## Title

Deciphering the Proteome of *Escherichia coli* K-12: Integrating Transcriptomics and Machine Learning to Annotate Hypothetical Proteins

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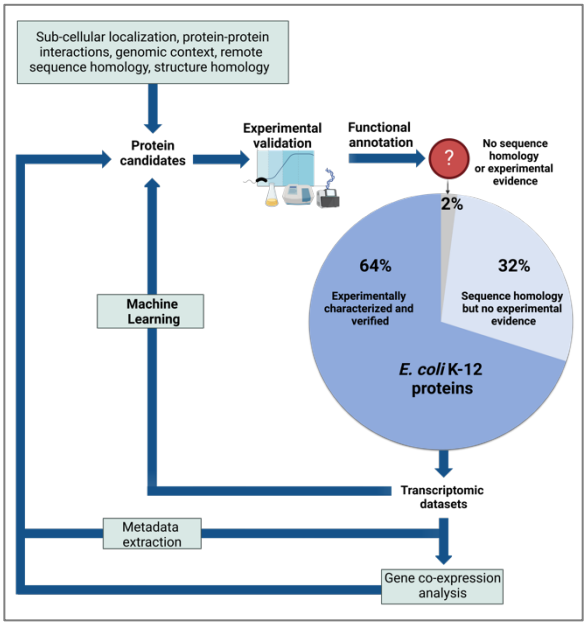
## Graphical Abstract



**Abstract**

Omics technologies have led to the discovery of a vast number of proteins that are expressed but have no functional annotation - so called hypothetical proteins (HPs). Even in the best studied model organism *Escherichia coli* K-12, 2% of the proteome remains uncharacterized, with no sequence homologies available in public databases. This knowledge gap becomes even worse when looking at microbial dark matter. However, knowing the functions of proteins is crucial for elucidating cellular and metabolic processes and harnessing biotechnological potentials. In this study, we employed machine learning (ML) to decipher the transcriptional regulatory network of *E. coli* K-12, deep learning for the prediction of structural homologs as well as other ML and bioinformatic tools, with the goal to assign functions to uncharacterized HPs. We further provide a proof-of-concept for experimental validation of function for three HP-encoding genes (*yhdN*, *yeaC* and *ydgH*) hinted from the *in silico* methods, by analyzing growth patterns of *E. coli* K-12 deletion mutants compared to the wild type, as well as their transcriptional responses to specific conditions. This study demonstrates that the use of Big Omics Data in combination with Artificial Intelligence and experimental controls is a powerful approach to illuminate functional dark matter.

**Keywords**: Functional Dark Matter, Big Omics Data, Artificial Intelligence, Independent Component Analysis (ICA), Functional Annotation of Proteins

## Introduction

Due to the advents in Next Generation Sequencing technologies, the volume of omics data has massively increased over the past years. As of today, gold-standard knowledgebases like the National Center for Biotechnology Information (NCBI)'s Reference Sequences Database (RefSeq) and the Universal Protein Resource Database (UniProt) [1] provide freely accessible sequences and information for over 163 million unique prokaryotic proteins **(Figure 1)**. This rapid accumulation of sequencing data has long surpassed the rate of possible experimental characterization *in vivo* and *in vitro*. Hence, researchers often rely on functional analysis of proteins from model organisms. These findings are then extrapolated *in silico* to closely related sequence homologues. Consequently, the definition of cut-off values plays a crucial role in protein annotation. To manage this challenge, automated annotation pipelines, such as InterPro have become a valuable tool [2]. Here, analysis of protein sequences is provided by classifying them into families and integrating predictive models from multiple protein databases such as e.g. Pfam [3], ProSite [4], SMART [5] and/or CDD [6] to infer functions for experimentally uncharacterized proteins. However, only 83% of all UniProt sequences have so far been functionally annotated by InterPro [7] The remaining 17% of proteins are designated with terms such as 'putative uncharacterized', 'uncharacterized', 'unknown protein families (UPFs)' or proteins bearing 'domains of unknown functions' (DUFs). These proteins are summarized by the term hypothetical proteins (HPs), since they are expressed but could so far not be functionally characterized by classical *in vivo*, *in vitro*, and/or *in silico* methods (8,9). In addition, there is also no information on these proteins available in widely-used knowledge-databases such as BioCyc [10], RegulonDB [11], and EggNOG [12] **(Figure 1).**

While there have been notable advancements in methodologies for protein function prediction in the recent past [13–16] several challenges persist [17,18]: These include different functionalities across homologous proteins, proteins performing different functions in distinct cellular locations, proteins with multiple three-dimensional structures, the lack of conserved proteins across different species to identify shared functional relationships and a heavy

reliance on pre-existing data for inferential annotations [19]. Even in the best studied model organisms *Escherichia coli* K-12, 31.8% of the genome comprises of protein encoding genes that lack experimental validation, commonly referred to as 'Putative HPs' [8], and 2.1% that even lack sequence homologies (this study) (**Figure 1**). The problem of annotating HPs becomes even larger when looking at microbial dark matter(9, 20).In uncultivated microorganisms, the proportions of genes with unknown functions can comprise up to 60% in bacterial and 80% in archaeal genomes [21]. However, elucidating the function of these proteins is essential for understanding cellular processes, metabolism and evolution, as well as for harnessing their biotechnological potential [22].

Transcriptional profiling has helped to gain insights on protein functions by monitoring gene expressions and clustering genes based on their responses to varying environmental conditions and stimuli [23]. Here, the entire set of RNA transcripts produced by a genome under specific conditions provides a dynamic representation of the organisms' operational state. However, standard bioinformatics-based methods for transcriptomic data analysis are low-throughput and require quite extensive computing power and time due to the high degrees of complexity and heterogeneity of the data [24]. Today, artificial intelligence (AI)-based methodologies have made significant advancements over traditional bioinformatics approaches, offering the capacity to process and analyze data at a scale and speed that were previously unattainable (24, 25). They are particularly suited for deciphering complex interactions among genes and transcription factors or proteins that regulate them, as well as revealing the regulation of gene expression in response to diverse environmental conditions [27]. Among various techniques for analysis, module-detection methods have proven to efficiently predict the functions of co-regulated groups of genes from large gene expression datasets [28]. Independent Component Analysis (ICA), introduced by Comon in 1994 [29], employs an unsupervised machine learning (ML) approach, categorizing the input data without any prior information. This technique is especially relevant in the context of transcriptomics, where the goal is to understand the complex interplay of gene expression signals and to

85 decipher co-regulated gene sets. ICA therefore allows to reveal the regulatory patterns and
86 activity levels of genes governed by specific regulators (i.e. transcription factors controlling the
87 expression of genes) across diverse experimental conditions, so called transcriptional
88 regulatory networks (TRNs), thereby disclosing the organizational and functional architecture
89 of genes. Expanding upon this, McConn et al. developed a variant of ICA, termed OptICA,
90 which can be applied to large TRNs, analyzing interactions between transcription factors and
91 their target genes. Here, discrete groups of genes are clustered into robust independent
92 components by avoiding over-clustering of datasets (leading to loss of biological information)
93 or under-clustering (leading to too few clusters and a loss of output resolution), and therefore
94 providing an optimal representation of the organism's underlying TRN [27]. This results in
95 revealing independently regulated gene groups, so called iModulon from transcriptomics
96 datasets (29, 30). iModulons are therefore the data-driven analogs of so-called regulons - sets
97 of genes governed by regulators [30]. The OptICA algorithm along with high-quality RNA-seq
98 data has recently been used to decipher the TRN of *Bacillus subtilis* [32] and *E. coli* [27] to
99 uncover the detailed responses to environmental conditions and genetic perturbations, e.g.
100 deletion mutants. However, these studies focused on the identification of regulons that were
101 not experimentally described before rather than the functional characterization of HPs.
102
103 In order to decipher the function of HP-encoding genes in the *E. coli* K-12 proteome, we
104 adapted the workflow originally designed for *Bacillus subtilis* [32] and analyzed the gene
105 expression data of the *E. coli* K-12 sub-strains MG1655 and BW25113. Functional
106 characterization of HP-encoding genes was inferred by identifying co-regulated genes under
107 the influence of a common regulator from publicly available transcriptomic datasets. This
108 characterization was then further validated by annotating these genes using specific Gene
109 Ontology (GO) categories through the PANTHER tool (Protein ANalysis THrough Evolutionary
110 Relationships) [33]. Additionally, metadata conditions for the transcriptomic datasets were
111 extracted. Furthermore, other *in silico* methods were employed to determine the sub-cellular
112 localization [34], protein-protein interactions (PPIs) [35], genomic context [36] and sequence-

113 based remote homologies [37]. Structural homology information was obtained by using the
114 AlphaFold Protein Structure Database (AFDB) clusters webserver, which is based on a deep
115 learning (DL) method [38].
116
117 Since AI-predicted functions are usually not experimentally verified, raising the possibility of
118 untrue annotations [9], we also provide an experimental validation of three candidate HP-
119 encoding genes (*yhdN*, *yeaC* and *ydgH*). The growth curves and transcriptional responses of
120 *E. coli* K-12 deletion mutants in comparison to the wild-type strain under specific conditions
121 were analyzed, showing that the *in silico* predictions were indeed true. Hence, the here
122 presented pipeline not only facilitates the discovery of previously unidentified regulators, but
123 also sheds light on our understanding of the function of HPs **(Figure 1).**
124

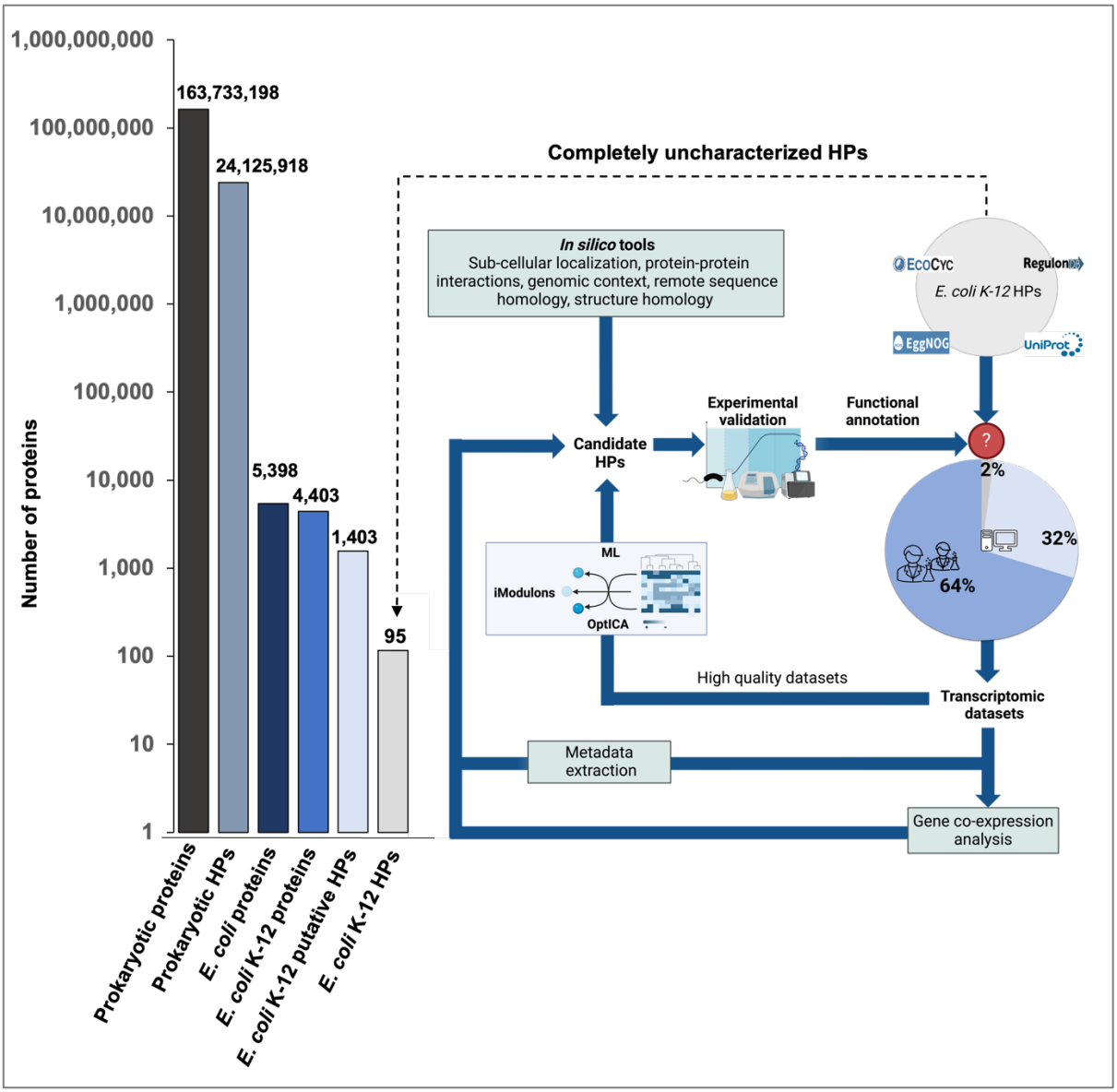



**Figure 1. Total number of sequences for all unique prokaryotic and *Escherichia coli* proteins deposited in the National Center for Biotechnology Information (NCBI) as of April 2024 and methodological set-up of this study.** Of the 4403 *E. coli* K-12 protein encoding genes, 1403 (31.8%) encode for unique proteins with functions predicted only *in silico* based on homologous sequences but lacking *in vivo* or *in vitro* experimental evidence (termed "putative hypothetical proteins"). 95 protein encoding genes (2.1%) of *E. coli* K-12 are completely uncharacterized with no sequence homologues according to the four knowledge databases - EcoCyc [39], RegulonDB [11], EggNOG [12] and UniProt (1) (termed "hypothetical proteins"). Transcriptomic datasets from NCBI were filtered and processed using the OptICA approach to generate iModulons [32]. Metadata information was curated in parallel using manual or semi-automated approaches(39–41).Bioinformatics, machine learning and deep learning tools along with the presence of relevant metadata then resulted in potential functions for HP candidates for *in vitro* testing. HPs, hypothetical proteins; ICA, independent component analysis; ML, machine learning.

