility, we tested if priming is able to decrease the mutant frequency and hence lethality due to mutagenesis. Preserving the conditions of the previous experiments, we treated the bacterial cultures with the same priming and triggering concentrations at low cell density, the basal mutant frequency without H_2O_2

treatment was also determined. We found that the priming response decreases H_2O_2-induced mutant frequency close to one order of magnitude if compared with naïve cells ($p<0.001$ primed vs naïve, Welch's test, figure S2). In addition, no difference was found between primed bacteria and the basal level of mutagenesis ($p=0.03$ primed vs basal, Welch's test), indicating that priming is able to supress most of the mutagenesis caused by H_2O_2. In our experiment, naïve and primed populations showed different death rates (figure 1). We also showed previously that different inoculum size on similar conditions to our experiment has no influence on mutagenesis [34]. This experiment allows us to deduce that one of the most important consequences of the priming response to H_2O_2 is a drastic decrease in lethal mutagenesis, indicating that although hydrogen peroxide damages most of the cellular components [7], damage to DNA is likely a major contributor to lethality.

In principle, an increase of mutation rate is a parameter that increases the evolvability of asexual populations [35–37]. How is it possible that naïve populations show lower evolvability compared to primed populations despite a higher mutation rate? A possible explanation is that evolvability is also influenced by the population size and the mutation supply that is increased by mutagenesis is balanced by the decrease in population size of the naïve population. We also found that the mutational spectra of our evolving populations are different. Mutational spectra are a qualitative property of mutation rate that could enhance or hinder the access to beneficial mutations [38–40]. It is possible that the observed changes in mutational spectra between primed and naïve evolving populations could play a role in balancing the ratio of deleterious/beneficial mutations but to confirm this fact in our experiments is not a trivial task.

Conclusions

We found that priming by hydrogen peroxide in exponentially growing bacteria is quantitatively driven by genes that are mostly under OxyR control. Some other genes such as *recA* also seem to play an important role. The memory of

the priming response can last up to four generations upon first exposure to H_2O_2 taking into account the time after addition of the stressor and number of divisions. We also showed high stability of hydrogen peroxide detoxifying proteins which play a significant role in survival. Moreover, we found that priming plays a role in evolving populations by enhancing the survival under oxidative stress, which supports a link between the phenotype as a target of selection during evolutionary processes and provides a validation for the phenotype first hypothesis [4]. The type and number of mutations indicates that scavenger systems against oxidative stress are optimally evolved since no mutations affecting these systems were found under H_2O_2 stress selective pressure. Our work also contributes to understanding how the adaptation of early life with oxygen could have taken place.

Material and methods

Bacteria and growth conditions. The *E. coli* MG1655 was used as bacterial model for all experiments with hydrogen peroxide. For genetic manipulation, *Escherichia coli* DH5α strain was used and routinely cultured in Lysogeny Broth (LB medium), supplemented with antibiotics when appropriate. All bacterial strains were cultured in Lysogeny broth lenox (Carl Roth, Germany). For all experiments with hydrogen peroxide the LB was fresh prepared and kept in the dark until use.

Construction and verification of deletion mutants. All mutants used in this work (table S6) were generated in *E. coli* K-12 strain MG1655 following the a modified methodology described elsewhere [41]. Briefly, transformants carrying a red recombinase helper plasmid, pKD46, were grown in 5-ml SOB medium with ampicillin (100 µg/ml) and L-arabinose at 30°C to an O.D.600 of ~0.5 and then made electrocompetent. PCR products with homology regions were generated using specific primers (table S7) to amplified the region of interest from using the corresponding mutants of the Keio collection [42]. The PRC-generated fragments were purified (MinElute PCR Purification Kit, Qiagen). Competent cells in 50 µl aliquots were electroporated with 100 ng of PCR product. Cells were added immediately 0.9 ml of SOC, incubated 1 h at 37°C, and then 100 µl aliquots spread onto LB agar with kanamycin (30 µg/ml). The mutants were verified by PCR and the antibiotic resistance cassette was removed using the plasmid pCP20. The correct inactivation of genes was verified by PCR. To construct double mutants, singles mutants obtained in MG1655 were transduced using the P1vir phage procedure as previously described [43].

Hydrogen peroxide susceptibility testing. When working with hydrogen peroxide, we carried out all the experiments with fresh-made LB to avoid the accumulation of chemically formed H_2O_2 by joined the action of light and flavin present in the medium during storage. Susceptibility to H_2O_2 was determined by exposing exponentially phase bacteria at a density of ~ $2x10^7$ CFU/ml to different concentrations and times, taking as a reference previous works.

Priming experiments with H_2O_2. Starting from $2x10^7$ CFU/ml, where *E. coli* the bacteria were exposed (stimulus) to 0.1 mM of hydrogen peroxide during 30 minutes at at 37°C with shaking. Catalase was added to a final concentration of 4 µl/ml, centrifuged at 4000 g during 10 minutes and allow to recover during 90 minutes, keeping the cell density constant by removing the needed volume and replace it with appropriate amount of fresh LB every 30 minutes. The trigger (H_2O_2 1 mM) was added 90 minutes after removal the

463 stimulus. The challenge lasted for 30 minutes. At this point catalase (4 µl/ml)
464 was added and cultures were diluted and plated to determine cell viability. Non
465 treated cells were used as control. Five cultures per group were used.
466
467 **Priming experiments in multi-well plates.** The 96-multiwell plates were
468 prepared under aseptic conditions. All experiments were carried out in a final
469 volume of 100 µL of fresh LB. Bacteria were exposed to serially diluted
470 concentrations of H_2O_2 from 256 mM by adding 50 µL of peroxide containing LB
471 first 50 µL of mid-exponential phase bacteria rendering approximately 10^7 CFU/
472 ml suspension. The plate was placed into a Synergy H1 plate reader (BioTek,
473 USA) and kinetics of killing or growth were followed during two hours. Once
474 those relevant non-lethal concentrations in these conditions were established,
475 a second experiment was carried out. In a similar plate and conditions, bacteria
476 were exposed to serial dilutions of H_2O_2 starting from 4 mM during 30 minutes.
477 The plate was centrifuged, the supernatant removed and fresh LB transferred.
478 The cells were allowed to recover for two hours. After this period, 50 µL were
479 removed per well and replaced with 50 µL LB containing 32 mM of H_2O_2 for a
480 final concentration of 16 mM. The killing and survival were followed for 8 hours
481 by absorbance at 600 nm.
482
483 **Memory of the priming response.** After applying a stimulus (0.1 mM H_2O_2)
484 during half an hour, the cells were allowed to recover for 30, 60, 90, 120 and
485 150 minutes before the addition of the trigger (1 mM H_2O_2). During the
486 experiment, the D.O.600 of all cultures were kept around 0.05 by adding
487 suitable volume and removal of equivalent quantity to maintain the population
488 in exponential phase and the same population size. Bacteria remained exposed
489 to the trigger during 30 minutes before starting the dilution in 1 ml of LB
490 containing 4 µg of catalase (Sigma-Aldrich, Germany). Appropriated dilutions
491 were made and plated onto LB agar to determine the survival rate.
492
493 **Global proteomics by mass spectrometry.** *E. coli* strain MG1655 was
494 grown at 37°C in LB medium to OD600 0.5. The cultures were diluted ten times
495 in fresh LB. H_2O_2 was added to tubes at 0.1 and 1 mM. Non treated samples

