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Adaptations of *Escherichia coli* strains to oxidative stress are reflected in properties of their structural proteomes

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Abstract

Background: The reconstruction of metabolic networks and the three-dimensional coverage of protein structures have reached the genome-scale in the widely studied *Escherichia coli* K-12 MG1655 strain. The combination of the two leads to the formation of a structural systems biology framework, which we have used to analyze differences between the reactive oxygen species (ROS) sensitivity of the proteomes of sequenced strains of *E. coli*. As proteins are one of the main targets of oxidative damage, understanding how the genetic changes of different strains of a species relates to its oxidative environment can reveal hypotheses as to why these variations arise and suggest directions of future experimental work.

Results: Creating a reference structural proteome for *E. coli* allows us to comprehensively map genetic changes in 1764 different strains to their locations on 4118 3D protein structures. We use metabolic modeling to predict basal ROS production levels (ROStype) for 695 of these strains, finding that strains with both higher and lower basal levels tend to enrich their proteomes with antioxidative properties, and speculate as to why that is. We computationally assess a strain's sensitivity to an oxidative environment, based on known chemical mechanisms of oxidative damage to protein groups, defined by their localization and functionality. Two general groups - metalloproteins and periplasmic proteins - show enrichment of their antioxidative properties between the 695 strains with a predicted ROStype as well as 116 strains with an assigned pathotype. Specifically, proteins that a) utilize a molybdenum ion as a cofactor and b) are involved in the biogenesis of fimbriae show intriguing protective properties to resist oxidative damage. Overall, these findings indicate that a strain's sensitivity to oxidative damage can be elucidated from the structural proteome, though future experimental work is needed to validate our model assumptions and findings.

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Conclusion: We thus demonstrate that structural systems biology enables a proteome-wide, computational assessment of changes to atomic-level physicochemical properties and of oxidative damage mechanisms for multiple strains in a species. This integrative approach opens new avenues to study adaptation to a particular environment based on physiological properties predicted from sequence alone.

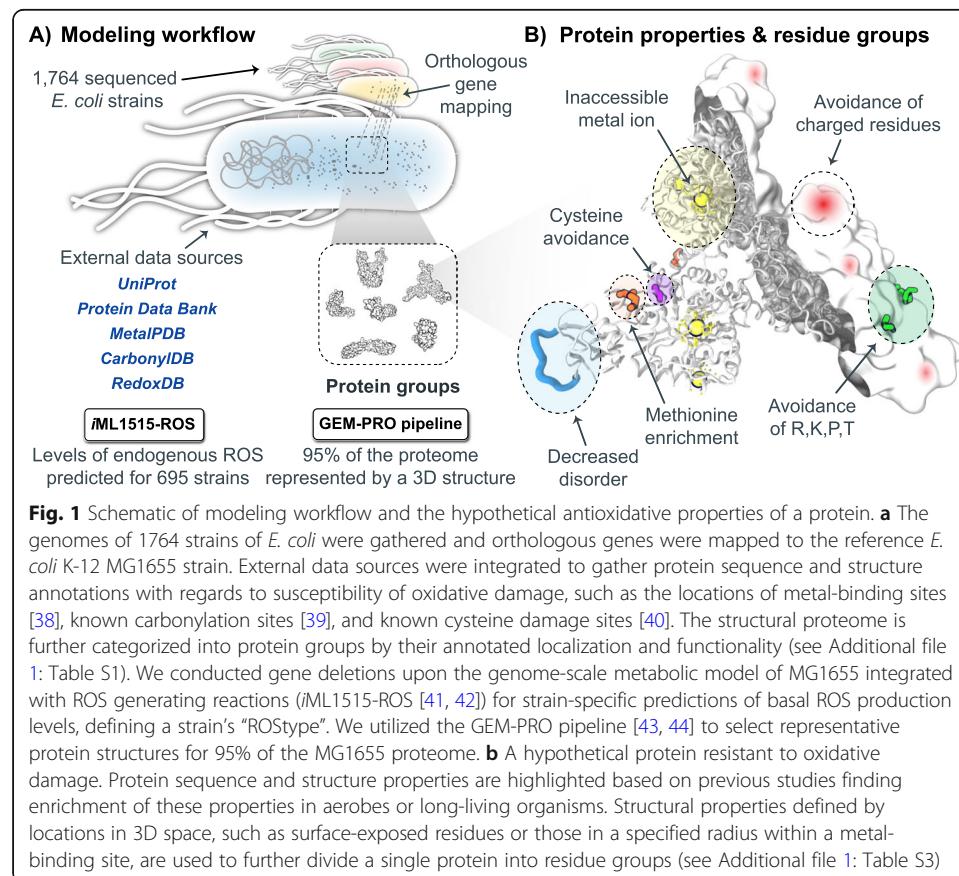
Keywords: Structural systems biology, Oxidative stress, Structural proteome, Physicochemical properties, Oxidative damage, Metabolic model

Background

Reactive oxygen species (ROS) can cause severe oxidative damage to cellular proteins, which often results in chain reactions that spread the damage to neighboring macromolecules and leads to systems-level changes of cellular function [1]. While ROS can be beneficial - and even necessary in some contexts [2–5] - at a high concentration, oxidative damage must be responded to and repaired. As a result, cells are equipped with a number of mechanisms to quench ROS, directly repair the damaged components, or manage ROS indirectly [6]. Certain amino acids that constitute the structures of proteins are principal sites of oxidative damage [7]. This damage can be divided into two groups - reversible or irreversible modifications. The sulfur-containing residues methionine and cysteine incur reversible modifications, while irreversible damage impacts histidine, arginine, lysine, proline, threonine (RKPT), and tyrosine (with reactive nitrogen species) [8]. The damage to the RKPT amino acids is a post-translational modification known as carbonylation, and is commonly used as an experimental measurement of oxidative damage with mass spectrometric methods [9–11].