## Results and Discussion

### Identification of uncharacterized HPs in *E. coli*

In 2019, Ghatak et al. had identified HP-encoding genes in *E. coli* K-12, however only by employing keywords such as ‘possibly’, ‘predicted’ or ‘hypothetical’ from the EcoCyc database, or annotation scores of two or below in UniProt [8]. This approach did, however, not establish a stringent threshold for accurate identification of completely uncharacterized HP-encoding genes. Hence, a more rigorous approach was used in this study, which included additional keywords for HPs (such as ‘Uncharacterized’, ‘Putative uncharacterized’, ‘DUF’ etc.) and information on the absence of any functional characterization using multiple gold-standard databases (EcoCyc, UniProt , RegulonDB and EggNOG). 158 of the 1600 HP-encoding genes previously listed by Ghatak et al. have now been functionally characterized with experimental evidence in the EcoCyc database. Notably, the HP-encoding gene *ydfX*, curated as one of the HPs in this study, was missing from Ghatak et al.'s list since it was previously categorized as a pseudogene/phantom gene. Its exclusion, however, could not be verified in the current EcoCyc database. We identified 1403 of 4403 genes (31.8%) of the *E. coli* K-12 genome as 'putative’ HPs, implying that their functions were inferred based on sequence homologies in the gold-standard databases, but lacking an experimental evidence for function. A recent study using the EggNOG tool also identified about 30% in a different *E.* strain (O157:H7 strain Sakai) as putative HPs [18].

95 HP-encoding genes (2.1%) could be identified with no sequence homologies in the *E. coli* K-12 genome. These could be further sub-categorized in 'uncharacterized proteins' (53), ‘putative uncharacterized’ (5), ‘(DUF)' domain containing proteins (21) and ‘UPF’ (HPs with unknown protein families, 3). 13 HPs had the characteristic ‘Protein Y…’ naming scheme. The distribution of those 95 HP-encoding genes within the genome can be found in the Supplementary (*Supplementary Figure 1)*.

171 **iModulon generation and analysis**

172 In order to investigate the functions for the 95 uncharacterized HP-encoding genes, publicly
173 available RNAseq datasets from *E. coli* K-12 substrain MG1655 were downloaded from NCBI
174 and quality filtered. 779 high-quality datasets from the MG1655 substrain were then used as
175 input for the OptICA algorithm [27]. For the substrain BW25113, a separate OptICA analysis
176 was not conducted due to the very small size of the dataset. The iModulons were identified by
177 utilizing the PyModulon package, specifically employing the *Inferring iModulon Activities*
178 function [32] for the BW25133 substrain. In total, 131 iModulons were obtained for the entire
179 *E. coli* K-12 transcriptome. Three single gene iModulons were discarded from the analyses,
180 since they were considered a result of artificial knockouts or overexpression of single genes
181 in the dataset. Therefore, a total of 128 iModulons were selected for further analysis.
182 Functional information on the HP-encoding genes was obtained by mapping the iModulons to
183 existing experimentally derived regulons (groups of genes regulated by the same transcription
184 factor), using data from the EcoCyc and RegulonDB databases. Additionally, Gene Ontology
185 (GO)-based functional annotations for member genes of an iModulon (co-regulated gene sets)
186 were identified using the PANTHER tool (Protein ANalysis THrough Evolutionary
187 Relationships) [33]. A total of 51 HP-encoding genes were clustered into iModulons using
188 OptICA. Of these, 46 genes were assigned putative functions based on information from
189 regulator proteins and/or Gene Ontology (GO) categories. (**Figure 2**). Two genes, *yfeS* and
190 *yffL* had information on their regulators (RpoE and NarL, respectively) but no assigned GO
191 categories, while five genes (*ybaA, yfaP, yjgZ, ymgI and ymgJ*) (white) could not be associated
192 with a regulator or a GO category. 44 of the HP-encoding genes could not be clustered into
193 distinct iModulons (grey) (**Figure. 2**).

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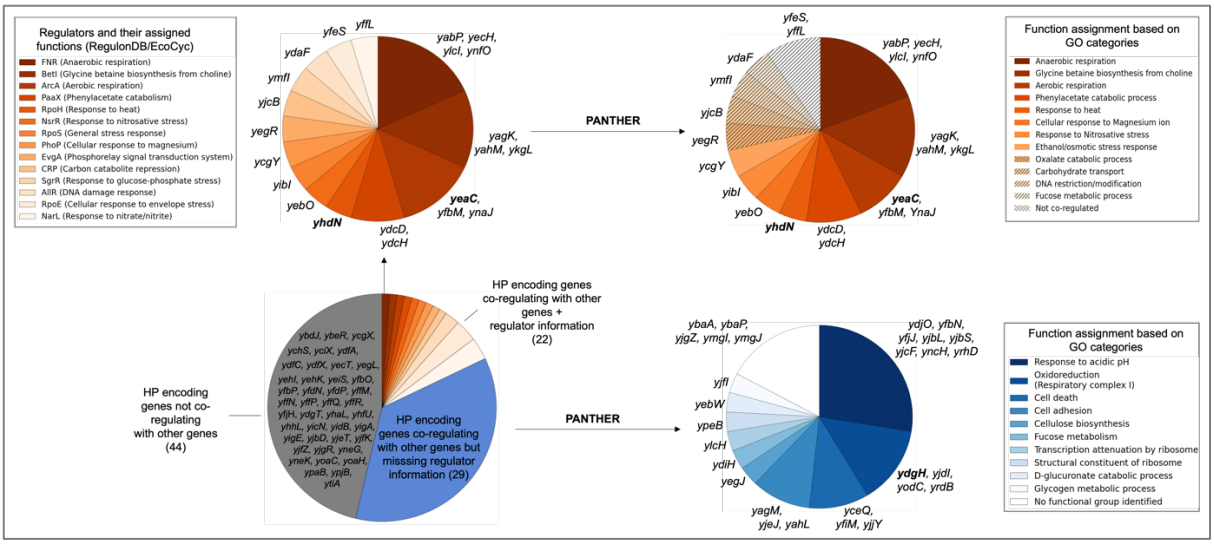


**Figure 2. Regulator and functional classification for 95 HP-encoding genes in *Escherichia coli K-12*.** 44 HP-encoding genes could not be clustered by the OptICA method [27] (grey), since they did not co-regulate with any other genes. 22 genes were characterized based on information on their regulators as obtained from RegulonDB and/or EcoCyc, as well as by Gene Ontology (GO) categories based on the co-regulating genes using the PANTHER tool (Protein ANalysis THrough Evolutionary Relationships) (orange) [33]. 29 genes co-regulated with other genes, but no information on their regulators could be obtained (blue). 24 out of these 29 genes could be assigned putative functions based on GO annotations of co-regulating genes derived from the PANTHER tool. The striped regions denote genes where the regulator-associated function from EcoCyc/RegulonDB did not match a GO-derived functional category obtained from PANTHER. Highlighted in bold are the three HP-encoding genes which were selected for *in vitro* testing.

In addition to the 95 HP-encoding genes, all other *E. coli K-12* genes were also analysed using this method. 2630 of the 2905 (91%) genes which have experimental evidence of function could also be characterized into iModulons. 1697 (64%) co-regulated with other genes, i.e. they were part of characterized iModulons with information on their regulator on RegulonDB and/or EcoCyc, while 933 of the genes were part of uncharacterized iModulons. For genes with known regulators, only 121 (5%) had either a non-matching functional category when compared with the PANTHER categories or a non-significant ($p$>0.05) functional category, and 95% had a matching PANTHER GO category. For genes which were part of uncharacterized iModulons, with no regulator information, 546 (58%) could be functionally classified using PANTHER. 1061 of the 1403 putative HP-encoding genes (75%), i.e. genes with functional characterisation based on sequence homology information, could be characterized into iModulons. 342 genes (25%) did not co-regulate with other genes. For genes that had co-regulation, 539 (51%) had information on their regulator on RegulonDB

223 and/or EcoCyc. The other 522 (49%) were part of uncharacterized iModulons. For genes with
224 known regulators, only 20 genes (4%) had either a non-matching function when compared
225 with the PANTHER categories or a non-significant ($p$>0.05) functional category in PANTHER.
226 For genes with no information on their regulator, 366 (67.9%), could however be characterized
227 by PANTHER. Hence, about 95% of the iModulon functional categories matched the
228 PANTHER GO categories, demonstrating the robustness of the method. Moreover, the
229 inclusion of experimentally characterized genes within uncharacterized iModulons shows
230 potential for the discovery of new regulons or co-regulated genes.
231
232 **Functional analysis of HP-encoding genes using *in silico* tools**
233 All 95 HP-encoding genes were analysed based on multiple criteria including the generated
234 iModulons from the OptICA algorithm, functional information based on the regulators of the
235 co-regulated gene sets obtained *via* RegulonDB/EcoCyc and their GO categories from
236 PANTHER. Information on metadata conditions pertaining to the highest level of gene
237 expression relative to the reference gene *frr* (encoding the ribosome recycling factor) [42] and
238 annotation of the most significant co-expressed gene were also extracted. Additionally, the
239 sub-cellular localization, protein-protein interactions, genomic context, remote sequence
240 homology as well as structural homology aided in forming hypothesises for assigning functions
241 to the respective HPs. 33 of the protein encoding genes (highlighted in green) could be
242 assigned functions with a high confidence. 28 of the genes were assigned functions based on
243 lower confidence, since only information from a maximum of two sources could be well-
244 correlated (highlighted in yellow). A clear inference could not be made for 34 of the genes
245 (highlighted in red). Information retrieved for each gene and functional inference (if applicable)
246 is detailed in **Table1**. A pictorial overview of Table 1 based on the functional annotations for
247 the 95 HPs can be found in *Supplementary Figure 2*.

**Table1. A comprehensive functional interference of HPs based on different *in silico* tools.** Annotations are based on regulator information from RegulonDB/EcoCyc and/or GO category based on co-regulated genes obtained from PANTHER) [33](as shown in Fig. 2). The highest gene expression condition, obtained from the Tjaden dataset [43], with metadata conditions categorized by the type of medium, oxygen availability (normal $O_2$ conditions unless stated otherwise), temperature etc. in comparison to the reference gene '*frr'* are described. Functional annotations of co-expressed genes were retrieved from EcoCyc [36]. 'SCL' or sub-cellular localization inferred from the BUSCA tool [34] are abbreviated as either 'NUC' for nuclear 'CYT' for cytoplasmic, 'ECS' for extra cellular space or 'PM' for plasma membrane. Functional information from protein-protein interaction (PPI) analyses was obtained from STRINGdb [35]. Annotations for neighbouring upstream (⟵) and downstream (⟶) genes in context of the HP-encoding gene were retrieved from the EcoCyc database. Additionally, remote homology was assessed using the Uniref30 database using the Hhblits tool [37], and structural homology was inferred from the AlphaFold Protein Structure Database [38]. A functional inference can be made for genes supported by multiple lines of evidence when similar functional information was provided by at least two sources. Proteins with well correlated information from at least three sources are highlighted in green (with higher confidence) and proteins with information that can be correlated using at least two sources are highlighted in yellow (with lower confidence). Protein encoding genes highlighted in grey were selected for *in vitro* testing. An unclear inference suggests that, based on the different knowledge bases, the extracted information did not provide enough confidence or could not be well correlated to assign a function (red).

| **Gene** | **Function based on regulator and/or co-regulated genes** | **Condition with highest gene expression (growth medium)** | **Annotation of most significant co-expressed gene** | **SCL** | **PPI: function of partner protein** | **Annotation of neighbouring genes** | **Remote homology** | **Structural homology** | **Overall functional inference** |
|---|---|---|---|---|---|---|---|---|---|
| *1. yabP* | Anaerobic respiration | M9, 37°C; 100 µg/ml Ampicillin (inhibits cell wall synthesis) [44] | Growth related protein (*yabQ)* | CYT | No interaction partner | *djlA* ⟵ Co-chaperone protein; ***yabP*** ⟶; *yabQ* ⟶ Growth related protein | Putative Undecaprenyl pyrophosphate phosphatase (cell wall biosynthesis) [45] | Type III secretion system (bacterial pathogenecity) [46] | Cell wall biosynthesis |
| *2. yagM* | Cell adhesion | LB, 37°C; 20 µg/ml Bicyclomycin (produces ROS, blocks cell division) [47] | Transcriptional regulator (fimZ) (involved in fimbriae production) [48] | CYT | DNA-binding protein (YagL) | *yagL* ⟵ resolvase like prophage; ***yagM*** ⟵; *ykgV* ⟵ Growth related protein | Unknown function | Unknown function | Cell adhesion under antibiotic stress |
| *3. yagK* | Glycine betaine bio-synthesis from choline (osmoprotectant ) [49] | Na-Phosphate buffer, 37°C; *ΔthiI* (*thiI* is responsive to UV) [50] | Putative fimbrial usher protein (*yhcD)* | NUC | Putative ligase (GtrS) | *yagJ* ⟶ Response to X-radiation; ***yagK*** ⟵; *yagL* ⟵ resolvase like prophage | CP4-57 prophage protein (cell growth/biofilm formation/ motility/ transport) [51] | Unknown function | Protection against radiation-induced stress |