were used as control. All tubes were incubated during five minutes with shaking at 37°C. Remaining hydrogen peroxide was removed by adding 4 µg/ml of catalase first and by centrifugation at 10000 g during 2 minutes. After removal of the supernatant, equivalent amount of fresh LB was added. For the memory decline experiment, the procedure was identical, except that only 0.1 mM of hydrogen peroxide was used and the samples were treated during 30 minutes. After removal of the treatment, time-point samples were taken after 30, 60, 90, 120 and 150 minutes. Each experimental condition consisted of six replicas. Two millilitres per sample were pelleted by centrifugation at 10000 g for 2 minutes. The cells were re-suspended in 50 ml of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) that contained chicken lysozyme (1 mg/ml, Sigma) and DTT (10 mM) and incubated at room temperature for five minutes with occasional swirling. A volume of 250 µl of denaturation buffer (6M urea/2 M thiourea in 10 mM HEPES pH 8.0) was added into each sample. Then, 10 µl (which approximately corresponds to 50 µg of protein) of the resulting lysate were mixed with 150 µl of the denaturation buffer and used for in-solution protein digestion as described previously [44]. Briefly, proteins, demobilised in denaturation buffer, were reduced by addition of 1 ml of 10 mM DTT dissolved in 50 mM ammonium bicarbonate (ABC) and incubation for 30 minutes, followed by alkylation with 1 µl of 55 mM iodacetamid dissolved in 50 mM ABC for 20 minutes, digestion with 1 µg Lysyl endopeptidase (LysC, Wako, Japan), resuspended in 50 mM ABC, for 3 hours. After pre-digestion with LysC, protein samples were diluted four times with 50 mM ABC and subjected to overnight trypsin digestion using 1 µg of sequencing grade modified trypsin, Promega), diluted in 50 mM ABC. All in-solution protein digestion steps were performed at room temperature and after addition of iodacetamid the samples were protected from the light until the digestion was stopped by adding 10 ml of Buffer A* (5% acetonitrile, 3% trifluoric acid). After trypsin digestion overnight at room temperature, samples were diluted with 200 µl of buffer A (5% acetonitrile, 3% trifluoracetic acid) treated as described elsewhere and vacuum dried. Re-dissolved samples were loaded on a ReprosilPur C18 reverse phase column and peptides were analyzed using a nano-HPLC Dionex Ultimate 3000 system (Thermo scientific) coupled to an Orbitrap Velos mass spectrometer

(Thermo scientific). MS and MS/MS data from each LC/MS run were analysed with MaxQuant software [45]. Identification of proteins was performed using the MaxQuant implemented Andromeda peptide search engine.

Quantification of gene expression. To assess the quality of hydrogen peroxide stress response related genes in *E. coli*, treated bacteria comparison with 0.1 mM of hydrogen peroxide were compared to non-treated control. Preparation of each of the six treatments and the control samples was carried out by two operators three times on three consecutive days, thereby comprising three biological replicates for each of the six experimental and one control groups. Overnight (ON) cultures were diluted 1:100 in fresh LB, subdivided into six 10 ml aliquots and incubated in 50 ml-Falcon tubes at 37°C for 2.5 hours at 220 rpm. When the OD600 of bacterial cultures reached 0.4-0.5, the cultures were diluted ten times with LB and hydrogen peroxide was added to the final concentrations of 0.1 mM. These bacterial cultures were incubated for 30 minutes at 37°C with shaking. Catalase was added to each tube to a final concentration of 4 µg/ml. Ten millilitres of treated cultures and from the control tubes were collected, immediately centrifuged at 10 000 g for 2 minutes and the supernatant was fully removed. The bacterial pellets were resuspended in 1 ml LB, followed by addition of 1ml of RNAprotect Bacteria Reagent (Qiagen, Germany), incubated during 2 minutes and centrifuged at 10000 g for 2 minutes at room temperature. The supernatant was discarded and the bacterial pellet was immediately frozen and stored at -80°C until RNA extraction.

RNA was isolated using RNeasy kit (Qiagen) according to the manufacturer’s instructions and eluted in 50 µl of RNase-free water. The nucleic acid yield and purity were determined by measuring the optical density at A260/280 using Nanodrop spectrophotometer (Thermo Scientific). RNA samples were treated with TURBO DNase (Life Technologies). Briefly, 10 µg of RNA were used in a total volume of 500 µl containing 20 units of TURBO DNase, incubated for 30 minutes at 37°C, immediately followed by RNeasy (Qiagen) clean-up and elution in 30 µl of RNase-free water. Following DNase treatment, RNA integrity

562 was assessed using Agilent RNA 6000 Nano kit and 2100 Bioanalyzer
563 instrument (both Agilent Technologies). All samples had RIN values above 8.
564
565 For cDNA synthesis, total RNA (250 ng per reaction) and random primers were
566 used for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit
567 with RNase Inhibitor (Applied Biosystems). Initially, to ensure linear conversion
568 of the transcripts, the dynamic range of reverse transcription reaction was
569 tested by performing a standard curve with cDNA, synthesised using various
570 input amounts of pooled RNA. To obtain sufficient amount of cDNA, several
571 batches of 20 µl RT reactions were pooled, diluted 50-fold with RNase-free
572 water and stored in single-use aliquots at -80°C until further use. All 21
573 samples were tested for presence of contaminating genomic DNA by running
574 the *mdoG* assay with cDNA and the respective no reverse transcription (-RT)
575 controls. There was no amplification in the majority of –RT controls. In –RT
576 samples with detectable amplification, difference in Ct values when compared
577 with +RT varied between the samples, but was no less than 10 cycles for all of
578 them with the lowest Ct values in –RT control samples ≥ 30.
579
580 For primer design, *Escherichia coli* strain K-12 MG1655 complete genome
581 (accession U00096) sequence was downloaded from NCBI database
582 (www.ncbi.nlm.nih.gov) and used as reference. Target sequence accession
583 number, and primer sequences for each assay can be found in table S8.
584 Primers were designed using Primer Express software (Applied Biosystems) and
585 optimized for annealing temperature of 60°C. Each primer pair and amplicon
586 were checked for secondary structure formation using Oligo Tool (Integrated
587 DNA technologies, http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/).
588
589 Quantitative Real-time PCR reactions were prepared manually in a total volume
590 of 10 µl by mixing 5 µl 2x KAPA SYBR FAST ABI Prism master mix (KAPA
591 Biosystems), 0.2 µl forward and reverse primer mix (10 µM each primer), 2.8 µl
592 RNase-free water and 2 µl cDNA in MicroAmp Fast Optical 48-wells reaction
593 plates (Applied Biosystems). PCR reactions were run in the Fast mode using
594 StepOne thermocycler (Applied Biosystems) with the following cycling

conditions: 95°C 3’ /40x(95°C 3’’/60°C 20’’)/melting curve analysis. Each assay was run in duplicate. No template controls (NTC) were run each time. Presence of a single specific product was verified by running a melt curve analysis followed by visualisation of the qPCR product in a 2% agarose gel stained with SYBR Safe DNA Gel stain (Life Tecnologies). Additionally, each PCR assay was tested for reaction efficiency as follows: equimolar amounts of cDNA from all 21 samples were pooled together and used for the preparation of the standard curve by serial dilution (1:3) over five dilution points and run in triplicate. Expression of target genes was normalized to the expression levels of three reference genes (*arcA*, *mdoG* and *tus*), selected based on the assessment of the expression stability across all experimental conditions using BestKeeper software. Reaction efficiency information inferred from the standard curve data was used to correct for differences in amplification efficiencies in the REST 2009 software [46]. Default settings (2000 iterations) were used for randomisation and bootstrapping analysis to test significance of gene expression. Expression values with p-values ≤0.05 were assigned as differentially expressed. We followed the Minimum Information for Publication of Quantitative Real-Time PCR experiments (MIQE) guidelines-compliant check-list [47].

For data analysis, qPCR Amplification curves were first visually examined in the StepOne software (Applied Biosystems). No baseline and threshold line adjustments were necessary. Ct values of the technical replicates were averaged and used for relative gene expression analysis in the REST 2009 software (Qiagen, Germany) [46]. Expression of target genes was normalized to the expression levels of three reference genes (*arcA*, *mdoG* and *tus*), selected based on the assessment of the expression stability across all experimental conditions using BestKeeper software. Reaction efficiency information inferred from the standard curve data was used to correct for differences in amplification efficiencies in the REST 2009 software. Default settings (2000 iterations) were used for randomisation and bootstrapping analysis to test significance of gene expression. Expression values with p-values ≤0.05 were assigned as differentially expressed. We followed the

Minimum Information for Publication of Quantitative Real-Time PCR experiments (MIQE) guidelines-compliant check-list [47].