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|---|---|---|---|---|---|---|---|---|---|---|
| *4. yahE* | Not co-regulated | M9, 37°C; 0.3 M NaCl + 0.6% n-butanol (cause cellular stress) [52] | Putative acyl-CoA synthetase (*yahF)* (energy metabolism) | CYT | Putative NAD(P)-binding succinyl-CoA synthase (SucD) (energy metabolism) | *yahD* → Response to UV | ***yahE*** → | *yahF* → Putative acyl-CoA synthetase | Unknown function | Unknown function | Energy metabolism under cellular stress |
| *5. yahL* | Cell adhesion | LB, 37°C | Membrane protein (*yahC)* | NUC | Unknown function (YahE) | *yahK* → Aldehyde reductase (involved in biofilm formation) [53] | ***yahL*** → | *xtpA* → Putative transcr. regulator | Unknown function | Unknown function | Cell adhesion |
| *6. yahM* | Glycine betaine bio-synthesis from choline (osmoprotection ) [49] | M9, 37°C; 25 µg/ml Kanamycin | Putative amino acid exporter (*yahN)* (Efllux of toxic sunstances) [54] | CYT | Putative replicase (YidX) | *xtpA* → Putative transcr. regulator (confers antibiotic resistance) [55] | ***yahM*** → | *yahN* ← Putative amino acid exporter | Unknown function | Unknown function | Protection against stress |
| *7. ybaA* | Co-regulated (unknown regulator or function) | LB, 37°C | Putative oxido-reductase (*yohF)* | CYT | Double strand break repair protein (YegP) | *ffs* → Signal recognition particle | ***ybaA*** → | *pdeB* ← Cyclic di-GMP phospho-diesterase | RNA signal recognition protein [56] | Unknown function | Oxidoreduction/ DNA damage repair |
| *8. ybaP* | Co-regulated (unknown regulator or function) | LB, 37°C | DNA binding transcriptional activator (*AaeR)* (role in biofilm formation) [57] | CYT | Cysteine-tRNA deacylase (YbaK) | *chiX* → Small regulatory RNA (role in biofilm formation) [58] | ***ybaP*** ← | *ybaQ* → DNA binding transcr. regulator (regulates biofilm based response) [59] | Conjugal transfer protein | Unknown function | Biofilm formation |
| *9. ybdJ* | Not co-regulated | LB, 37°C | Putative transporter (*yeeA)* (aromatic acid exporter) [60] | PM | DUF419 family protein (YbdF) | *ybdF* ← Defense mechanism | ***ybdJ*** ← | *ybdK* ← Amino acid bio-synthesis | Unknown function | Unknown function | Stress response |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *10. ybeR* | Not co-regulated | M9, 37°C; 0.3 M NaCl + 0.6% n-butanol (cause cellular stress) [52] | Putative oxamate carbomyl-transferase (*fdrA)* | CYT | Putative co-chaperone (DjlB) (heat stress response) | *ybeQ* ← Oxidative stress response | ***ybeR*** → | *djlB* → J-domain type protein | Unknown function | Unknown function | Multi-stress response |
| *11. yceQ* | Cell death | LB, 37°C | DNA binding transcriptional repressor (*acrR)* – involved in efflux of toxic substances [61] | CYT | Endoribonuclease (RNE) – adaptation to different growth conditions [62] | *Rne* ← Ribosomal nuclease | ***yceQ*** → | *rluC* → 23S rRNA pseudo-uridine synthase | Unknown function | Unknown function | Cell death prevention |
| *12. ycgX* | Not co-regulated | LB, no $O_2$, 37°C | Membrane protein (*yigF*) | CYT | Repressor of blue light-responsive genes (BluR) | *ymgL* ← Consti-tutively expressed protein in rich medium | ***ycgX*** ← | *bluR* ← DNA binding transcr. regulator | Prophage protein | Unknown function | Unclear |
| *13. ycgY* | Stress response in stationary phase | LB, no $O_2$, 37°C | Outer membrane protein (*yaiO)* | CYT | Uncharacterized protein (YncH) | *ymgE* → DNA Recomb-ination | ***ycgY*** → | *treA* ← Trehalase protein (nutrient stress in stationary phase) [63] | Unknown function | Unknown function | Growth-associated response during stationary phase |
| *14. ychS* | Not co-regulated | LB, 37°C; 1 ug/mL of Ciprofloxacin+ 500 µM of ET2DA (cause oxidative/ cellular stress) [64] | Putative amino acid de-hydrogenase (*ykfG)* – role in oxidative stress [65] | CYT | Not found | *narI* → Nitrate reductase (oxido-reductase activity) (UniProt) | ***ychS*** → | *tpr* ← Protamine like protein | Beta-galactosidase | Protein YchS (Unknown function) | Oxidative stress response |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *15. yciX* | Not co-regulated | M9, 37°C; *yqjD* over-expression (inhibits cell growth) [66] | Membrane-associated regulatory protein (*ymiA)* (in exponential or tationary phase) [67] | CYT | Membrane-associated regulatory protein (YmiA) | *ymiA* → Membrane-associated regulatory protein | ***yciX*** → | *ymiC* → Stationary phase adaptation protein | Unknown function | Unknown function | Growth-associated response during stationary phase |
| *16. ydaF* | Fucose metabolism/DNA damage response (Fucose metabolism is disrupted by prophage) [68] | LB, no $O_2$, 37°C | Prophage protein (*ydaG)* | CYT | Prophage protein (YdaG) | *sieB* → Phage infection exclusion protein | ***ydaF*** ← | *ydaG* ← Prophage protein | Prophage protein | Unknown function | Prophage protein involved in disruption of fucose metabolism |
| *17. ydcD* | Phenylacetate catabolic process (prevalant under anaerobic conditions) [69] | LB, no $O_2$, 37°C | Cytoplasmic trehalase (*treF)* (osmotic stress) [70] | CYT | Putative peri-plasmic protein (YjbT) | *yncH* → | ***ydcD*** → | *yncO* ← growth-related protein expressed in rich medium) | dCTP de-aminase | Unknown function | Anaerobic respiration |
| *18. ydcH* | Phenyl acetate catabolic process (prevalant under anaerobic conditions) [71] | LB, no $O_2$, 37°C | Transcription (*ygeV)* | CYT | Putative structural protein (YaeH) | *opgD* → Glucans synhesis protein | ***ydcH*** → | *rimL* → Ribosomal transferase | Unknown function | Unknown function | Anaerobic respiration |
| *19. ydfA* | Not co-regulated | LB, no $O_2$, 37°C | Putative transcriptional regulator (*ygeV)* | CYT | Qin prophage (constitutively expressed, unknown function) (YdfB) | *dicA* → DNA binding transcr. regulator | ***ydfA*** → | *ydfB* → Consti-tutively expressed protein | Phage protein | Unknown function | Unclear |
| *20. ydfC* | Not co-regulated | M9, 37°C; 0.3 M NaCl, + 0.6% n-butanol (causes osmotic stress) [52] | Putative carbamate kinase (*ycbF)* (biofilm formation) [72] | CYT | Qin prophage (constitutively expressed, unknown function) (YdfB) | *ydfB* → Consti-tutively expressed protein | ***ydfC*** → | *dicB* → Cell division inhibition protein | Putative phage protein | Unknown function | Cellular stress response |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *21. ydfX* | Not co-regulated | LB, 37°C | Putative glycosyl-transferase (*yefI)* | CYT | Qin prophage (transcriptional regulator) (DicA) | *ydfW* ← Response to antibiotics | ***ydfX*** ← | *dicC* ← DNA-binding transcr. regulator | Bacterial toxin protein | Unknown function | Unclear |
| *22. ydgH* | Oxidoreduction (Respiratory complex I) (NADH: ubiquinone oxidoreductase, oxidative stress protection) [73] | LB, 37°C; 1ug/ml of Ciprofloxacin (FC=0.38, relative to *frr* gene) | Beta-glucosidase (*bglA)* (Bgl operon confers fitness advantage under stress) [74] | ECS | Dihydro-monapterin reductase (FolM) (role in oxidative stress) [75] | *pntA* ← Pyrimidine nucleotide hydro-genase – role in oxidative stress [76] | ***ydgH*** → | *ydgI* → Putative Arginine: ornithine antiporter | YdgH/BhsA/McbA-like domain-containing protein (involved in stress response/biofilm formation and patho-genesis) | Unknown function | Oxidative stress response |
| *23. ydiH* | Co-regulated (Unknown/ regulator or function) | M9, 37°C; 10% n-hexane (oxidative stress) [77] | Cyclopropane fatty acyl phospholipid synthase (*cfa)* | CYT | Putative ferrodoxin-like lipoprotein (YdjY) (oxidative stress response) [78] | *menI* ← Mena-quinone bio-synthesis | ***ydiH*** ← | *ydiK* → Putative trans-porter | Unknown function | Unknown function | Oxidative stress response |
| *24. ydjO* | Response to acidic pH (upregulation of oxidative stress response genes) [79] | MOPS, 37°C; 0.4% Glycerol + 0.5% 2-Deoxy-D-glucose | Cysteine/ selenocysteine cation transporter (*tcyP)* [80] | CYT | Cell division modulator (CedA) (induced in oxidative stress) | *tcyP* → Cation transporter | ***ydjO*** ← | *cedA* ← Cell division modulator | Unknown function | Unknown function | Oxidoreduction under acidic pH conditions |
| *25. yeaC* | Aerobic respiration | LB, 37°C | Methionine sulphoxide reductase (*msrB)* (oxidative stress) response [81] | CYT | Methionine sulfoxide reductase B (MsrB) | *ydjL* ← Putative Zn-binding dehydro-genase (oxidative stress response) (UniProt) | ***yeaC*** ← | *msrB* ← Meth-ionine sulphoxide reductase | Transcriptional regulator | Unknown function | Oxidative stress response during aerobic conditions |

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| *26. yebO* | Cellular response to Magnesium ion (during aerobic respiration) [82] | M9, 37°C; double mutant for genes involved in aerobic respiration *(Δndh ΔcyoB)* [83] | PhoQ kinase inhibitor (*mgrB)* | PM | PhoP/PhoQ signaling protein (MgrB) (induced by low $Mg2^{+}$) [84] | *yobF* ← General stress response protein | ***yebO*** ← | *mgrB* ← Kinase inhibitor | Unknown function | Unknown function | Response to magnesium ions during aerobic respiration. |
| *27. yebW* | Not co-regulated | LB, 37°C | Phospho-protein phosphatase (*pphA)* | CYT | Lipopolysaccharide biosynthesis protein (WbbK) | *yebV* → Protein binding | ***yebW*** ← | *pphA* → Phospho-protein phos-phatase | Lipoprotein | DUF1482 family protein | Unclear |
| *28. yecH* | Anaerobic respiration | M9, 37°C; triple mutant of genes involved in aerobic respiration *(ΔndH, ΔcydB, ΔappC)* [83] | DNA gyrase inhibitor (*sbmC)* (involved in SOS response) [85] | CYT | Putative nucleoside tri-phosphatase (SgcQ) | *ftnA* → Ferritin iron storage protein (oxidative stress response) [86] | ***yecH*** ← | *tyrP* → Tyrosine symporter | Metal-binding protein | YecH family protein | Switching from aerobic to anaerobic respiration under stress |
| *29. yecT* | Not co-regulated | M9, 37°C; 0.3 M NaCl + 0.6% n-butanol (causes osmotic stress) [52] | Multidrug/cho-line/betaine efflux transporter (*emrE)* (osmotic stress response) [87] | CYT | Putative DNA-binding transcriptional regulator (YhfZ) | *yecV* ← Expo-nential phas protein (in rich medium) | ***yecT*** → | *flhE* ← Flagellar protein | Lysozyme inhibitor | LprI domain-containing protein (lipo-protein) | Osmotic stress response |
| *30. yegJ* | Cellulose biosynthetic process | LB, no $O_2$, 37°C | Reactive chlorine species responsive protein (*rclC)* | ECS | Putative non-specific DNA-binding protein (YegI) | *yegI* ← Ser/Thr Phos-phatase | ***yegJ*** → | *pphC* ← Ser/Thr Phosp-hatase | Unknown function | Unknown function | Unclear |
| *31. yegL* | Not co-regulated | LB, no $O_2$, 37°C | Zinc-finger domain protein (*yehQ)* | CYT | Ser/thr phosphatase realted protein (YegK) | *pphC* ← Ser/Thr phos-phatase | ***yegL*** ← | *yegR* → | Homeostasis protein | Mediator of transcription | Unclear |
| *32. yegR* | Phosphorelay signal transduction system / Oxalate catabolic | M9, 37°C; double mutant for genes involved in aerobic respiration *(Δndh ΔcyoB)* [83] | Putative oxido-reductase (*ydeP)* | ECS | Orphan protein (OrgK) | *ogrK* ← Trans-cription regulation | ***yegR*** ← | *yegS* → Lipid kinase | Lipoprotein | Unknown function | Oxidoreduction |

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| | process (involved in acid stress resistance) [88] | | | | | | | | |
| *33. yehI* | Not co-regulated | LB, no $O_2$, 37°C | ATP-binding lipopolysaccharide transport protein (*msbA)* | CYT | Putative ATPase (YehL) | *yehF* → Molybdate transport regulator; ***yehI*** →; *yehK* → | Putative regulator | DUF4132 domain-containing protein | Transport protein |
| *34. yehK* | Not co-regulated | MOPS, 37°C; 0.4% Glycerol + 0.5% 2-Deoxy-D-glucose | Putative fimbrial adhesin protein (*sfmH)* | CYT | Putative ATPase (YehL) | *yehI* →; ***yehk*** →; *orzP* → Small RNA | Unknown function | Unknown function | Unclear |
| *35. yeiS* | Not co-regulated | LB, 37°C | Vanomycin responsive protein (*sanA)* | CYT | Cell membrane barrier (SanA) | *sanA* → Response to Vanomycin; ***yeiS*** →; *preT* → dehydro-genase | Vancomycin high temperature exclusion protein | Unknown function | Maintaining cell membrane integrity against drugs (e.g. Vanomycin) |
| *36. yfbM* | Aerobic respiration | M9, no $O_2$, 37°C | Hemolysin E (*hlyE)* (pore forming protein) [89] | CYT | Unknown function (YfbN) | *yfbL* → Putative peptidase (proteo-lysis) (EcoCyc); ***yfbM*** →; *yfbN* ← | Unknown function | DUF1877 family protein | Protein lysis |
| *37. yfbN* | Response to acidic pH | MOPS, 37°C; 0.4% Glycerol + 0.5% 2-Deoxy-D-glucose | DNA binding transcriptioal regulator (*ydeO)* | CYT | Unknown function, YfbO | *yfbM* →; ***yfbN*** ←; *yfbO* → | Unknown function | Unknown function | Unclear |
| *38. yfbO* | Not co-regulated | MOPS, 37°C; 0.4% Glycerol + 0.5% 2-Deoxy-D-glucose | Function unknown (*yfbP)* | ECS | Unknown function (YfbP) | *yfbN* ←; ***yfbO*** →; *yfbP* → | Immunity protein (cyto-toxicity) | Unknown function | Unclear |
| *39. yfbP* | Not co-regulated | M9, 37°C; 0.3 M NaCl + 0.6% n-butanol | Inner membrane protein (*ynjI)* | CYT | Putative amino-peptidase (YfbL) (Proteolysis) (EcoCyc) | *yfbO* ←; ***yfbP*** →; *nuoN* ← Oxido-reductase | Protein folding and transport | Unknown function | Protein quality control under stress |
| *40. yfdN* | Not co-regulated | LB, no $O_2$, 37°C | Oxidative stress response protein (*yfdO)* | CYT | Prophage protein (phage-related function) (YfdT) | *yfdM* ← Putative methyl-transferase; ***yfdN*** ←; *yfdO* ← Oxidative stress response | Transcriptional activator family protein | Unknown function | Oxidative stress response |