Transcriptome sequencing. The transcriptome sequence was carried out on samples treated with 0.1 mM of hydrogen peroxide during the experiment of memory decline. The time point used corresponded to 120 minutes after removal of the treatment. Bacterial cells that were kept in low density (0.05 O.D.600) were concentrated ten times and the pellets were resuspended in 1 ml of lysis buffer. Both, small RNA fraction and the large one were isolated using the microRNA & small RNA Isolation kit (Thermo Fisher Scientific, Germany). Traces of genomic DNA were removed from 10 µg of RNA by digestion in a total volume of 500 µl containing 20 units of TURBO DNase, incubated for 30 minutes at 37°C, immediately followed by RNeasy (Qiagen) clean-up and elution in 30 µl of RNase-free water. Following DNase treatment, RNA integrity was assessed using Agilent RNA 6000 Nano kit and 2100 Bioanalyzer instrument (both Agilent Technologies, USA). Both fractions were depleted from ribosome RNA using the Ribo-Zero depletion Kit for Gram negative bacteria (Illumina, USA). Libraries were prepared using a TruSeq Stranded Total RNA library preparation kit (Illumina, USA) and were sequenced on a MiSeq platform. All sequences are available are available from the NCBI SRA under BioProject accession PRJNA485867.

Evolution experiment. Before the beginning of the experiment, five independent clones of *E. coli* MG1655 were pre-adapted to the experimental conditions such as culture medium, temperature and oxygen level and other parameters. The pre-adaptation was carried out by diluting an overnight culture of each clone 1:1000 and incubated at 37°C in the plate reader with the same shaking conditions used during the rest of the experiment in 0.2 ml in fresh-made Lysogeny Broth Lenox(LB). The bacteria we cultured by serial passage every 24 hours using a bottleneck of 1% (1/100 dilution) to allow for approximately fifty generations during ten days. Contamination checks by plating out on LB and cryopreservation of culture aliquots at -80°C in LB containing 15% glycerol solution were carried out periodically. All lines showed

661 similar fitness and five independent colonies (one per line) were selected for
662 the evolution experiment. The experiment was performed at 37°C with
663 periodical shaking in a microplate reader (Synergy 2, Biotek). We used flat
664 bottom polypropylene non-binding 96-well plates from Greiner Bio-One GmbH
665 (Germany). The plates were covered with polystyrene lids with anti-evaporation
666 rings also from Greiner Bio-One GmbH. A final volume of 200 µl per well was
667 used. Growth curves were generated by taking measurements of OD600 every
668 20 minutes (preceded by a brief shaking of 5 seconds). Every day, the
669 experiment consisted of two stages. Every morning a V-bottom plate
670 containing 190 µl of LB was inoculated with 2 µl of overnight culture and
671 incubated for two hours to reach an OD between 0.2 to 0.6. At this point, 3 µl of
672 LB containing the corresponding priming concentration of H_2O_2 (for populations
673 to be primed) or only LB (control populations) were added to each well. The
674 plate was then incubated for 30 minutes, centrifuged and the supernatant
675 removed using an 8-channel vacuum line provided of disposable tips. The
676 pellets were resuspended in 200 µl of fresh LB and 190 µl were transferred to a
677 new flat-bottom plate, allowed the cell to recover for 30 minutes at 37 °C and
678 challenged with a ten-fold higher concentration of the priming one. The dish
679 was located in the plate reader and the growth was followed as described
680 above during 20 hours. The serial passage started at 50µM of H_2O_2. Next day,
681 the procedure was identical by both, priming and challenging concentrations
682 were doubled to reach a final challenging concentration of 32 mM of H_2O_2
683 where all bacterial population went extinct. Besides, 10 µl of the remaining
684 cultures were added to 180 µl of sterile 0.9% NaCl and then serially diluted,
685 plated and inspected for contamination.
686
687 **DNA isolation.** Genomic DNA samples for whole genome sequencing were
688 isolated using in house method based on fast phenol:chloroform extraction,
689 removal of RNA and ethanol precipitation, capturing the DNA in paper filter as
690 previously described. The DNA quantity and quality were estimated by
691 measuring the optical density at A260/280 using the Nanodrop
692 spectrophotometer (Thermo Scientific) and agarose gel electrophoresis.
693

Genome re-sequencing. We sequenced the total genomic DNA coming from sixty populations from the evolution experiment, all from the previous passage before extinction (20 evolved from priming regime, 20 from non-priming regime and 20 control strains). TruSeq DNA PCR-free libraries were constructed according to the manufacturer's instructions and sequenced for 600 cycles using a MiSeq at the Berlin Center for Genomics in Biodiversity Research. Sequence data are available from the NCBI SRA under BioProject accession PRJNA485867.

The haploid variant calling pipeline snippy [48] was used to identify mutations in the selection lines. Snippy uses bwa [49] to align reads to the reference genome and identifies variants in the resulting alignments using FreeBayes [50]. All variants were independently verified using a second computational pipeline, breseq [51].

Determination of H_2O_2-induced mutagenesis. This procedure was carried out following similar protocols to previous studies with some modifications [52,53]. Five independent cultures (5 ml each one) of *E. coli* MG1655 was grown in fresh-made LB medium to an OD of ~0.2. At this point, each culture was diluted 10 times (volume was increased to 50 ml) with LB and divided into two separate sets of 25 ml per culture each one. One set, consisting of five cultures of 25 ml was treated with 0.1 mM of H_2O_2 (primed) while the second set of culture remained untreated (naïve). After 30 minutes, both cultures were treated with 1 mM of H_2O_2 during another 30 minutes. Then, H_2O_2 was removed, first, by quickly adding 4 µl/ml of catalase and, second, by collecting the cells by centrifugation and washing them with one ml of fresh LB. An additional set of tubes received no treatment all and was used to determine the basal frequency of mutants. For determination of H_2O_2-induced mutation frequency, 4 ml of fresh LB was inoculated with 1 ml of washed bacteria from each culture. The cultures were allowed to recover overnight 16 h at 37°C. Serial dilutions of each culture were plated next day onto LB plates containing 100 ug/ml of rifampicin or without antibiotic to estimate the viability. The three groups were compared among them using a Welch's test.

Statistical analysis. For point to point comparisons in sensitivity and priming killing curves a Welch's test was used. For survival analysis we used a log-rank test. Some additional analyses are mentioned elsewhere in the manuscript. All tests were performed with software package R [54].

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Legend to the figures

Figure 1. Hydrogen peroxide killing curves of *E. coli* MG1655. Panel A shows bacterial sensitivity to the 1 mM of hydrogen peroxide with a strong dose response effect ($p=2.1x10^{-16}$, DRC model fitted based on maximum likelihood). Panel B shows the priming effect or improved response when cells are treated in advanced with a non-lethal dose (0.1 mM). Curves represent the mean of five independent cultures per time point. Asterisks represents significant differences (Welch's test, one asterisk for $p < 0.05$ and two asterisks for $p < 0.01$). Only comparison between primed and non-primed groups are shown.

Figure 2. During memory decline experiment, the viability of the cells remains unaltered before the addition of the trigger (A). The priming memory declined over time after induction of priming response with H_2O_2 0.1 mM (trigger) (B). Asterisks indicate significant differences between each time-point pair (Welch's test, one asterisk for $p < 0.05$ and two asterisks for $p < 0.01$).

Figure 3. Heatmap of relative protein expression based on quantitative lable-free quantification by liquid chromatography/mass spectrometry (LC-MS). Hierarchical clustering of the intensities was performed by using Euclidean distances between means. Row indicate the fluctuation of protein (by gene names) level at 5 minutes after addition of 0.1 and 1 mM H_2O_2. Intensity ranges of the log2 fold-changes are given from highest intensity (green) to lowest (red). Only the 50 statistically most significant upregulated proteins are shown, taking as a reference the 0.1 mM concentration.

Figure 4. Heatmap of relative protein expression based on quantitative label-free quantification by liquid chromatography/mass spectrometry (LC-MS). Hierarchical clustering of the intensities was performed by using Euclidean distances between means. Row indicate the fluctuation of protein (by gene names) level at different time points. Intensity ranges of the log2 fold-changes are given from highest intensity (green) to lowest (red). Only the most 50 statistically significant upregulated proteins at time point T1 (30 minutes after priming) are represented to follow their fluctuation in the other time points with 30 minutes between them.

Figure 5. Projection of differentially translated proteins 30 minutes after addition of H_2O_2 on protein-protein interaction network of *E. coli* K12. Pale green nodes indicate upregulated proteins while pale red represents downregulated ones. Note that the most critical nodes (DnaK and GloL) of the protein-protein interaction network are affected. The interactions among nodes show the wide-proteome impact of H_2O_2.

Figure 6. Sensitivity of a selected set of mutants in relevant proteins of *E. coli* MG1655 that presented an increased level of expression being part of priming response. Asterisks represents significant differences between the wild-type (wt) strain and its derivatives mutants (Welch's test, one asterisk for $p < 0.05$ and two asterisks for $p < 0.01$)

Figure 7. Priming experiments of different mutants of *E. coli* MG1655 in responsive genes to oxidative stress. Asterisks represents significant differences (Welch's test, one asterisk for $p < 0.05$ and two asterisks for $p < 0.01$). Only comparison between primed and non-primed groups are shown.

Figure 8. Proportion of population extinctions (20 populations per treatment) due to exposure to growing concentration of H_2O_2 during experimental evolution of 40 individual populations. Both, priming and triggering doses were increased twofold daily up to 32 mM, where total extinction was reached.

Evolvability differs between the two treatments, naïve and primed populations (Log-rank test, $p < 0.01$).

Figure 9. Mapping of mutations that are unique to each H_2O_2-evolved population shared ones. The positions indicate the approximate locations of mutations in the *E. coli* MG1655 chromosome relative to the wild-type. Three generic main type of changes are indicated.

Figure 10. Mutational spectra. Frequencies of different types of mutations among evolved populations of both primed and no-primed conditions. The number of mutations (nucleotide substitutions and indels) is plotted against respective nucleotide positions within the gene fragment. In this analysis, the mutations were taken by type regardless of the targets by generating two frequency datasets to compare frequency by type of mutation of evolved populations under priming and non-priming regimes. The mutational spectra are significantly different between the two conditions according to the spectrum analysis software iMARS [32].

Supporting Information Legends

Figure S1. Relative gene expression for E. coli MG1655 OxyR regulon selected genes in treatments with 0.1 mM H 2 O 2 vs. non-treated bacteria. Cells were collected 30 minutes after exposure. Error bars represent the standard error of the mean of three independent biological replicas, each one being the average of three technical repetitions.

Figure S2. Boxplot showing the H 2 O 2 -induced mutant frequency. Naïve and primed cells (pre-treated with 0.1 mM, 30 minutes in advanced) cultures challenged with 1 mM, allowed torecover and plated in rifampicin (100 µg/ml). The basal level of mutagenesis for non pre-treated, non-challenged cells is also shown. Every sample consisted in five independent replications. Equal letter indicates significant difference while same express no differences (Welch’s

test, p=0.03 for basal level vs primed, p<0.01 for both basal vs naïve and primed vs naïve).

Table S1. Output table of the proteomic experiment reporting time-lapse decline of the *E. coli* response to 1 mM H_2O_2 during 30 minutes. Bacteria were sampled 30, 60, 90, 120 and 150 minutes after removal of the treatment. Each treatment group consisted of six independent replications and bacteria before treatment (T0) were used as control. Statistical analysis used student t-test and false discovery rate for correction of the p-values (data analysis using Maxquant and Perseus software for label-free quantification of proteins with LC-MS).

Table S2. Output table of the proteomic experiment reporting *E. coli* response to H_2O_2 treatment of 0.1 and 1 mM during 5 minutes. Each treatment group consisted of six independent replications and bacteria before treatment (T0) were used as control. Statistical analysis used student t-test and false discovery rate for correction of the p-values (data analysis using Maxquant and Perseus software for label-free quantification of proteins with LC-MS).

Table S3. Transcripts differentially expressed (±2 log2) in the fraction of small of RNA (<200 nt) during the decay of H_2O_2 response (120 minutes after removal the treatment).

Table S4. Transcripts differentially expressed (± 2.5 log2) in the fraction of large RNA (>200 nt) during the decay of H_2O_2 response (120 minutes after removal the treatment).

Table S5. Relative gene expression (qPCR) for *E. coli* MG1655 selected responsive genes after a treatment with 0.1 mM H_2O_2 vs. non-treated bacteria during 30 minutes.

Table S6. Strains used in this work and their relevant phenotypes

Table S7. Primers used for amplification of kanamycin insertion of mutants from the Keio collection which PCR product was used to transfer the mutations to the *E. coli* MG1655 strain.

Table S8. Primers used for relative gene expression quantification by real time PCR. *E. coli* MG1655 was treated with 0.1 mM H_2O_2 during 30 minutes.

Figure 1

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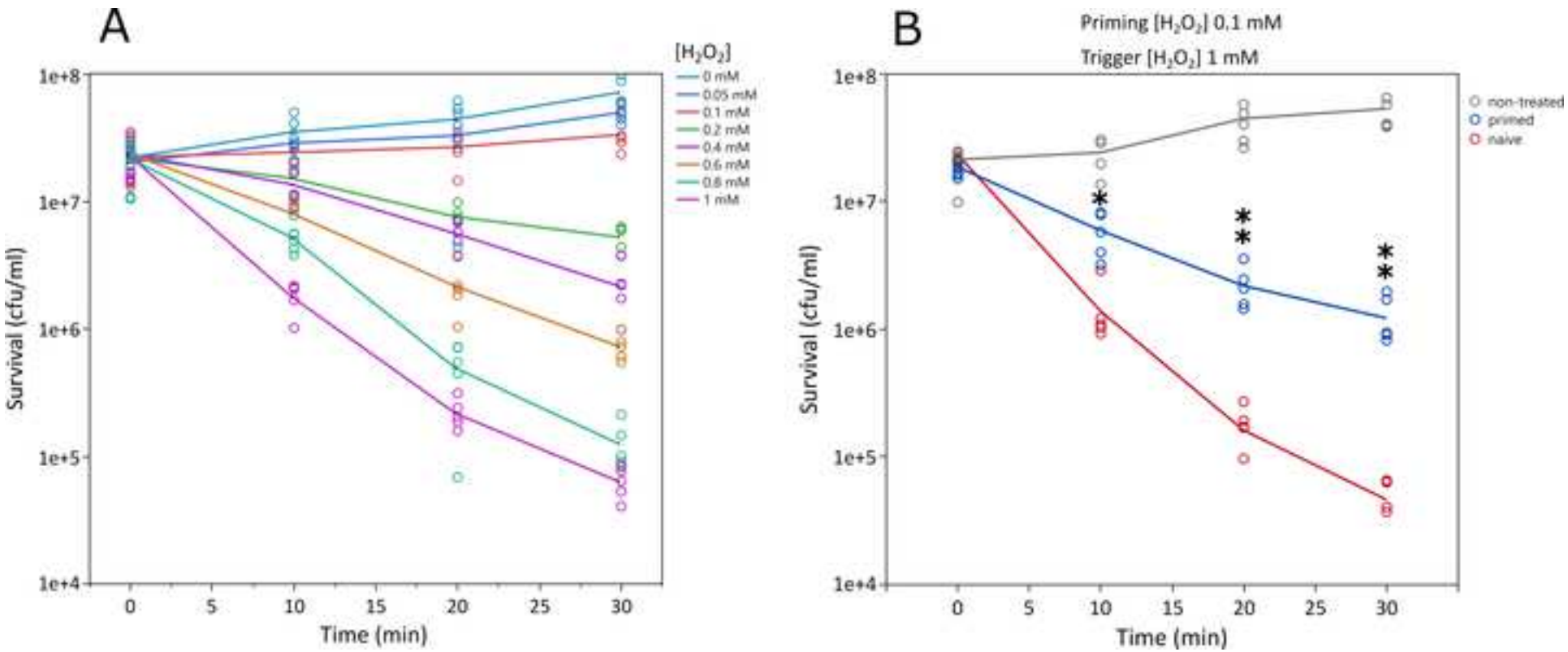