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| *41. yfdP* | Not co-regulated | LB, no $O_2$, 37°C | Putative auto-transporter adhesin (*ycgV)* | CYT | Prophage protein (response to oxidative stress) (YfdS) | *yfdO* ← Oxidative stress response | ***yfdP*** → | *yfdQ* → Phage-related function | Prophage protein | Unknown function | Oxidative stress response |
| *42. yfeS* | Cellular response to envelope stress | MOPS, 37°C | Putative lipoprotein (*yidX)* | CYT | Putative efflux protein (YdhK) | *yfeK* → Transcr. regulation | ***yfeS*** → | *cysM* ← Cysteine synthase | Molybdate metabolism protein | Unknown function | Envelope stress response |
| *43. yffL* | Response to Nitrate/ Nitrite (anaerobic respiration) | M9, 37°C | Anaerobic glycerol-3-phosphate dehydro-genase *(glpB)* [89] | CYT | Prophage protein (Integrase) (IntZ) | *intZ* → Putative integrase | ***yffL*** → | *yffM* → | Prophage protein | CPZ-55 prophage (involved in general stress response) [90] | Anaerobic respiration |
| *44. yffM* | Not co-regulated | LB, no $O_2$, 37°C | Alpha-mannosidase (*mngB)* | CYT | Prophage (unknown function) (YffN) | *yffL* → | ***yffM*** → | *yffN* → | Unknown function | Unknown function | Unclear |
| *45. yffN* | Not co-regulated | MOPS, 37°C; 0.4% Glycerol + 0.5% 2-Deoxy-D-glucose | Tail fiber protein (*stfR)* | CYT | Prophage (unknown function) (YffM) | *yffM* → | ***yffN*** → | *yffO* → | Unknown function | Unknown function | Unclear |
| *46. yffP* | Not co-regulated | M9, 37°C; 0.3 M NaCl + 0.6% n-butanol | Function unknown (*yffO)* | CYT | Prophage (unknown function) (YffO) | *yffO* → | ***yffP*** → | *yffQ* → | Unknown function | Unknown function | Unclear |
| *47. yffQ* | Not co-regulated | MOPS, 37°C; 0.4% Glycerol | CRISPR-associated endo-ribonuclease (*cas2)* (defense response to virus) (EcoCyc) | CYT | Prophage (unknown function) (YffP) | *yffP* → | ***yffQ*** → | *yffR* ← | Unknown function | CPZ-55 prophage (involved in general stress response) [90] | Stress response |
| *48. yffR* | Not co-regulated | M9, 37°C; triple mutant of genes involved in aerobic respiration *(ΔndH, ΔcydB, ΔappC)* [83] | Bacterial adhesion/ biofilm formation (*yhjY*) | CYT | Prophage (unknown function) (YffQ) | *yffQ* → | ***yffR*** → | *yffS* → DNA bindin | Unknown function | Unknown function | Stress response |

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| *49. yfiM* | Cell death | LB, 37°C | Alpha-ketoglutarate: $H^+$ symporter (*kgtP) (*efflux transporter for sugar-phosphate stress) [91] | ECS | Phospholipid biosynthesis protein (PssA) | *pssA* → Phos-phatidyl-serine synthase | ***yfiM*** → | *kgtP* ← $H^+$ symporter | Lipoprotein | Unknown function | Lipoprotein with a role in mitigating the effects of cellular stress |
| *50. yfjH* | Not co-regulated | MOPS, 37°C; 0.4% Glycerol + 0.5% 2-Deoxy-D-glucose | Growth related protei (*htrL)* | CYT | Prophage (transcriptional activation) (AlpA) | *intA* → Integrase protein | ***yfjH*** ← | *alpA* → Transcr. regulator | Unknown function | Unknown function | Unclear |
| *51. yfjJ* | Response to acidic pH (upregulation of genes involved in cell adhesion) [92] | MOPS, 37°C; 0.4% Glycerol and 0.5% 2-Deoxy-D-glucose | Putative fimbrial usher protein (*htrE)* – cell adhesion or pilus assembly protein [92] | CYT | Prophage (cytosolic protein) (YfjI) | *yfjI* → Prophage protein | ***yfjJ*** → | *abpB* ← Putative helicase | Inovirus-type Gp2 protein (viral DNA replication) | Unknown function | Cell adhesion |
| *52. ygdT* | Not co-regulated | LB, no $O_2$, 37°C | Putative lipoprotein (*yidX)* | CYT | Not found | *rppH* ← RNA phospho-hydrolase | ***ygdT*** ← | *mutH* → DNA repair protein | Transposase protein | Unknown function | Unclear |
| *53. yhaL* | Not co-regulated | LB, 37°C; *Δhns* (stress adaptation) | DNA binding transcriptional activator (*fucR)* | CYT | Ser/thr phosphatase-related protein (Yegk) | *yhaK* → bicupin related protein (acid stress/oxidative stress response) [93] | ***yhaL*** → | *cyuA* ← L-cysteine di-sulphidase | Cytoplasmic protein | Unknown function | Stress response |

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| *54. yhdN* | Response to heat | LB, 30°C (stationary phase) | DNA binding transcriptional activator (*zntR)* (stress responsive transcription factor) [94] | CYT | Transcriptional activator (ZntR) | *zntR* ← DNA binding transcr. regulator | ***yhdN*** ← | *rplQ* ← 50S ribosome subunit protein (protein bio-genesis) [95] | Conserved protein | DUF1992 domain-containing protein (conserved protein belonging to the DNAJ homolog family, heat shock response) (UniProt) | Regulation of heat shock response, expressed during stationary phase |
| *55. yhfU* | Not co-regulated | M9, 37°C; 0.3 M NaCl + 0.6% n-butanol (osmotic stress) [53] | Aromatic carboxylic acid efflux pump (*aaeA)* (involved in defense mechanisms) [96] | CYT | Putative transport protein (YhfT) | *yhfT* ← Membrane protein | ***yhfU*** ← | *php* ← Putative hydrolase | Unknown function | Unknown function | Osmotic stress response |
| *56. yhhL* | Not co-regulated | LB, 37°C; *ΔdsrA* (gene involved in stress response, biofilm formation) [97] | 16S rRNA methyl-transferase (*rsmD)* | CYT | 16S rRNA methyl-Transferase (RsmD) | *rsmD* → 16S rRNA methyl transferase | ***yhhL*** → xs | *yhhM* ← Quorum sensing protein – (stress response, biofilm formation) [98] | rRNA small subunit methyl-transferase protein | Unknown function | Biofilm formation |
| *57. yibI* | Response to nitrosative stress (redox stress) [99] | M9, 37°C; 0.3 M NaCl + 0.6% n-butanol (osmotic stress) [52] | Inner membrane protein (*yibH)* (stress response) [100] | PM | Inner membrane protein (YibH) | *yibH* ← Inner membrane protein | ***yibI*** ← | *mtlA* → mannitol-specific PTS enzyme | Inner membrane protein | Unknown function | Stress response protein. |
| *58. yicN* | Not co-regulated | LB, 37°C; *Δhns* (stress adaptation) [101] | DNA binding transcriptional regulator (*frlR)* | CYT | Putative Transporter (NepI) | *nepI* ← Purine ribo-nucleoside exporter | ***yicN*** ← | *adeQ* ← Adenine trans-porter | Unknown function | Unknown function | Nucleotide transport |

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| *59. yidB* | Not co-regulated | LB, 37°C; triple mutant of genes involved in respiration *(ΔndH, ΔcydB, ΔappC)* [83] | Bitopic inner membrane protein (*ypfJ)* | CYT | Sugar phosphate phosphatas (YidA) (phosphate metabolism) [102] | *yidA* ← Sugar phos-phatase | ***yidB*** ← | *gyrB* ← DNA gyrase | Radical SAM domain-containing protein | Unknown function | Unclear |
| *60. yigA* | Not co-regulated | LB, 37°C | Diamino-pimelate epimerase (*dapF)* (cell wall biosynthesis) [103] | CYT | Diamino-pimelate epimerase (DapF) (cell wall structure) [104] | *dapF* → diamino-pimelate epimerase | ***yigA*** → | *xerC* → Tyrosine recombi-nase | Diamino-pimelate epimerase protein | Not found | Cell wall biosynthesis |
| *61. yigE* | Not coregulated | LB, no $O_2$, 37°C | Putative magnesium chelatase (*yifB)* (chaperonic activity) [105] | CYT | Inner membrane protein (YedI) | *uvrD* → DNA helicase | ***yigE*** ← | *ysgD* → Consti-tutively expressed protein (in rich medium) | Phospho-diester glycosidase family protein | Anaerobic nucleoside-tri-phosphate reductase-activating protein (DNA synthesis and repair) [106] | Stress response during anaerobic conditions |
| *62. yjbD* | Not coregulated | M9, 37°C; 10% n-hexane | Putative methyl-transferase (*yafS)* | CYT | Cysteine cluster protein (YcgN) | *rluF* → Pseudo-uridine synthase | ***yjbD*** ← | *lysC* ← Aspartate kinase | Unknown function | Unknown function | Unclear |
| *63. yjbL* | Response to acidic pH | LB, no $O_2$, 37°C | Cell envelope stress response protein (*yqcG)* | CYT | Uncharacterized protein (YjbM) | *zur* ← Transcr. regulator | ***yjbL*** → | *yjbM* → Cell-envelope related protein | Unknown function | Unknown function | Envelope stress response under stress |
| *64. yjbS* | Response to acidic pH (upregulation of oxidative stress response genes) [107] | M9, 37°C; 10µg/ml Mitomycin-C (genotoxic agent, ROS producing) [108] | Pathogenicity-related (*yqeK)* | CYT | Putative oxido-Reductase (YqiJ) ([109] | *tyrB* → Tyrosine amino-transferase | ***yjbS*** ← | *aphA* → Acid phos-phatase | Unknown function | Unknown function | Oxidative stress response. |
| *65. yjcB* | Carbohydrate transport | M9, no phosphate, 37°C; *ΔphoB* (phosphate transport gene) | Capsule synthesis regulator (*rcsB)* | CYT | Putative peri-plasmic protein (YpeC) | *ssb* → ss-DNA binding protein | ***yjcB*** ← | *pdeC* → Cyclic di-GMP Phospho-diesterase | Membrane protein | Not found | Unclear |

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| *66. yjcF* | Response to acidic pH | LB, 37°C | Pathogenicity-related (*ygeH)* | CYT | Ribosomal silencing factor (RsfS) | *yjcE* → Putative transporter | ***yjcF*** ← | *actP* ← Cation trans-porter | Unknown function | Unknown function | Unclear |
| *67. yjdI* | Oxidoreduction (Respiratory complex I) | LB, 37°C; triple mutant of genes involved in respiration *(ΔndH, ΔcydB, ΔappC)* | Putative N-acetyl-Transferase (*yjdJ)* | CYT | Putative N-acetyl-Transferase (YjdJ) | *dcuS* ← Sensor histidine kinase | ***yjdI*** → | *yjdJ* → Putative acetyl-trans-ferase | Unknown function | Fer4_19 domain-containing protein (involved in iron-sulphur binding) | Aerobic respiration |
| *68. yjeJ* | Cell adhesion | LB, 37°C; HS, 50°C for 7' | 6-phospho-gluconate phosphatase (*yieH)* | CYT | Lysylation protein (EpmB) | *yejI* → Lipoprotein (heat shock response) [110] | ***yjeJ*** ← | *epmB* ← Lysine amino-mutase | Unknown function | Unknown function | Cell adhesion under heat shock response |
| *69. yjeT* | Not coregulated | LB, 37°C; HS, 50°C for 15' | Lipo-polysaccharid e core heptose kinase *(waaP)* | CYT | HflB protease modulator (HflC) (in heat shock response) [111] | *hflC* → ftsH protease regulator (heat shock response) [111] | ***yjeT*** → | *purA* → Adenylo-suucinate syn-thetase | Unknown function | Unknown function | Heat shock response |
| *70. yjfI* | Coregulated (unknown regulator or function) | LB, 37°C, reaction buffer (with Lysine) | Inner membrane protein (*yjgN)* | CYT | Membrane chaperone family protein (YjfJ) [112] | *rlmB* → Methyl-transferase | ***yjfI*** → | *yjfJ* → Transcr. regulator | Cytoplasmic protein | Unknown function | Unclear |
| *71. yjfK* | Not coregulated | LB, no $O_2$, 37°C | Membrane chaperone family protein (*yjfJ)* | CYT | Inner membrane protein (YjfL) | *yjfJ* → Transcr. regulator | ***yjfK*** → | *yjfL* → Inner membrane protein | Unknown function | Unknown function | Unclear |
| *72. yjfZ* | Not coregulated | LB, 37°C | Acyl transferase (*yihG)* | CYT | Putative inner membrane poly-saccharide flippase (YghQ) | *rpiI* → Ribosomal subunit protein | ***yjfZ*** ← | *ytfB* ← Cell division | Unknown function | Unknown function | Unclear |

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| *73. yjgR* | Not coregulated | LB, no $O_2$, 37°C | NADPH dependent aldehyde reductase (*ahR)* | PM | L-idonate and D-gluconate transporter (IdnT) | *iptG* → LPS Transport system | ***yjgR*** ← | *idnR* ← DNA binding transcr. regulator | Ornithine amino-transferase | Conjugal transfer protein | Transport |
| *74. yjgZ* | Coregulated (unknown regulator or function) | M9, 37°C; 0.3 M NaCl + 0.6% n-butanol | Putative multidrug efflux pump (*mdtO)* | CYT | Putative receptor protein (YhhM) | *insD6* → Trans-posase | ***yjgZ*** → | *insG* ← Putative transposase | Transposase | Unknown function | Unclear |
| *75. yjjY* | Cell death | LB, 37°C | DNA binding transcriptional regulator (*arcA)* | CYT | Uncharacterized protein YniD | *arcA* ← DNA binding transcr. regulator | ***yjjY*** → | *ytjE* → Growth related protein | Unknown function | Unknown function | Unclear |
| *76. ykgL* | Glycine betaine biosynthesis from choline [49] | M9, 37°C; *ΔkdpE*; Tris-maleic acid+115 mM KCl (disrupts potassium uptake under osmotic stress) [113] | 50S ribosomal subunit protein (*ykgM)* | CYT | Carnitinyl-CoA dehydratase (CaiD) – osmoprotectant protein [114] | *matA* ← DNA binding transcr. regulator | ***ykgL*** → | *ykgO* ← 50S ribosomal subunit protein | Unknown function | Unknown function | Osmotic stress response. |
| *77. ylcH* | Trans-criptional attenuation | M9, 37°C; 0.3 M NaCl + 0.6% n-butanol | Ionic detergents response protein (*yfbK)* | No inf. | Uncharacterized protein (YlcG) | *ybcM* → DNA binding transcr. regulator | ***ylcH*** → | *ybcN* → DNA base-flipping protein | Unknown function | Unknown function | Unclear |
| *78. ylcI* | Aerobic respiration | LB, 37°C; double mutant of genes involved in respiration *(ΔndH, ΔcyoB)* | Phage related protein (*ybcW)* | CYT | Oxidoreductase (NuoM) – involved in aerobic respiration [115] | *ybcW* → Prophage protein | ***ylcI*** ← | *nohD* → DNA packing protein | Unknown function | DUF3950 domain-containing protein | Aerobic respiration |
| *79. ymfI* | DNA restriction/ modification system | LB, 37°C; 1 ug/mL of Ciprofloxacin+ 500 µM of ET2DA | Phage related protein (*ymfJ)* | CYT | Uncharacterized protein (*ymfJ)* | *ymfH* ← Prophage protein | ***ymfI*** → | *ymfJ* ← Prophage protein | Phage protein | Unknown function | Unclear |
| *80. ymgI* | Not co-regulated | M9, no phosphate, 37°C; *ΔphoB* (phosphate transport gene) | Heat shock responsive protein (ymgG) | CYT | Cell division topological specificity protein (MinE) | *ymgG* ← Heat shock response protein | ***ymgI*** ← | *ymgJ* → | Unknown function | Unknown function | Unclear |