Figure 2

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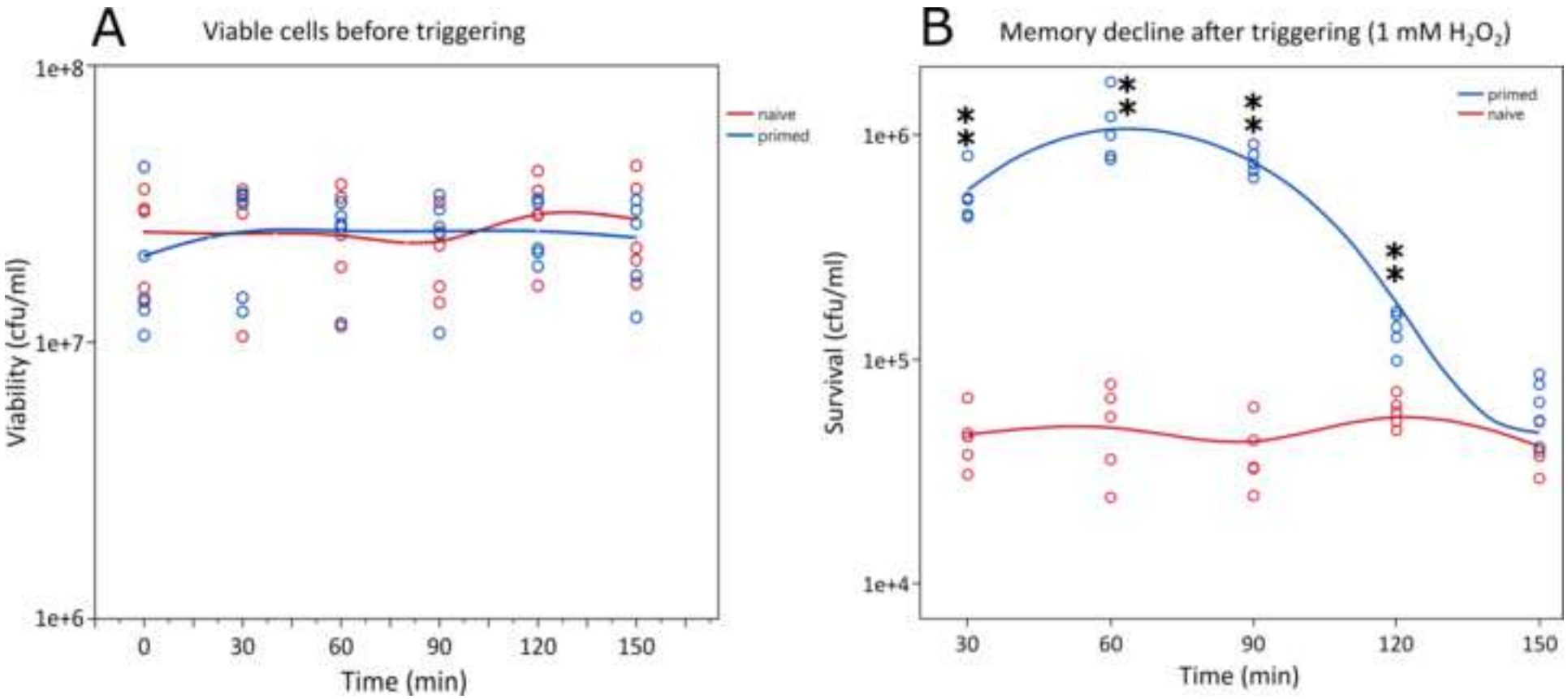

Figure 3

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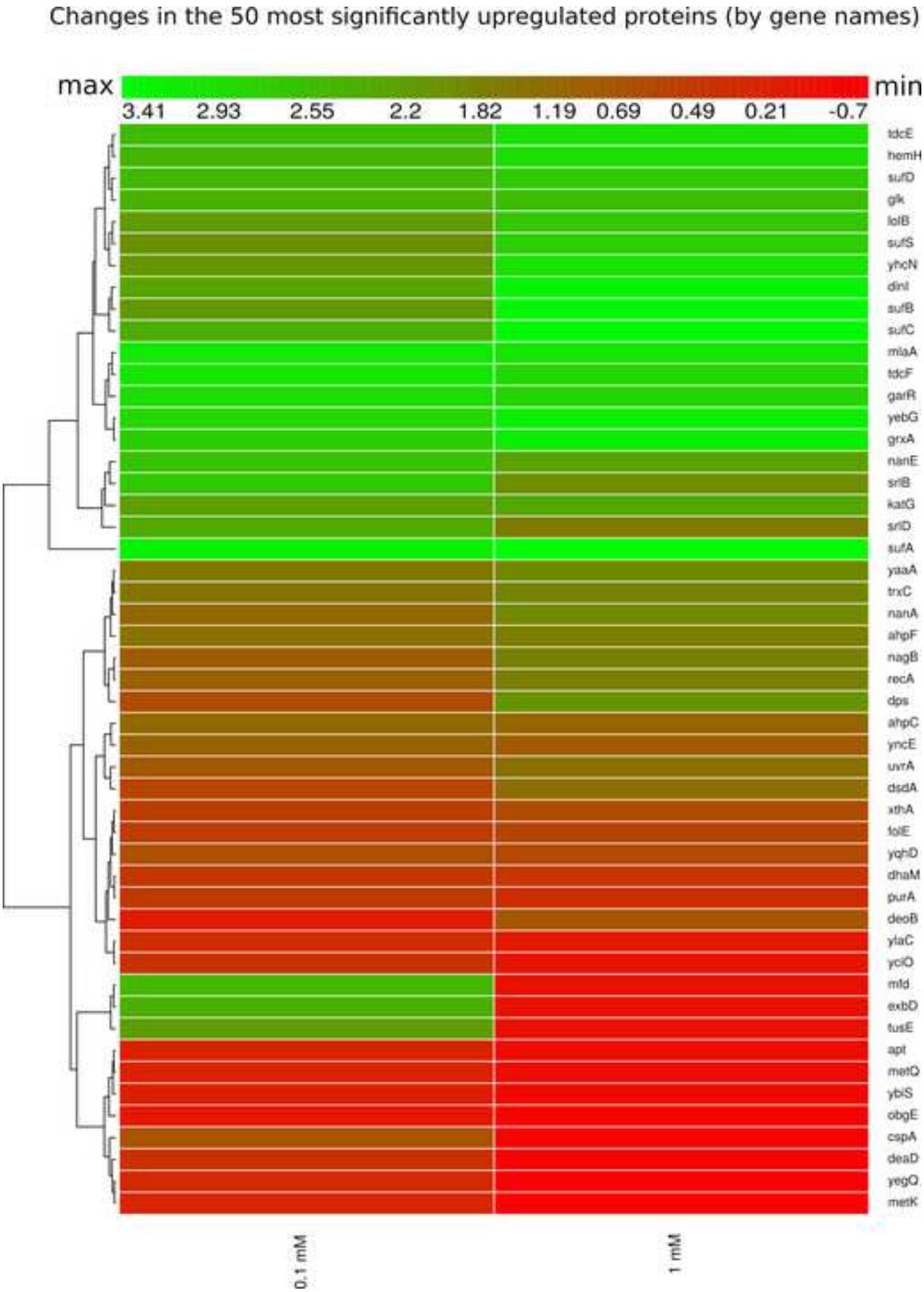

Figure 4

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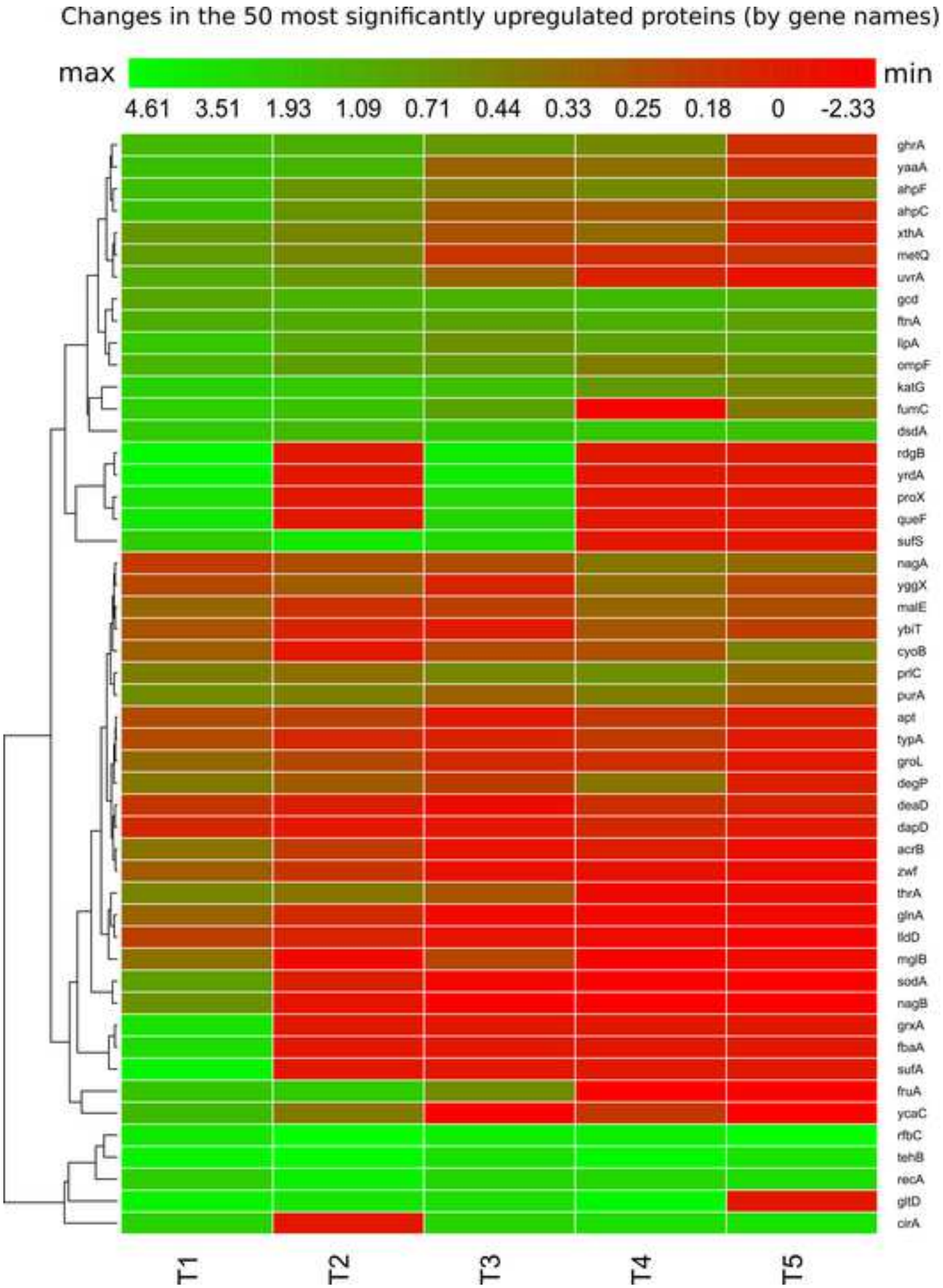

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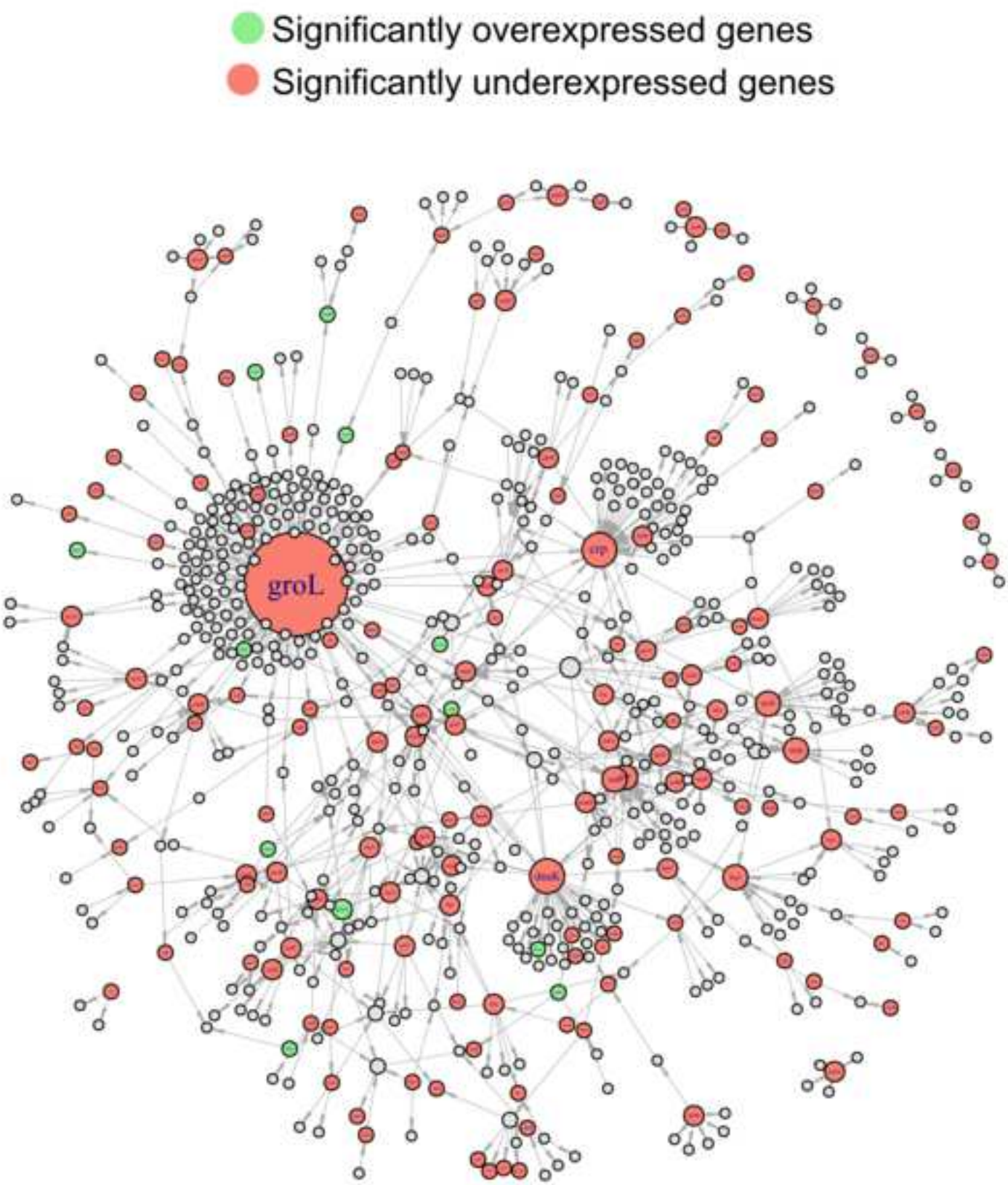

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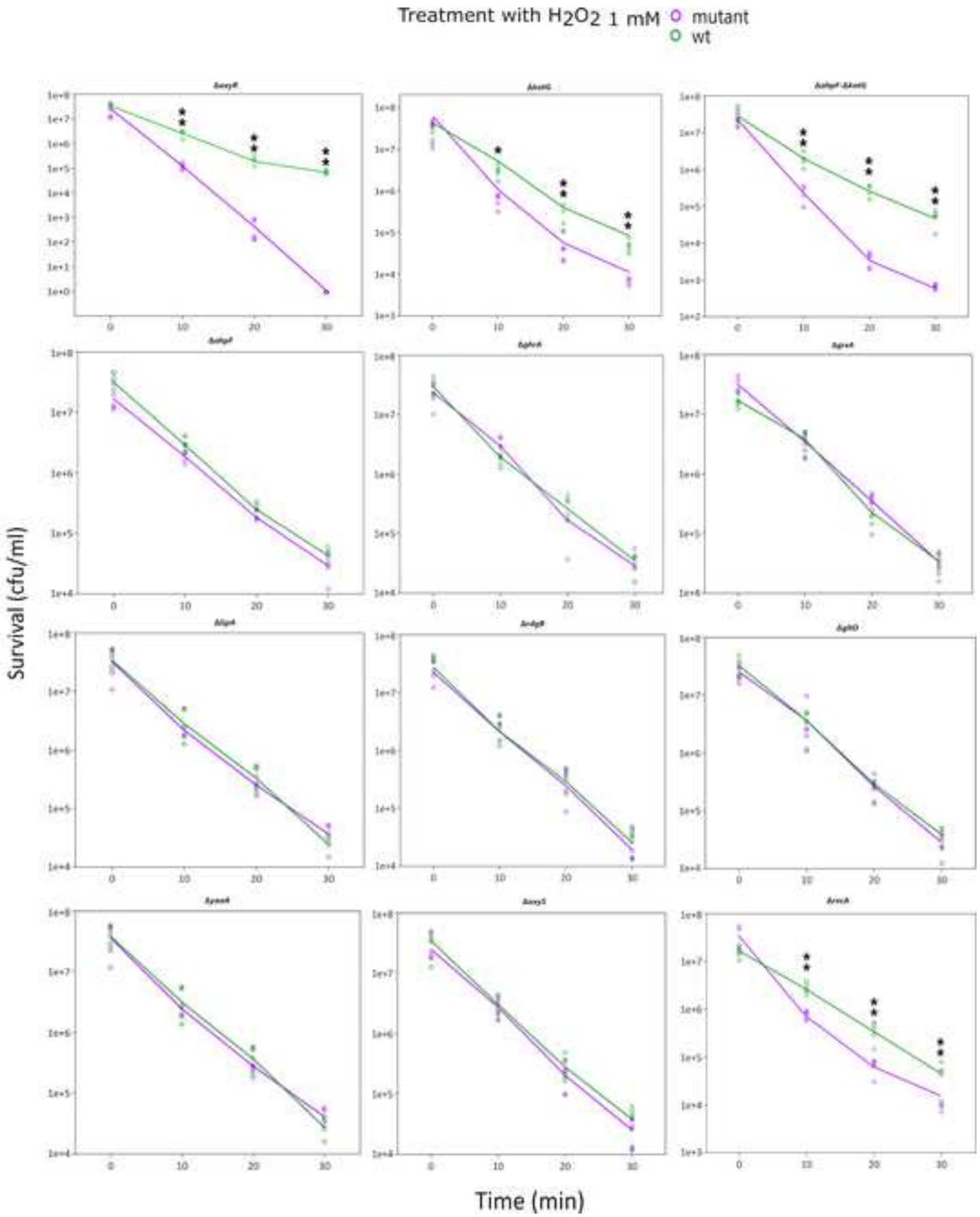