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| *81. ymgJ* | Not co-regulated | M9, 37°C; 0.3 M NaCl + 0.6% n-butanol | Putative fimbrial usher protein (*htrE)* (heat shock response) [116] | CYT | Heat shock response protein (YmgG) | *ymgI* ← | ***ymgJ*** → | *minE* ← Cell division | Transposase protein | Unknown function | Heat shock response |
| *82. ynaJ* | Aerobic respiration | LB, 37°C | ADP-sugar diphosphatase (*nudE)* | CYT | Translation-realted protein (YciU) | *insH4* → Trans-posase | ***ynaJ*** → | *uspE* ← Universal stress protein | Inner membrane protein | Unknown function | Unclear |
| *83. yncH* | Response to acidic pH | LB, 37°C; *rpoD* overexpression (response to heat) [117] | Cell envelope stress response protein (yqcG) | CYT | Glutathione S-transferase homolog (YncG) | *yncG* → Putative Glutata-thione-S transferase | ***yncH*** → | *yncO* ← Consti-tutively expressed protein (in rich medium) | Unknown function | Unknown function | Stress response |
| *84. yneG* | Not co-regulated | M9, no ammonium, 37°C | Glutaminase (*glsB)* Ammonium assimilation [118] | CYT | Putative Glutaminase (GlsB) (amino acid metabolism) [119] | *dgcF* ← Putative Di-guanylate cyclase | ***yneG*** ← | *glsB* ← Glutaminase | Cytoplasmic protein | Unknown function | Nitrogen metabolism during ammonium limitation |
| *85. yneK* | Not co-regulated | MOPS, 37°C; 0.4% Glycerol + 0.5% 2-Deoxy-D-glucose (metabolic stress) [120] | Putative fimbrial usher protein (*htrE)* (heat shock response) [116] | CYT | Putative oxido-reductase (YdhF) | *ptrR* → L-glutamate meta-bolism | ***yneK*** → | *ydeA* → L-arabinose exporter | Unknown function | Unknown function | Stress response |
| *86. ynfO* | Anaerobic respiration | LB, 37°C; 100 µg/ml Ampicillin | Function unknown (*ylcI)* | CYT | Putative (D)-galactarate transporter (GarP) | *nohA* ← Prophage protein | ***ynfO*** → | *ydfO* → Prophage protein | Unknown function | DUF3950 domain-containing protein | Unclear |
| *87. yoaC* | Not co-regulated | LB, 37°C | Lipoprotein (*yfiL)* | CYT | Protein export (MsyB) | *yoaB* → Biofilm based activity | ***yoaC*** → | *yoaH* ← | Unknown function | Unknown function | Unclear |

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| *88. yoaH* | Not co-regulated | LB, 37°C; *Δhns* (stress adaptation) | DNA-binding transcriptional repressor *(acrR)* | CYT | DUF1889 family protein (YoaC) | *yoaC* → | ***yoaH*** ← | *pabB* → Amino-deoxy-choris-mate synthase | Unknown function | Unknown function | Unclear |
| *89. yodC* | Oxidoreduction (Respiratory complex I) | LB, 37°C | Putative adenosine mono-phosphate *(fic)* | CYT | Ribosome modulation protein (RmF) (response to oxidative stress) [121] | *dgcQ* ← Di-guanylate cyclase | ***yodC*** ← | *yedI* ← Inner mem-brane protein | Cytoplasmic protein | Unknown function | Oxidoreduction |
| *90. ypaB* | Not coregulated | M9, 37°C; 0.3 M NaCl +0.6% n-butanol | Putative fimbrial chaperone *(yadV)* | CYT | Not found | *yfaL* ← Putative auto-transporter adhesin | ***ypaB*** ← | *nrdA* → Ribo-nucleoside reductase | Transposase | Unknown function | Unclear |
| *91. ypeB* | Ribosome structure | M9, 37°C; 50 mM isoprenol | Tetraacyl-disaccharide 4'-kinase *(lpxK)* | CYT | DNA ligase (LigA) | *yfeH* → Putative $Na^+$ symporter | ***ypeB*** ← | *ligA* ← DNA ligase | Cytoplasmic protein | Cyto-plasmic protein | Unclear |
| *92. ypjB* | Not coregulated | LB, no $O_2$, 37°C | Putative type III secreted effector *(yqeK)* (bacterial patho-genecity) [122] | CYT | Not found | *ypjA* ← auto-transporter | ***ypjB*** ← | *ypjC* ← Patho-genicity | Uncharacteriz ed protein | Unknown function | Pathogenecity |
| *93. yrdB* | Oxidoreduction (Respiratory complex I) | LB, 37°C | Shikimate dehydro-genase *(aroE)* | CYT | Dehydro-shikimate reductase (AroE) - oxidative stress response) [123] | *yrdA* → carbonic anhydrase | ***yrdB*** ← | *aroE* ← shikimate anhydrase | DUF1488 domain-containing protein | Unknown function | Oxidoreduction |
| *94. yrhD* | Response to acidic pH | LB, no $O_2$, 37°C | Putative type III secreted effector *(yqeK)* (bacterial patho-genecity) [122] | CYT | Not found | IS1E → Insertion sequence | ***yrhD*** → | *yrhB* → Putative HS chaperone | Unknown function | Unknown function | Unclear |
| *95. ytiA* | Not co-regulated | LB, no $O_2$, 37°C | Putative transporter *(yjiJ)* | CYT | Not found | *yjiK* ← Membrane | ***ytiA*** ← | *yjiL* ← Putative de- | DUF3343 domain-containing | Unknown function | Unclear |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | protein | hydratase | protein | |

Abbreviations: ATP, Adenosine triphosphate; dCTP, Deoxycytidine triphosphate; ET2DA, Ethyl trans-2-decenoic acid; HS, Heat shock; KCl, Potassium chloride; LB, Luria Bertani; LPS, Lipopolysaccharide; M9, Minimal medium; MOPS, 3-(N-morpholino) propanesulfonic acid; NADP, Nicotinamide dinucleotide phosphate; PTS, phosphotransferase system; ROS, Reactive oxygen species; SAM, S-adenosylmethionine; Ser/Thr, Serine/Threonine; Transcr. , Transcription

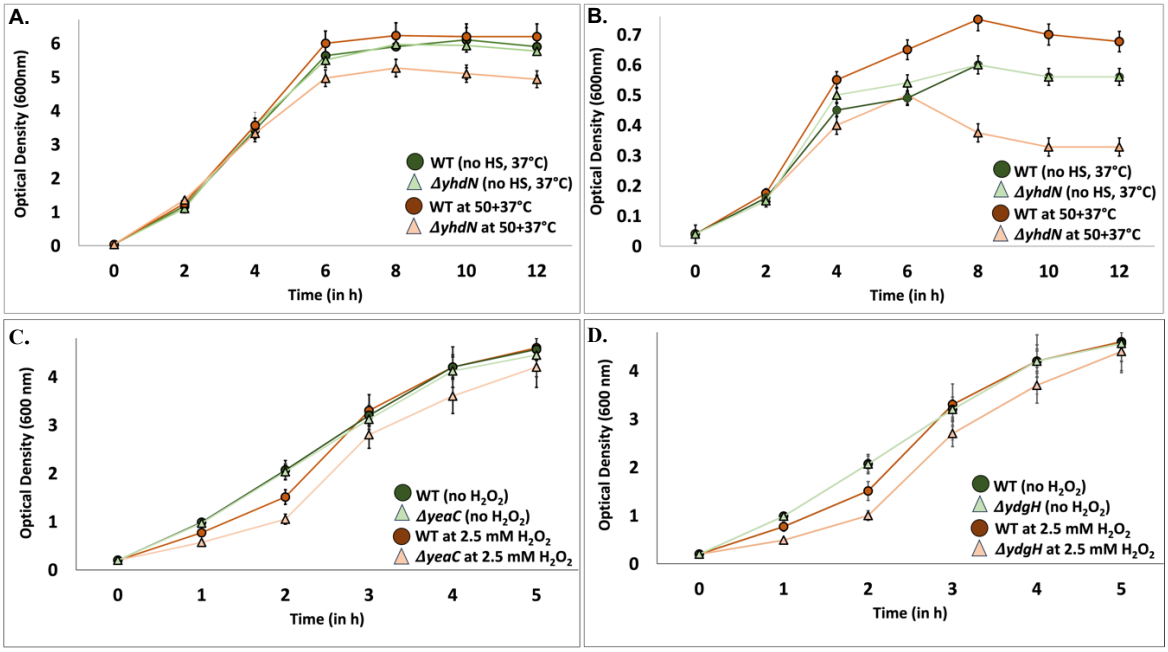
252 Genes with a higher confidence in the inference section (green) were analysed for candidates
253 to be used for *in vitro* testing. Three HP-encoding genes, namely *yhdN*, *yeaC*, and *ydgH*, were
254 selected for further analysis (highlighted in grey) (**Table 1).**
255
256 **Protein encoding gene *yhdN***
257 Protein YhdN, characterized by the presence of a domain of unknown function (DUF1992),
258 was predicted to be involved in heat shock response. The gene encoding *yhdN* is regulated
259 by the RNA polymerase sigma factor RpoH (σ32), which serves as the primary heat shock
260 transcriptional regulator. Under normal conditions, RpoH levels are kept low due to chaperone-
261 mediated degradation; however, these levels increase significantly upon heat shock [124].
262 Gene Ontology (GO) annotation for this cluster is categorized under 'response to heat'. Based
263 on publicly available transcriptomic data [43], the *yhdN* gene exhibited significant expression
264 in cells grown to the stationary phase, with a $Log_2FC$ of 5.5 relative to the reference gene *frr*,
265 signifying a 45 times higher expression of *yhdN* gene. This observation suggests that
266 the *yhdN* gene is highly expressed during the stationary phase and therefore might have a
267 role in nutrient stress response. Additionally, *yhdN* expression was upregulated following
268 exposure to a transient heat shock at 50°C for 15 minutes, with a $Log_2FC$ of 4.8 and 27 times
269 higher expression, suggesting that the gene might also play a role in heat shock response.
270 Co-expression analysis using Spearman correlation of the gene resulted in a high correlation
271 with the gene *zntR* (0.93) encoding an HTH (helix-turn-helix)-type transcriptional regulator
272 [125] which is involved in protein stability and prevention of aggregates [126,127]. Genes *yhdN*
273 and *zntR* are also part of the same transcription unit (EcoCyc database). Other genes with
274 high correlations to *yhdN* were *relB* (0.84)*, pspB* (0.84) *and pspC* (0.84), all of which are
275 involved in stress response [128] (*Supplementary Figure 3A.*). Gene *relB* encodes for an
276 antitoxin protein involved in regulation of growth [129] while genes *pspB* and *pspC* encode for
277 phage shock proteins that are known to be induced by infection with bacteriophages or
278 ethanol, osmotic and/or nutrient stress as well as heat shock conditions [130]. Protein-protein
279 interaction (PPI) analysis showed highest interaction confidence with ZntR. The local genomic

280 context analysis showed genes *zntR* and *rplQ* (a ribosomal subunit protein) as neighbouring
281 genes. Protein YhdN showed remote sequence homology to a protein cluster comprising of
282 uncharacterized conserved proteins and was structurally homologous to conserved proteins
283 with similarity to J-domains of chaperones, regulating the activity of heat-shock proteins [131].
284 To elucidate the potential function of *yhdN*, we hypothesized that its absence would affect
285 bacterial growth under heat shock conditions and possibly the cells stationary phase under
286 nutrient limitation. This hypothesis was tested by comparing the growth patterns of the
287 *E. coli* K-12 substrain BW25113 wild type and its isogenic *ΔyhdN* knockout mutant under a
288 transient heat shock condition (50°C) and recovery at 37°C in LB and nutrient-limited M9
289 medium.
290
291 **Protein encoding gene *yeaC***
292 Protein YeaC is characterized by the presence of a DUF1315 domain. Gene *yeaC* is predicted
293 to be governed by the regulator ArcA, which regulates a group of proteins involved in redox
294 homeostasis and aerobic respiration [132]. The Gene Ontology (GO) annotation for this cluster
295 was predicted as 'aerobic respiration'. Analysis of gene *yeaC* in publicly available
296 transcriptomic datasets showed slightly higher expression levels against gene *frr* in LB
297 medium at 37°C under normal $O_2$ conditions ($log_2$ FC 1.9) (**Table1**) and in knock-out mutants
298 for genes encoding type II NADH:quinone oxidoreductase (*ndh*), Cytochrome bd-I ubiquinol
299 oxidase (*cydB*) and Cytochrome bd-II ubiquinol oxidase (*appC*), involved in the Tricarboxylic
300 acid cycle or oxidative phosphorylation [133] ($log_2$ FC 1.7). Based on the co-expression
301 analysis of public data, the highest correlation was observed with a gene encoding for a
302 peptide methionine sulfoxide reductase (*msrB),* which is an oxidoreductase and known to be
303 highly expressed during oxidative stress to maintain the cell-redox homeostasis [134]
304 (*Supplementary Figure3B.*). Other highly correlated genes involved succinate dehydrogenase
305 cytochrome b556 subunit (*sdhC)*, succinate dehydrogenase hydrophobic membrane anchor
306 subunit (*sdhD)* and Fumarate hydratase class I, aerobic (*fumA),* which are all involved in the
307 tricarboxylic acid or TCA cycle [135,136] (*Supplementary Figure 3B.*). PPI analysis also