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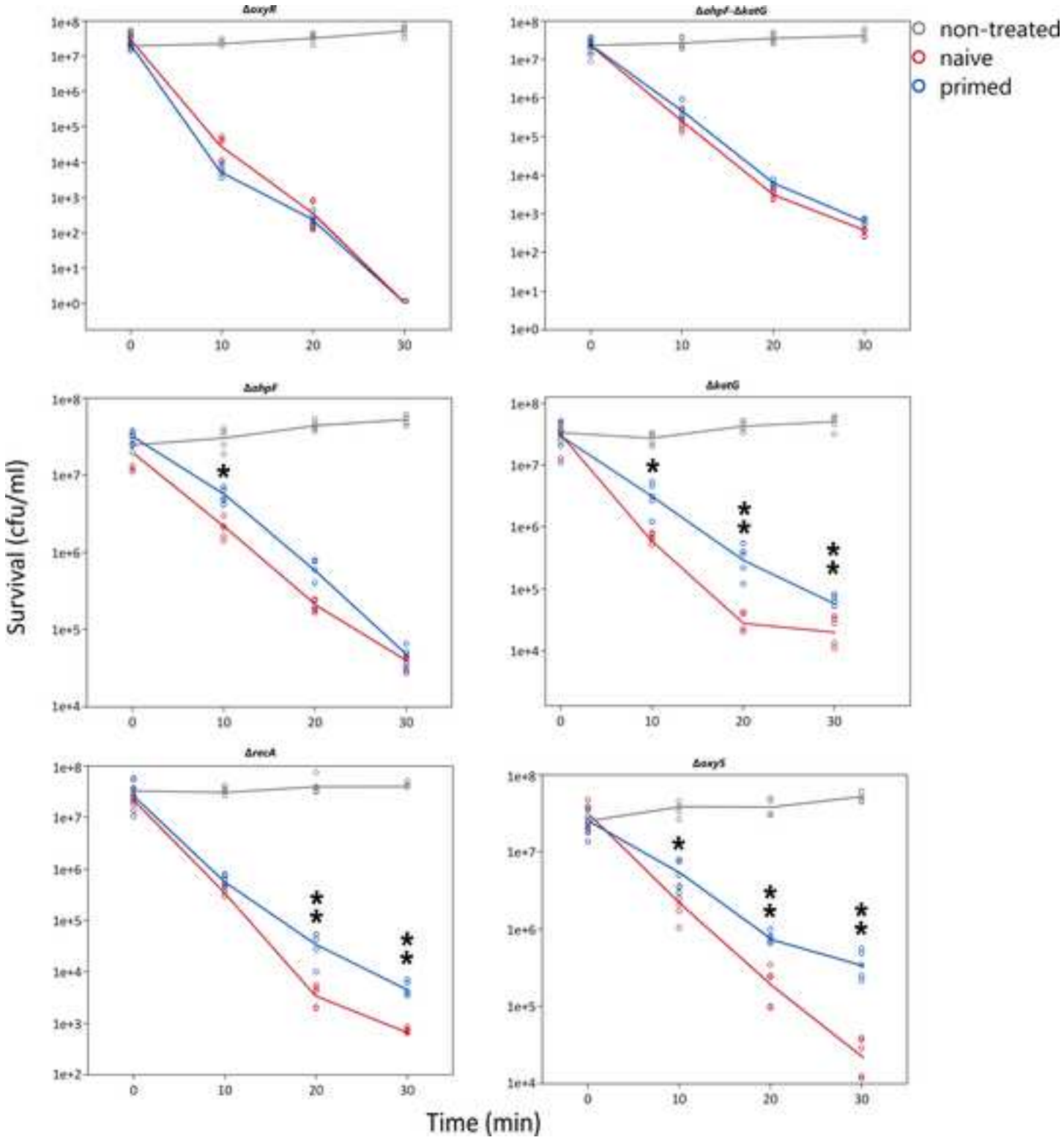

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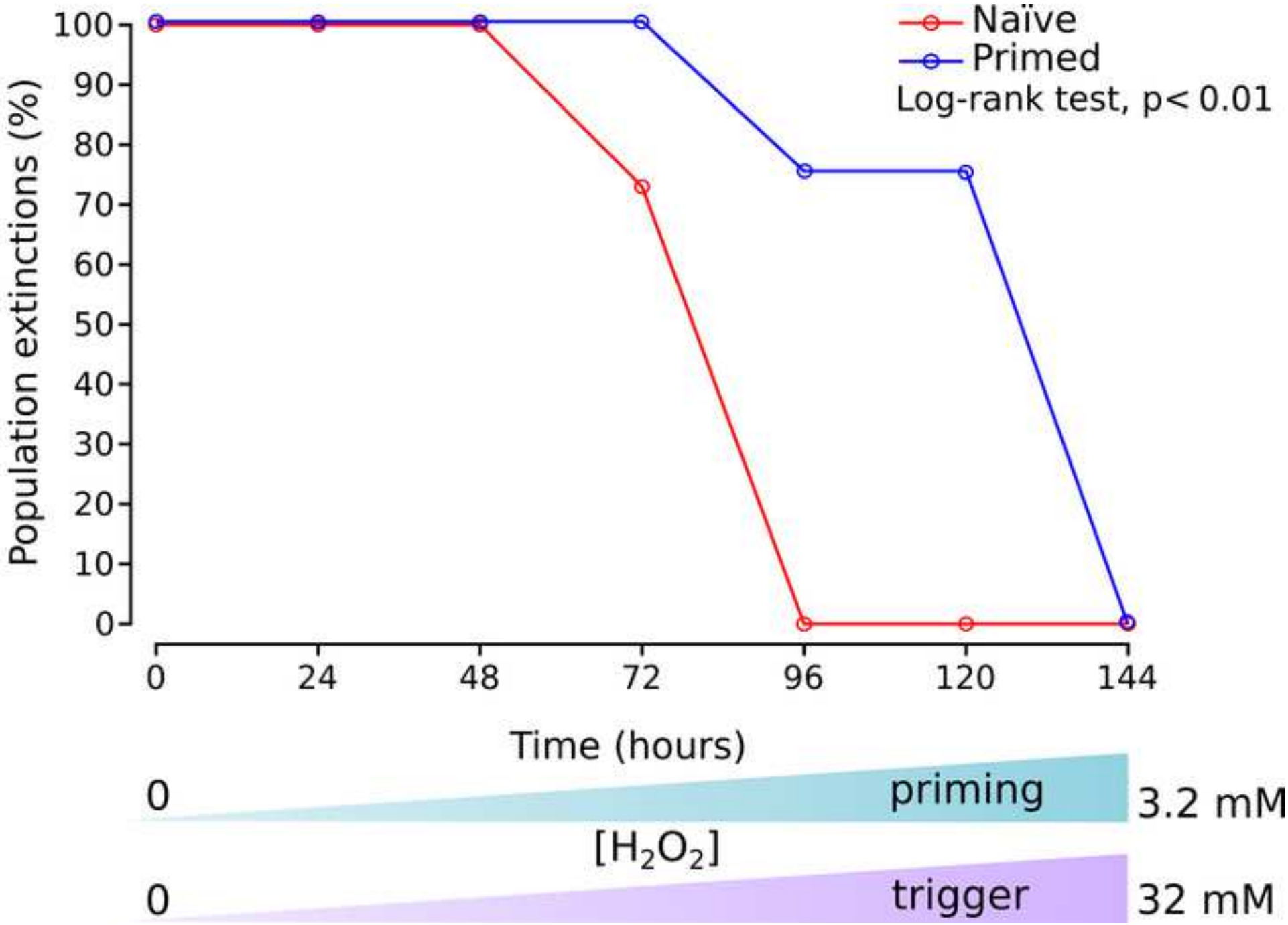

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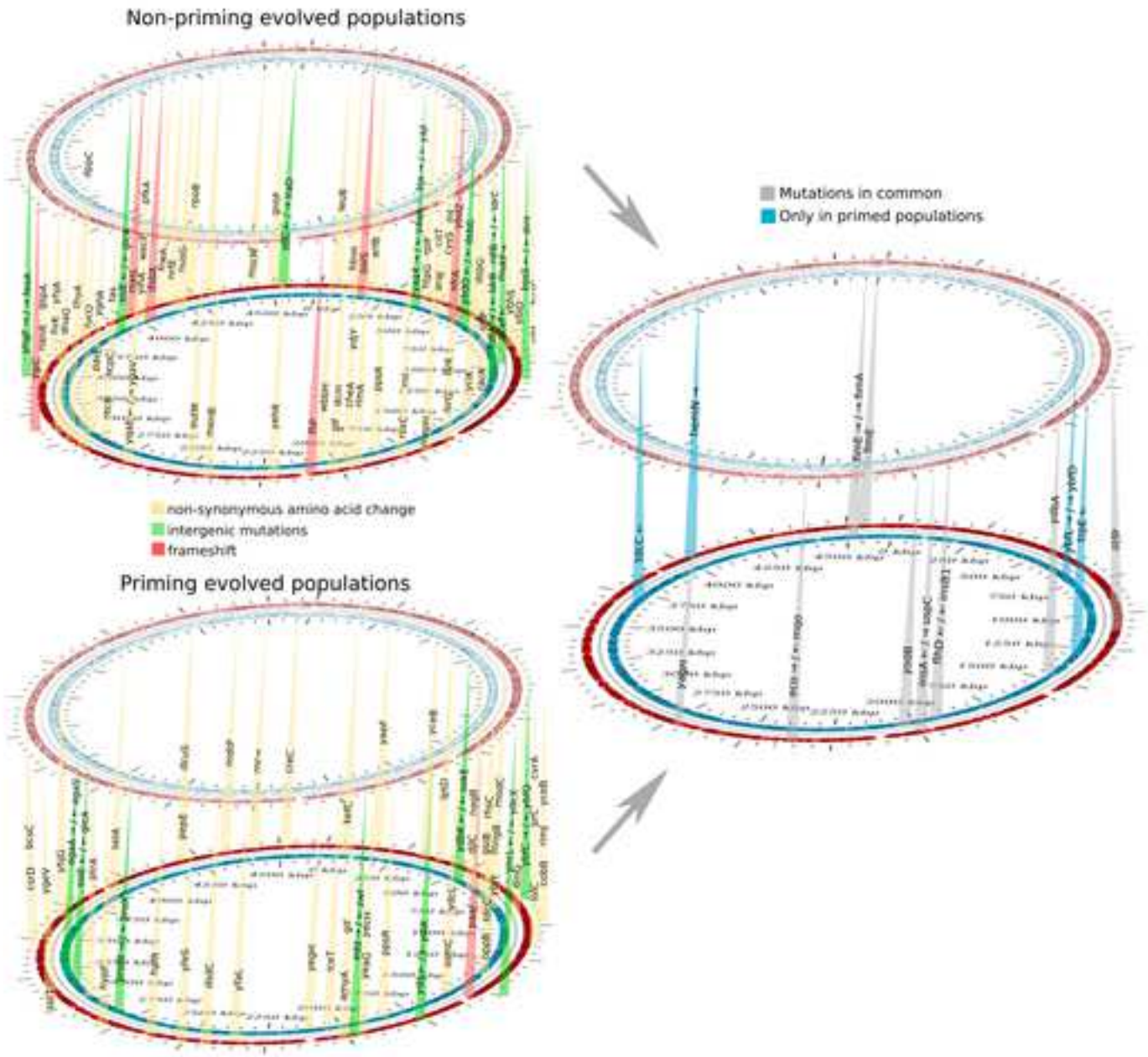

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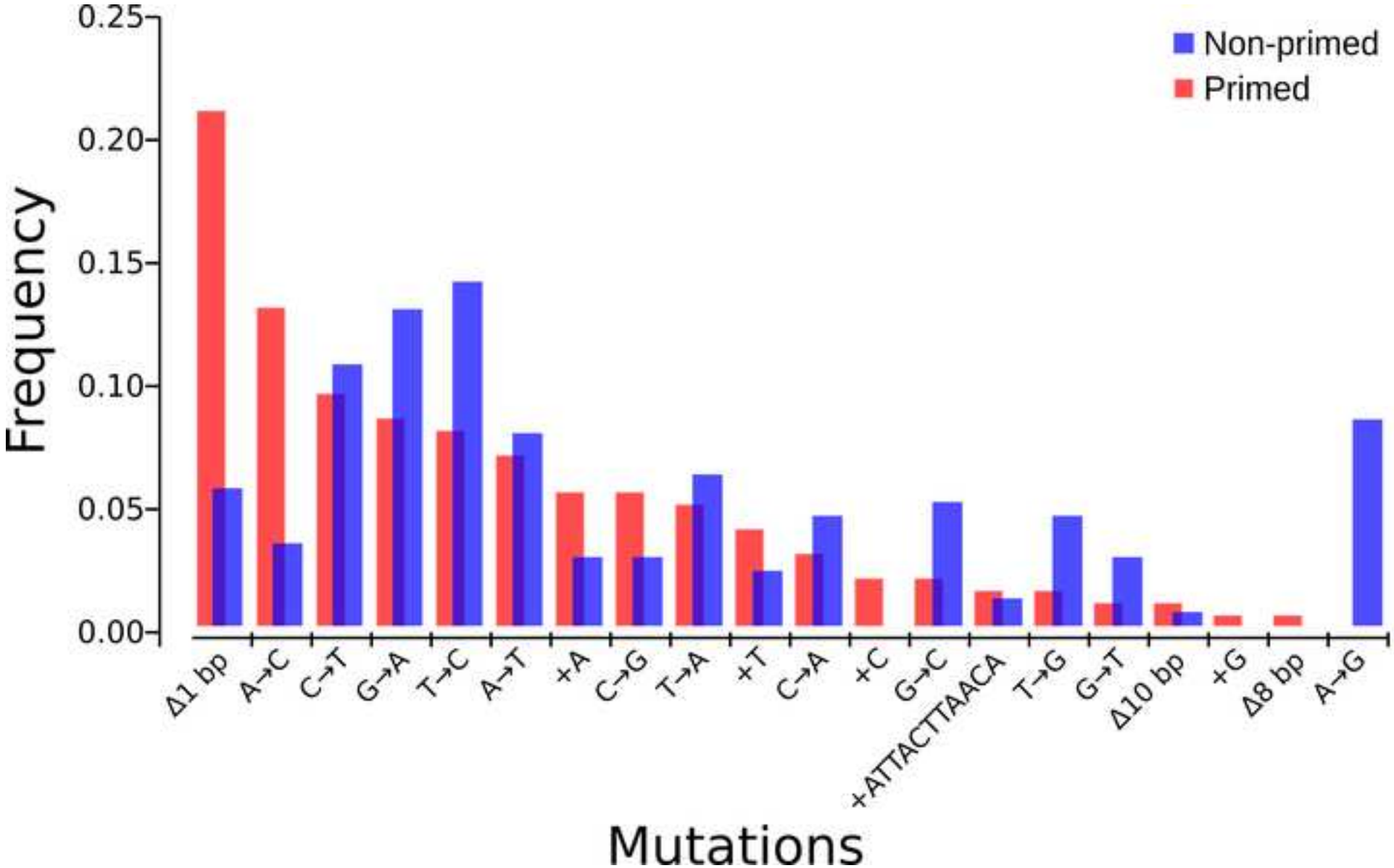
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