308 showed highest interaction confidence with MsrB. Additionally, it neighbours the *msrB* gene
309 and a putative zinc-binding dehydrogenase, *ydjL* which is also involved in oxidoreduction
310 (UniProt) (**Table 1**). Protein YeaC shows remote sequence homology to other proteins
311 involved in transcriptional regulation. Structural homology revealed similarities to a group of
312 uncharacterized proteins with unknown functions. We hypothesized that *yeaC* affects the cell-
313 redox homeostasis and cellular growth under oxidative stress. This hypothesis was tested by
314 comparing the growth patterns of the *E. coli* K-12 substrain BW25113 wild type and its isogenic
315 *ΔyeaC* knockout mutant with a sub-lethal concentration of $H_2O_2$ (2.5 mM) [137].
316
317 **Protein encoding gene *ydgH***
318 Protein YdgH has a DUF1471 domain of unknown function. It is predicted to be part of an
319 unknown cluster, with no information on its regulator. The GO annotation for this cluster,
320 however, is categorized under oxidoreduction (complex I). The energy-converting
321 NADH:ubiquinone oxidoreductase respiratory complex I, is the main entry point for electrons
322 from NADH into the respiratory chains in bacteria [138]. Based on the public datasets, we
323 could not obtain a gene-specific condition where gene *ydgH* had higher expression compared
324 to gene *frr.* Co-expression analysis of *ydgH* from the publicly available RNAseq datasets
325 revealed highest correlation with a 6-phospho-beta-glucosidase (*bglA*) encoding hydrolase,
326 involved in the hydrolysis of phosphorylated beta-glucosides [139]. Other highly correlated
327 genes were phosphoglycolate phosphatase *(gph)* [140] and ribulose-phosphate 3-epimerase
328 *(rpe)* [141], involved in oxidative stress response (*Supplementary Figure 3C.*). PPI analysis
329 showed highest interaction confidence with YjfY*,* a putative HP, with a possible role in stress
330 resistance (UniProt). Genomic context provided information on a neighbouring upstream gene
331 involved in $NAD(P)^+$ transhydrogenase activity, annotated as NAD(P) transhydrogenase,
332 *pntA*, which is known to play a role in oxidative stress [142]. The protein showed remote
333 sequence homology to a YdgH/BhsA/McbA-like domain involved in stress response/biofilm
334 formation and pathogenesis [143]. The structural similarity was only to uncharacterized
335 proteins with unknown function. Following a similar hypothesis to that proposed for *yeaC*, we

336 evaluated the growth patterns of the wild type and its isogenic *ΔydgH* knockout mutant under
337 oxidative stress.
338
339 ***In vitro* analyses of candidate proteins**
340 Effect of heat shock on growth
341 To determine the effect of a transient heat shock at 50°C (7 minutes), the wild type and the
342 mutant ***ΔyhdN*** *E. coli* BW25113 strains were grown in LB medium. The *yhdN* mutant was seen
343 to have a growth defect of ~18% after 6h of growth based on the OD values obtained (**Figure**
344 **3A**). It was observed that after 6h of growth, the mutant could not resume normal growth
345 compared to the wild type. Additionally, the effects of a transient heat shock in Nitrogen-limited
346 M9 medium showed a significant growth defect of ~50% (**Figure 3B**). Growth curves for WT
347 and ***ΔyhdN*** in different temperatures can be found in the supplementary (*Supplementary*
348 *Figure 4A.*). Growth defects were also observed in the *yhdN* mutant compared to the wild type
349 when both the strains were grown continuously at 50°C. Since growth at 50°C is however
350 severely inhibited and cell started dying after 3h (*Supplementary Figure 4B.*), it was difficult to
351 say whether this was solely based on the absence of the protein YhdN in the mutant cells.
352
353 Effect of sublethal $H_2O_2$ concentration on growth
354 To determine the effect of sublethal $H_2O_2$ concentration, the wild type (WT), ***ΔydgH*** and ***ΔyeaC***
355 *E. coli* BW25113 strains were grown in LB medium until an OD of 0.2 and then exposed to an
356 $H_2O_2$ concentration of 2.5 mM, which is known to causes DNA damage and oxidative stress
357 [143] (**Figure 3C & D**). To study the responses due to oxidative stress, the absorbance values
358 ($OD_{600}$) after each hour over a 5h time period were measured. $H_2O_2$ was added to the cell
359 culture during the exponential phase of growth, which is characterized by rapid cell division
360 and metabolic activity and where the quorum-sensing signalling, i.e. process which allows the
361 bacteria to communicate and adjust gene expression according to cell density, is minimal.
362 Under non-stress conditions, the WT and mutant strains (*ΔydgH* and *ΔyeaC*) exhibited
363 comparable growth, with no significant differences observed. A growth lag was observed in

364 the mutants (*ΔydgH* and *ΔyeaC*) as compared to wild type when the medium was
365 supplemented with 2.5 mM $H_2O_2$. A growth defect of ~25% was observed in the mutant when
366 WT was compared to *ΔyeaC* at the 1h timepoint. This was reduced to ~7% after 5h of growth
367 underscoring the probability of the gene being expressed at this time point to counterfeit the
368 oxidative stress. Similarly, a growth defect of ~36% was observed in *ΔydgH* as compared to
369 WT at 1h, which decreased to ~2% after 5h, again probably due to stress adaptation and
370 expression of gene *ydgH*.
371




372
373
374 **Figure 3. Growth curves of *E.* coli K-12 BW25113 wild type (WT) and isogenic deletion mutants.**
375 **(A, B)** Effect of transient heat shock on WT and *ΔyhdN* cells. WT and the respective mutant strain were
376 exposed to a transient heat shock at 50°C for 7 minutes, (at $OD_{600}$ nm = 0.04) and grown for 12 h in **A.**
377 LB medium and **B.** in Nitrogen-limited M9 medium. **(C, D)** Bacterial growth of WT and respective
378 mutants at 37°C exposed to sub-lethal concentration of 2.5 mM $H_2O_2$, added during the exponential
379 phase ($OD_{600}$ = 0.2) and grown for 5h. **C.** WT and *ΔyeaC* and D. WT and *ΔydgH* cells. WT are indicated
380 by filled circles (●) and mutants by filled triangles (▲). Green lines represent controls, orange lines stress
381 conditions. Average of three independent readings taken for each specified condition. Error bars on the
382 graph indicate standard deviation from the mean.
383

384 **Differential gene expression analysis**

385 For a comparative analysis of differentially expressed genes (DEGs) and their functions in WT
386 *vs.* the mutant strains, all up- and down-regulated genes were analysed (*Figshare* > *DEGs* >
387 *Table1*). For positively regulated DEGs, representing genes upregulated in the WT strain, a

388 $Log_2$ fold change ($Log_2FC$) threshold of ≥1.5 was applied, corresponding to a minimum 2.8-
389 fold increase in expression levels. This cut-off ensured the inclusion of genes with significant
390 upregulation while minimizing noise [144]. Conversely, for negatively regulated DEGs,
391 corresponding to genes upregulated in the mutant strains, a Log2FC threshold of ≤-1.5 was
392 used.
393
394 WT *vs. ⊿yhdN*
395 Analysis of the WT strain at the 2h and 6h time points showed that *yhdN* was only significantly
396 expressed after 6h, hence this time point was chosen. In total, 484 DEGs ($p_{adj}$≤0.1) were
397 identified, (181) 213 genes with a positive $Log_2$ FC and 271 with a negative $Log_2FC$ of 1.5.
398 Within the positive DEGs, an analysis of the top 10 genes, resulted in one gene involved in
399 protein folding, five in stress response and four in motility/chemotaxis (**Figure 4A**).
400 Significantly upregulated genes in the WT strain were *tdcB* ($Log_2$ FC=7.37) and *pyrB* ($Log_2$
401 FC=6.14). *tdcB*, encoding for threonine dehydratase is known for being expressed during
402 nutrient deprivation (EcoCyc) and gene *pyrB* is involved in pyrimidine metabolism and
403 associated with motility under stress [145]. Gene *tnaA* ($Log_2$ FC=6.02), which encodes for
404 tryptophanase, has been recently studied for its role in protein folding and dissolution of protein
405 aggregates [146]. Motility and chemotaxis genes such as *fliL* ($Log_2$ FC=6.24)*, flgD* ($Log_2$
406 FC=5.99) are also known to be involved in response to environmental stresses [147].
407
408 In the deletion mutant (*⊿yhdN*), the top ten negative DEGs included two genes involved in
409 heat shock, six in some type of stress response and interestingly two other were
410 uncharacterized hypothetical proteins. *bolA* ($Log_2$ FC=-5.53)*,* a DNA binding transcriptional
411 regulator, plays a role in cellular stress response including heat stress [148] and *mgtS* ($Log_2$
412 FC=-5.01), a small inner membrane protein encoding gene, is known to be expressed upon
413 heat shock [149] (**Figure 4A**). Other upregulated genes in *⊿yhdN* involved *glgS* ($Log_2$ FC=-
414 5.34), encoding for a surface composition regulator for biofilms and *ymgA* ($Log_2$ FC=-4.44)
415 encoding a putative two-component system connector protein, known to play an important role

416 in biofilm formation (EcoCyc). Upregulation of these genes in the deletion mutant suggests a
417 compensatory mechanism to mitigate stress. Additionally, a HP-encoding gene, *yodC* was
418 also upregulated ($Log_2$ FC=-6.39) in *ΔyhdN*. Interestingly, this gene was predicted to play a
419 role in oxidoreduction (**Table1)**. The results of this transcriptomic experiment could further aid
420 in forming a hypothesis of this gene for *in vitro* testing. Another HP-encoding gene *yffR* ($Log_2$
421 FC=-4.85), was predicted to play a role in stress response (this study, Table 1) which aligns
422 with the fact that a majority of stress-response genes were upregulated in the WT, when gene
423 *yhdN* is present.
424
425 All observations highlight WT strain's prioritization of growth and motility, possibly facilitating
426 better environmental sensing and resource acquisition under stress while the absence of *yhdN*
427 gene seems to upregulate other heat shock and stress response genes which reorient
428 metabolic processes towards alternative survival strategies such as biofilm formation. Hence,
429 the protein encoding gene *yhdN* is predicted to be a stress response gene involved in the
430 regulation of heat shock or nutrient limitation response, possibly also playing a broader role in
431 general stress response.
432
433 WT *vs. ΔyeaC*
434 In WT strain, the *yeaC* gene exhibited a significant upregulation after the 5h, with a $log_2$ FC of
435 3 relative to the 1h time point, where its expression was undetectable. This $log_2$FC
436 corresponds to an 8-fold increase in expression. Hence, the 5h time point was chosen for the
437 analysis. A total of 156 DEGs for WT *vs. ΔyeaC* were obtained after filtering ($p_{adj}$≤0.1). In WT
438 *vs. yeaC*, 87 genes had a positive $Log_2$ FC while 69 had a negative $Log_2$ FC.
439
440 Analysis of the top ten upregulated DEGs in the WT strain resulted in three oxidative stress
441 response genes, two other stress response genes, two methionine biosynthesis genes and
442 three genes involved in bacterial secretion system and transposition (EcoCyc). The
443 upregulation of the latter three genes in WT was difficult to explain, since there was no

444 conclusive literature available (**Figure 4B**). Significantly upregulated DEGs involved *ymcE*
445 ($Log_2$ FC=6.81) which provides tolerance to n-butanol [145], and is also known to provide
446 protection against oxidative stress [150], *prpE* ($Log_2$ FC=4.75) which responds to hydrogen
447 peroxide [151] and *iprA* ($Log_2$ FC=4.34), which encodes for an inhibitor of hydrogen peroxide
448 [152]. *metF* ($Log_2$ FC=4.49), a methionine biosynthesis gene, is also known to provide
449 protection against oxidative stress: Methionine is a precursor in the synthesis of cysteine,
450 which is a component of glutathione—a major intracellular antioxidant. Glutathione is known
451 to neutralize reactive oxygen species [153].
452
453 All of the upregulated genes in *ΔyeaC* were involved in motility/chemotaxis-based functions
454 (**Figure 4B**). Significantly upregulated DEGs included genes like *fliF* ($Log_2$ FC=-9.86), *fliO*
455 ($Log_2$ FC=-9.26) and *fliJ* ($Log_2$ FC=-9.26). The upregulation of these genes in *ΔyeaC* signify
456 that the cells prioritize the expression of motility-based genes. They are known to aid the
457 bacterium in adapting to environmental changes by modulating their expression [154].
458 Moreover, there is often a trade-off between motility and stress resistance. Studies have
459 shown that hypermotile *E. coli* strains, which exhibit increased expression of motility genes,
460 may have reduced expression of stress resistance genes, as was also observed in the
461 downregulated DEGs in the WT. Hence, as opposed to WT, the deletion mutant prioritizes
462 motility and chemotaxis to mitigate oxidative stress.
463
464 WT *vs. ΔydgH*
465 The *ydgH* gene demonstrated an upregulation after the 5h, with a $log_2$FC of approximately 3.7
466 as compared to 1 hour, indicating a 13-fold increase in expression. Hence, also in this case
467 the 5h time point was chosen for further analysis. A total of 231 DEGs were obtained after
468 filtering ($p_{adj} \leq 0.1$), resulting in 111 genes with a positive $Log_2$ FC and 120 genes with a negative
469 $Log_2$ FC.
470

471 Top ten positive DEGs ($Log_2$ FC≥1.5) involved six stress response genes, one protein
472 transport gene, one membrane biosynthesis gene, one fatty acid oxidation gene and one HP-
473 encoding gene (**Figure 4C**). Significantly upregulated genes were *dsrB* ($Log_2$ FC=3.89),
474 encoding for a putative stress response protein [155] and *ghoS,* ($Log_2$ FC=3.65), an antitoxin
475 protein which prevents cell death [156]. Gene *fadM* ($Log_2$ FC=2.4), encoding a thioesterase,
476 is responsible for breaking down fatty acids into acetyl-CoA units, which can then be utilized
477 in energy production [157]. Another gene, *tatE* ($Log_2$ FC=2.69) encoding for a transporter, is
478 involved in transport of folded proteins and maintaining of cellular redox balance [158]. Gene
479 *yoaH,* a HP-encoding gene whose function was previously unclear from **Table 1**, might also
480 play a role in oxidative stress response and should be further investigated. The role of
481 lipopolysaccharide biosynthesis gene *ais* ($Log_2$ FC= 2.41) was, however, unclear.
482
483 In the deletion mutant (*ΔydgH*), the top ten negative DEGs included eight motility/chemotaxis
484 genes along with one sulphur metabolism and one urate transport gene (**Figure 4C**): *fliF* ($Log_2$
485 FC=-6.92) and *fliA* ($Log_2$ FC=-5.18) play a crucial role for motility (EcoCyc), *uacT* ($Log_2$ FC=-
486 6.22) is involved in urate transport, which has already been studied to reduce the effects of
487 oxidative stress in *E. coli* [159]. The role of gene *cysP* regarding oxygen stress, which is
488 involved in sulphur metabolism [160] is unclear at the moment and calls for further
489 investigation.
490
491 In WT*,* the upregulation of genes involved in response to stress, i.e. *ghoS* suggest that the
492 cells prioritize conservation of resources by entering a dormant state, i.e. formation of persister
493 cells [156]. Due to the absence of *ΔydgH* the cell seems to attempt to compensate for the loss
494 of oxidoreductive function by utilizing genes related to motility and chemotaxis (**Figure 4C**).
495 This increased expression could be an adaptive response to maintain survival under
496 continuous exposure to oxidative stress. Hence, similar to the previous case (WT *vs. ΔyeaC*),
497 more stress response genes were upregulated in WT while motility/chemotaxis genes were
498 upregulated in *ΔydgH*.

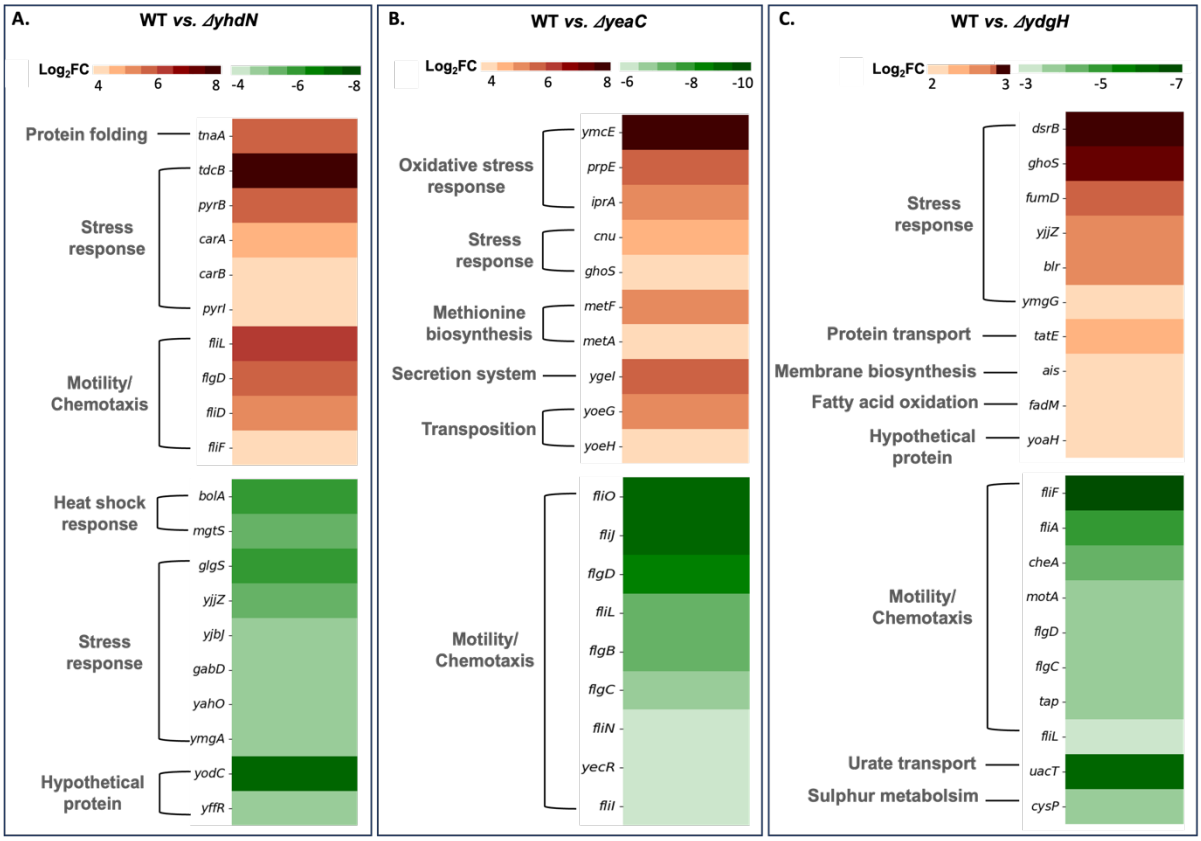



**Figure 4. Comparative differential gene expression analysis in A. wild type (WT) *vs. ΔyhdN*, B. WT *vs. ΔyeaC* and C. WT *vs. ΔydgH* strains.** Top ten positively and negatively expressed DEGs are shown. Each row represents a gene, and colour intensity represents the $Log_2$ Fold Change (Log2 FC), with red indicating upregulation in WT and green indicating upregulation in the mutant strains. Functional categories were based on information retrieved from Literature and EcoCyc.

## Conclusion and Outlook

The functional characterization of hypothetical protein (HP)-encoding genes remains a critical challenge, since the rapid accumulation of sequencing data has long surpassed the rate of possible experimental characterization *in vivo* and *in vitro*. While there have been notable advancements in methodologies for protein function prediction in the recent past, several challenges persist, including the heavy reliance on pre-existing data inferred from model organisms. Unfortunately, even in the best studied model organisms a significant amount of protein encoding genes could so far not been annotated.

516 AI-driven approaches, such as machine learning (ML) algorithms, now offer powerful tools to
517 analyze vast omics datasets. In this study we employed a comprehensive approach,
518 integrating independent component analyses to decipher transcriptional regulatory networks,
519 deep learning for structural homolog prediction, and various other bioinformatic tools to
520 characterize HPs that have no functional information available in public databases. With this
521 methodology putative/probable functions for at least 64% of 95 hypothetical HPs encoding
522 genes from *E. coli* K-12 could be extrapolated, facilitating the subsequent functional validation
523 of these proteins through *in vitro* and *in vivo* experiments in the future. For the remaining 36%
524 HPs, however, more high-quality datasets including metabolomics and proteomics are
525 probably needed, as these genes are often seen to be expressed under very specific
526 environmental conditions from which only one dataset was available. Nevertheless, for most
527 proteins it is possible to derive at least some characteristics from their general sequence
528 properties, whether there is evidence of expression, their possible cellular location, details of
529 homologs, or whether they are predicted or demonstrated to be expressed as part of an
530 operon and/or regulon. Taking these information into account in databases will aid in the
531 improvement of using AI tools for annotation in the future.
532
533 In this study we furthermore provided an experimental feedback loop for three HP-encoding
534 genes to verify the *in silico* prediction. Gene *yhdN* ought to play a role in the cells' responding
535 to heat shock and nutrient limitation, which could be confirmed by comparing the growth
536 curves and transcriptional responses of wild type and *ΔyhdN* deletion mutant strains. For the
537 second candidate *yeaC, in silico* predictions suggested a role in aerobic respiration.
538 Experimental evidence supported this hypothesis by the upregulation of oxidative stress
539 response and methionine biosynthesis genes, hence it is likely involved in scavenging reactive
540 oxygen species. In contrast, the significant upregulation of "Motility"/Chemotaxis" genes in the
541 deletion mutant suggest that during the absence of *yeaC*, bacteria may enhance motility
542 pathways as an adaptive mechanism under oxidative stress. The third candidate *ydgH*, was
543 predicted *in silico* to play a role in oxidoreduction, which could also be confirmed by the *in vivo*

544 experiments showing its role in the protection against oxidative stress. Similar to *yeaC,* in the
545 mutant an upregulation of motility/chemotaxis genes indicate that in the absence of *ydgH*, the
546 cell may compensate for reduced oxidoreductive function. However, it is important to
547 acknowledge that our understanding still remains incomplete since the functional landscape
548 of proteins is vast and complex. Future studies and more omics datasets are needed to further
549 refine and expand upon our findings, exploring the broader implications of these proteins
550 under different environmental conditions.
551
552 In conclusion, this study represents a significant step forward in elucidating the functions of
553 previously uncharacterized HPs with no information in existing knowledge databases.
554 Leveraging AI-driven annotations and integrating them with experimental laboratory work will
555 link new functional roles of genes, discover previously unknown cellular and metabolic
556 processes and maybe even biotechnological potentials. In the future this approach might also
557 aid in the isolation of new and so far uncultured microbial species, i.e. microbial dark matter,
558 by harnessing meta-omics datasets and identifying genes critical for growth and adaptation,
559 offering clues to optimize culture conditions.
560
561 **Methods**
562 **Identification and enumeration of hypothetical proteins (HPs)**
563 For the comprehensive analysis of *Escherichia coli* K-12 (*E. coli*) substrains MG1655 and
564 BW25113, the complete genome sequences available in the NCBI RefSeq database under
565 the accession number GCF_000005845.2 and GCF_004355105.2 were used, respectively.
566 Ribosomal RNA (rRNA), transfer RNA (tRNA), non-coding RNA (ncRNA) were filtered out.
567 CDS based annotations were used to find hypothetical proteins (HPs). HPs were identified
568 based on keywords like “Putative”, “Putative uncharacterized”, “Uncharacterized protein” and
569 “DUF (domain of unknown function)-domain containing protein”, “UPF” (unknown protein
570 family) as well as proteins having a ‘Protein Y…’ pattern. Further information was extracted

571 from the extensively curated databases EcoCyc version 26.1 [39], RegulonDB version 11.2
572 [11], EggNOG v6.0 [12] and UniProt release 2023_04 for proteome UP000000625 [1].
573 In order for a HP to be classified as a protein with no characterization (target group of this
574 study), it had to fulfill all of the following criteria:

575 1. The **EcoCyc** protein summary showed the statement 'No information about this'.
576 Additionally, the protein had to be marked as 'Uncharacterized'. Proteins which had
577 the statement but a 'Partial' characterization in EcoCyc were further investigated using
578 UniProt.
579 2. In **UniProt** the protein lacked any functional information in the 'Function' section and
580 was assigned an annotation score of '1', and a 'protein existence' score of '4' signifying
581 that the protein, although predicted to be expressed, lacked information based on
582 experimental evidence or orthologs in closely related species. For the "Function"
583 section, a score of '1' is the lowest rating in a five-point-scoring system and for the
584 "protein existing" category a score of '4' out of '5', where '5' indicates a higher level of
585 uncertainty regarding the protein's expression.
586 3. In the **RegulonDB** database it had the label "Weak" evidence for an annotation in the
587 evidence section and no additional information in the 'Note' subsection.
588 4. In the **EggNOG** database the protein was either classified under the cluster of
589 orthologous groups (COG) with a functional category 'S', where 'S' designates proteins
590 belonging to an unknown function category or it lacked an ortholog which was
591 displayed as 'No orthologs found'.
592
593 95 of the 4403 *E. coli* K-12 protein encoding genes fulfilled these criteria. All scripts for the
594 filtering and enumeration of uncharacterized HPs can be found on Figshare
595 (*Notebooks>HP_curation*). In addition, the here identified HP-encoding genes were compared
596 with the ones identified by Ghatak et al. in 2019 [8] to check for their current status.
597
598 **Transcriptomic data curation and processing**

599 RNA-sequencing data from NCBI for analyzing the expression patterns of genes was collected
600 for *E. coli* K-12 substrains MG1655 and BW25113 using an adapted workflow for *Bacillus*
601 *subtilis* [32]. For substrain MG1655 1854 datasets and for substrain BW25113 662 datasets
602 were downloaded and processed. Raw FASTQ files were obtained using sratoolkit 3.0.1 [161],
603 and subsequent read trimming was performed using Trim Galore 0.6.5 [162] with the default
604 settings. Trimmed reads were then subjected to FastQC 0.11.9 [163]. Alignments to the
605 genomes were done using Bowtie 1.2.3 [164]. The read direction was determined using
606 RSeQC 3.0.1 [165] before generating read counts using featureCounts [166]. Finally, all
607 quality control metrics were compiled using MultiQC [167] and the expression compendium
608 was obtained as log-transformed Transcripts per Million (log-TPM) values. Quality metrics
609 were established to filter and ensure the selection of high-quality datasets with the following
610 modifications [168]: Briefly, the datasets were considered to be of high quality if they passed
611 the fastqc parameters 'per_base_sequence_quality', 'per_sequence_quality_scores',
612 'per_base_n_content', 'adapter_content' and had at least 500,000 reads mapped to coding
613 sequences from the reference genome. To preserve biological variability and prevent loss of
614 information, samples with no replicates were also retained in the dataset. Short non-coding
615 transcripts (<100 nucleotides) and extremely low-expression transcripts (Fragments Per
616 Kilobase per Million mapped reads < 10) were removed to reduce noise. 779 datasets for
617 substrain MG1655 and 135 datasets for substrain BW25113 were then used for further
618 analysis. All other information on the *E. coli* K-12 genes including their transcription units,
619 descriptions etc. were sourced from EcoCyc by utilizing the SmartTables feature [39].
620 Annotations for clusters of orthologous groups (COG) were obtained from EggNOG v6.0 [12]
621 including Gene Ontology [169]. The transcriptional regulatory network (TRN) comprising
622 information on genes and their governing regulators (i.e. transcription factors controlling the
623 expression of genes) were sourced from RegulonDB version 12.0 [11] and EcoCyc [39] as
624 described by Sastry et al. [32] In order to mitigate any batch effects which might be caused
625 due to the usage of diverse datasets, a reference condition (wild type *E. coli* K-12 substrain

626 MG1655 in M9 minimal medium facilitated with glucose and essential micronutrients under
627 non-stressful conditions) was used [30]. This was done by subtracting the mean expression
628 of two replicates of the chosen reference condition from the entire gene expression dataset
629 and calculating the log2-fold-change i.e. relative changes in expression. This normalization
630 method allowed for the comparison of gene expression differences relative to a common
631 baseline as previously demonstrated in Sastry et al. [30]. The normalized data was then used
632 as input for machine learning (*Data processing>Nextflow*).
633
634 **Independent Component Analysis of Transcriptomic Data**
635 Independent Component Analysis (ICA) developed by McCon et al. -named OptICA- was
636 applied to prevent both over-decomposition and under-decomposition of transcriptomic
637 datasets [27]. The application of ICA to the normalized *E. coli* K-12 substrain MG1655 RNAseq
638 data resulted in two matrices: the first matrix, denoted as the M matrix, encompassing the
639 robust independent components named iModulons, while the second matrix, termed the A
640 matrix, comprised the corresponding activities of iModulons over different growth conditions.
641 Additionally, a PyModulon package was used in conjunction with Jupyter notebooks to
642 characterize the iModulons [32]. The scripts containing the specific utilization of resources and
643 curation of iModulons can be found on Figshare (*OptICA* and *iModulon curation*). The
644 information from substrain MG1655 was then used to infer the iModulons and their member
645 genes for substrain BW25113, since only for the latter strain single-gene deletion mutants
646 were available for *in vitro* experimentation. The HP-encoding genes, the regulators governing
647 them, and their derived functions can be found in **Table 1.**
648
649 Possible functions for the 95 HP-encoding genes were further identified through regulators
650 governing their gene expression, as provided by RegulonDB and EcoCyc and/or by Gene
651 Ontology (GO) terms derived from co-regulating genes using the PANTHER (Protein ANalysis
652 THrough Evolutionary Relationships) tool [33]. Here, member genes of an iModulon (including
653 the gene of interest, i.e. the HP-encoding gene) were used as input to obtain GO terms to see

654 if any were overrepresented. Statistically significant GO categories with the default false
655 Discovery Rate (FDR)-adjusted p-value (<0.05) were manually identified for each HP
656 (*Supplementary Table 1*). These gene-associated functional categories can be found in **Table**
657 **1** and **Figure 2**.
658
659 **Metadata extraction**
660 Metadata conditions for all datasets were retrieved either manually from the sequence read
661 archive (SRA), *via* a semi-automated approach [167] or by utilizing data from the Gene
662 Expression Omnibus Database using the GEOparse tool [170] to obtain experimental
663 information not available *via* the NCBI's Entrez Programming Utilities (E-utilities) [42]. As a
664 result, from 779 quality checked datasets for the substrain MG1655, metadata for 602 datasets
665 could be retrieved with confidence and termed as 'Explicit metadata'. 113 datasets were
666 termed as 'poor metadata', meaning that information on one or more experimental conditions
667 (such as media composition, temperature, pH, etc.) was missing or incomplete. Lastly,
668 datasets with 'unclear metadata' lacked all information. For the substrain BW25113, 128
669 datasets were 'Explicit' while seven were 'unclear' (*Notebooks>metadata_curation*).
670 Additionally, metadata condition for the highest expression of the HP- encoding genes against
671 the reference gene *frr* were extracted from the Tjaden dataset [44] in order to account for the
672 loss of datasets after quality filtering (also see below). The *frr* gene expression is typically
673 consistent across different conditions which was also observed when tested as reference
674 along with other genes like *rpoC*, *rpoD* for the uncharacterized HPs [this study, 171]. Hence,
675 gene *frr* was chosen as the reference gene serving as a control to validate the relative
676 expression levels of other genes. The extensive nature of this dataset ensured a broad
677 representation of gene expression profiles, in addition to the datasets procured for OptICA
678 analysis (*Notebooks> metadata_curation*).
679
680 **Co-expression analysis based on public RNA-Seq data**

681 To identify genes with expression patterns similar to those encoding HPs, a Spearman
682 correlation analysis on the Tjaden dataset was performed [172]. This dataset comprises a
683 comprehensive collection of 3.376 *E. coli* strain K-12 RNA-seq datasets, with expression
684 levels quantified in transcripts per million (TPM) [44]. For inferring gene-gene correlations
685 across the transcriptomic datasets for strain *E. coli* K-12, we identified the top ten co-
686 expressed genes to the respective 95 HP-encoding gene and used this information to get an
687 inference on their functions [10] **(Table 1)** (*Notebooks>gene_co-expression*).
688
689 **Sub-Cellular Localization**
690 For the purpose of determining the subcellular localizations of the 95 HP-encoding genes,
691 BUSCA (Bologna Unified Subcellular Component Annotator) was employed [173]. This tool
692 integrates various machine learning and bioinformatics tools [174–177] to analyze and
693 recognize patterns in protein sequences that are indicative of specific subcellular localizations.
694 The integrative webserver was used to obtain labels such as 'Nucleus', 'Periplasm/cytoplasm',
695 'Inner/outer membrane' or 'extracellular space'. All 95 HP-encoding genes with their predicted
696 sub-cellular localizations can be found in **Table1**.
697
698 **Protein-Protein Interactions (PPIs) Analysis**
699 The STRING database (version 12.0) (https://string-db.org) was employed for the
700 identification of PPIs of HP-encoding genes against the proteomic landscape of *E. coli*. The
701 tool incorporates data from diverse origins such as available publications, empirical evidence,
702 predictions derived from co-expression patterns, genomic context analysis, and curated
703 pathway databases. A 'confidence score' for each inferred association was derived,
704 quantitatively expressing the robustness of the predicted interaction [178]. In order to keep
705 only the high-scoring associations, a cut-off of 0.7 was utilized [179]. All 95 HP-encoding
706 genes and their interacting partner proteins are listed in **Table 1**.
707
708 **Analysis of Gene Context**

709 Shared functional relationships were analyzed using the genomic context of
710 *E. coli* strain K-12 (*Supplementary Figure 1*), particularly the genes located upstream and
711 downstream of the 95 genes of interest. A gene list comprising of gene names was used to
712 create a SmartTable in the EcoCyc database to examine the local genomic neighbourhood
713 and visualize the genes upstream and downstream, including their orientations [36]. (**Table**
714 **1)**.
715
716 **Detection of Remote Protein Homology**
717 In order to identify distantly homologous sequences, HP sequences were used as input for
718 the HHblits tool (version 3.3.0) available in the MPI Bioinformatics Toolkit using a Hidden
719 Markov Model (HMM) iterative sequence search [180]. The HHblits server
720 (https://toolkit.tuebingen.mpg.de/tools/hhblits) takes a single sequence or a multiple sequence
721 alignment as input and iteratively searches through the selected HMM databases [181,182].
722 HHblits performs sequence search and remote homology detection with higher sensitivity as
723 compared to gold-standard databases like InterPro [180]. Homologous sequences were then
724 searched against the default UniRef30 database [37]. Sequence based remote homologies
725 identified through the HHblits tool are catalogued in **Table 1**.
726
727 **Structure-Based Homology**
728 For identifying structural homologs, we used the AlphaFold Protein Structure Database
729 (AFDB) (https://cluster.foldseek.com/), a specialized computational database that includes
730 structural models for approximately 215 million protein sequences. This platform employs
731 deep learning algorithms to scan and match protein structures, enabling the discovery of
732 structural similarities, even among proteins with low sequence identity [183]. We analyzed
733 clusters to perform comparisons of HPs with all protein structures in the AFDB. UniProt
734 accession IDs were used as inputs to identify the cluster to which the HP belongs. The

735 homology-based function information for each of the 95 HP is listed in **Table 1**. A script to
736 extract the information can be found on Figshare (*Notebooks> HP_info_curation*).
737
738 **Bacterial strains and growth conditions**
739 *E. coli* K-12 substrain BW25113 was sourced from Horizon Discovery Ltd., United States.
740 Three single-gene knockout mutants from *E. coli* strain K-12 substrain BW25113 *ΔyhdN,*
741 *ΔyeaC*, and *ΔydgH* were obtained from the KEIO collection (NBRP, Japan). Glycerol stocks
742 were stored in an ultra-low temperature freezer (Thermo Fisher, United States) at −80°C until
743 further processing. Cells were cultured overnight on agar plates from glycerol stocks and
744 single colonies were picked from the agar plate and then used to Luria Bertani (LB) liquid
745 medium at 37°C [184].
746
747 **Media and reagents**
748 *E. coli* strains were cultured in LB broth (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl;
749 pH 7.0) at 37°C while shaking at 200 rpm [184]. M9 liquid medium for the pre-culture was
750 prepared using 42 mM $Na_2HPO_4$, 22 mM $KH_2PO_4$, 8.5 mM NaCl, 18.7 mM $NH_4Cl$, 2 mM
751 $MgSO_4$, 0.1 mM $CaCl_2$ and 11 mM glucose, supplemented with 0.05% casamino acids to
752 ensure non-limiting conditions. For nitrogen limited M9 medium, a $NH_4Cl$ concentration of 0.16
753 mM was used without the addition of casamino acids. For solid medium, 1.5% agar was
754 added. 30% $H_2O_2$ was sterile filtered through a 0.22 µm pore size filter to ensure sterility prior
755 to be used in the oxidative stress experiments. For all experiments with $H_2O_2$ the LB was
756 freshly prepared and kept in the dark until use. All chemicals were sourced from Sigma
757 Chemical Co. (St. Louis, MO, USA).
758
759 **Measurement of Bacterial Growth Curves**
760 Bacterial cultures were inoculated at a 1:100 dilution unless stated otherwise. Each
761 experiment included triplicates. All optical density values ($OD_{600}$) were measured *via* a cell
762 density meter (Ultrospec 10, biochrom). $OD_{600}$ was measured every 2h for heat shock in LB

763 and M9 or M9 modified medium and every 1h for oxidative stress experiments. Growth curves
764 were generated for each set of experiments including the wild type and mutant strain in
765 stressed and non-stressed conditions (controls).
766
767 Heat stress
768 A single colony (wild type and Δ*yhdN*) was picked from either LB or M9 agar plates and
769 inoculated into 5 mL LB broth for overnight cultivation at 37°C and shaken with 200 rpm. The
770 overnight culture was then used to inoculate fresh LB medium at a 1:100 dilution, which was
771 then cultured for ~2-3h at 37°C and 200 rpm until an $OD_{600}$ of 0.4 was reached. This was
772 repeated with the M9 liquid medium (preculture), at 37°C to ensure non limiting conditions.
773 For the nitrogen limitation assay, medium was prepared using 0.16mM $NH_4Cl$. To study the
774 effects of heat shock on bacterial growth, the culture was grown to mid-log phase ($OD_{600}$ 0.4),
775 diluted to $OD_{600}$ 0.04 and then heat shocked for 7 minutes in a shaker-incubator at 50°C. After
776 treatment, the bacterial suspension was incubated for 12 h at 37°C. Samples were measured
777 every 2h for 12h.
778
779 Oxidative stress
780 For studying the effects of oxidative stress, a single colony (wild type, *ΔyeaC* and *ΔydgH*) was
781 picked from the LB agar plate and inoculated into 5 mL LB for overnight cultivation at 37°C
782 and shaken with 200 rpm. The overnight culture was then used to inoculate fresh LB (dilution
783 of 1:100) and cultured at 37°C and 200 rpm until an $OD_{600}$ of 0.2 was reached. An OD of 0.2
784 was used since at this stage the cells were actively dividing, making them susceptible to the
785 effects of oxidative stress, allowing for better characterization and analysis of the stress
786 response[137]. $H_2O_2$ was added at a final concentration of 2.5 mM. After treatment, the
787 bacterial suspension was incubated for 5h at 37°C and the $OD_{600}$ was measured hourly.
788
789 **RNA Sequencing and data processing**

790 2 ml of cell suspension from the heat shock samples was collected after 2h and 6h of growth.
791 For the oxidative stress experiments, samples were collected after 1h and 5h of growth. The
792 medium was discarded, cell pellets were washed with Phosphate Buffer Saline and mixed with
793 DNA/RNA shield™ (Zymo Research Corp., Freiburg, Germany). All samples were processed
794 according to the manufacturer's instructions. Samples were preserved at -20°C until RNA
795 extraction. RNA was extracted using the Direct-zol RNA Miniprep Plus Kit (Zymo Research
796 Corp., Freiburg, Germany) according to the manufacturer's instructions. Total RNA was eluted
797 from columns with 100 µL nuclease-free water and quantified using the Qubit RNA Broad
798 Range Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The quality was
799 assessed by running an RNA-nano chip on an Agilent bioanalyzer (Agilent Technologies,
800 Waldbronn, Germany). Samples with an RNA integrity score ≥8 were used for further
801 processing [185]. The rRNA was removed from total RNA preparations using the NEBNext
802 rRNA Depletion (Bacteria) kit (New England Biolabs, Frankfurt, Germany). Paired-end library
803 preparation was done using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina
804 (New England Biolabs, Frankfurt, Germany) following the manufacturer's instructions with an
805 average insert size of 200 bp. Libraries were run on a NextSeq 550™ (Illumina, San Diego,
806 CA, United States).
807
808 The RNAflow pipeline [186] was implemented using a conda environment. Sequence reads
809 were checked for quality using FastQC v0.11.9 [187]. Raw reads were processed using fastp
810 v0.20.0 [188], which employs a sliding window approach for quality trimming and adapter
811 clipping, with the settings: a -5 to -3 cut for front and tail, a default window of 4 and mean
812 quality threshold of 20. Trimmed reads shorter than 15 bps were discarded. The remaining
813 reads were mapped onto the *E. coli* K-12 reference genome (NZ_CP009273.1) using HISAT
814 v2.1.0 [189]. The generated output was converted into a BAM format using samtools-1.20
815 [190], followed by the processes of sorting and indexing. A gene transfer format (GTF)
816 annotation file for *E. coli* substrain BW25113 was used to filter for gene and pseudogene
817 features. FeatureCounts v2.0.1 [191] was used to quantify the mapped reads based on the

818 gene annotations. Processed datasets were summarized in a MultiQC v1.9 [167] generated
819 file and were normalized by the DESeq2 module (v1.28.0) in R (version 4.3.2) using a
820 variance stabilizing transformation [192].
821
822 **Comparative gene expression analysis of wild type and isogenic mutant strains**
823 Sequencing reads were obtained from the transcriptomic datasets of wild-type (WT), *ΔyhdN*,
824 *ΔyeaC,* and *ΔydgH* mutants, respectively. Non-stressed control and heat shocked samples
825 from WT and *ΔyhdN* at the 6h timepoint and non-stressed control and oxidative stress samples
826 from WT, *ΔyeaC,* and *ΔydgH* at the 5h timepoint were used to identify up- or downregulated
827 genes. DESeq2 [192] was utilized to examine expression differences of genes in the WT and
828 mutant strains under both control and stress conditions. To focus on the specific effect of
829 stress on gene expression rather than the difference of WT *vs.* deletion mutants, read counts
830 under control conditions were subtracted from the read counts under stress condition [193].
831 Genes with an adjusted P-value (Padj) of 0.1 or less were classified as differentially expressed
832 to avoid false positives. A cut-off $\log_2$ fold change ($\log_2$FC) of 1.5 was chosen for further
833 analysis [194]. A $\log_2$FC of 1.5 means that a gene was 2.83 times more expressed in one
834 strain *vs.* the other, a $\log_2$FC of 2 means that a gene was 4 times more expressed and so on.
835 Significant positive $\log_2$FC indicated genes that were upregulated in WT relative to the mutant,
836 while negative values showed an upregulation in the mutant *vs*. WT. To determine the
837 functional categories of these genes, the EcoCyc database was used [36]. Information of the
838 differentially expressed genes can be found on Figshare (*DEGs>Table1*).
839
840 **Data availability**
841 All code and scripts used in this study are available on Figshare. These resources can be
842 accessed at https://figshare.com/s/0ede175c510cf201e7c2.
843 Raw sequencing data that supports the findings of this study have been deposited in the
844 Sequence Read Archive with the BioProject ID: PRJNA1209969.
845

## Competing interests

The authors declare no competing interests.

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## Authors' contributions

S.C, H.A. and Z.A did the data analyses, S.C. did the *in vitro* experiments, S.C and A.K.K. wrote the manuscript, A.K.K. provided the funding.

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