A number of studies have explored the adaptations of an organism's proteome to deal with an oxidative environment. These studies have examined how the amino acid usage of their proteomes differs between anaerobes versus aerobes [12], or between short- and long-living organisms [13–15]. The latter case has been of interest due to the proposed oxidative stress theory of aging [16] which states that the accumulation of oxidative damage to macromolecules is a major reason why organisms age. As a result of these studies, there are a number of proposed hypotheses regarding how aerobic organisms have evolved to live in oxygen-rich environments or why certain species have increased longevity. In the context of the structural proteome, these proposals include: 1) the use of "amino acid sponges" that can absorb oxidative damage by enriching cytosolic protein surfaces with methionine [17–20] or cysteine [21], and additionally tyrosine or tryptophan in transmembrane proteins [22]; 2) the avoidance of cysteines [23] or carbonylation-susceptible residues [10, 24] on the surface of a protein; 3) the avoidance of charged or disorder-prone residues [25–28] to favor a more stable folded state; 4) the protection of reactive cofactors such as transition metal ions or flavins with extended domains [29] or by altering global or local structural characteristics [30–32]; and 5) a number of other novel mechanisms [33–37]. The true impact of these adaptations remains unclear as patterns observed could be attributed to other factors [12], but it is clear from these studies that antioxidative protein properties have manifested themselves within the genetic code over time.

In this study, we use a combination of both structural and systems biology approaches to evaluate if the differential manifestation of these antioxidative properties in the proteomes of multiple *E. coli* strains reflects the varying oxidative environments they may encounter. We extend a pipeline to construct genome-scale models with protein structures (GEM-PROs) to the entire reference proteome of *E. coli* K-12 MG1655, with additional methods to select representative cofactor-bound structures and protein complexes. We use this information along with a set of 1764 sequenced *E. coli* strains to map DNA sequence variation to the reference proteome, with the goal of characterizing physicochemical changes in groups of proteins defined by their localization and functionality (Fig. 1). We additionally create strain-specific genome-scale metabolic models capable of predicting basal ROS production levels (henceforth referred to as “ROStypes”) [41, 42] to understand how adaptation may arise not only due to levels of ROS encountered exogenously, but also produced endogenously. With this information, we are able to pinpoint shared changes in relation to a strain’s phenotype and the antioxidative properties of its proteome. We unexpectedly find that strains with predicted higher levels of endogenous ROS relative to MG1655 (ROS^{hi}) share antioxidative properties in their proteomes to those with predicted lower levels (ROS^{lo}). We find that generally, metalloproteins and periplasmic proteins differ in these antioxidative properties, and detail two specific examples of molybdenum-binding enzymes and proteins involved in the biogenesis of fimbriae. This work demonstrates a structural systems biology approach



to explore patterns of protein sequence variation in relation to predicted or known phenotypes, specifically in the context of adaptation to an oxidative environment.

Results

Reconstructing the reference structural proteome of *E. coli* K-12 MG1655

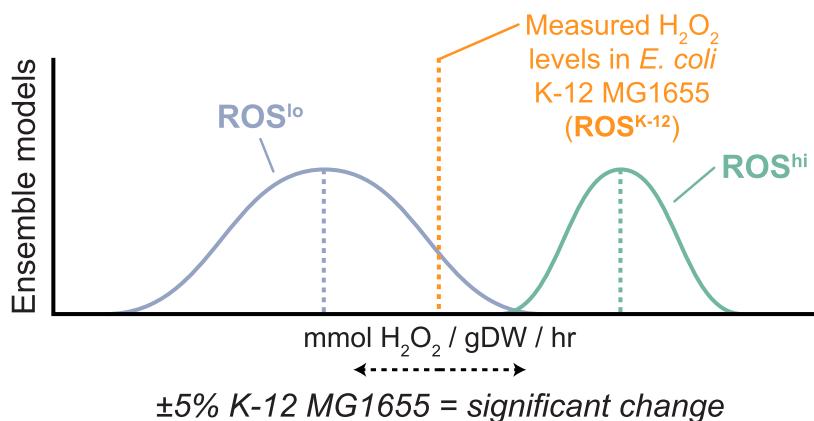
The construction of a reference structural proteome for the 4313 proteins of the *E. coli* K-12 MG1655 strain resulted in 1457 proteins that could be represented by an experimentally determined 3D structure, an additional 2661 proteins with a homology model, and 195 with no available structure. Proteins were segregated into functionally similar groups, first based on their localization within the cell and further using clusters of orthologous group (COG) categories and metabolic subsystem groupings, resulting in over 200 groups of proteins (henceforth referred to as *protein groups*) analyzed for differences in their structural properties that could potentially contribute to oxidative stress resistance (henceforth referred to as *antioxidative properties*) (Fig. 1) (see Additional file 1: Table S1). These antioxidative properties were selected on the basis of previous studies that characterized hypothesized resistance patterns seen in aerobes or in long-living organisms, and are listed in Additional file 1: Table S3.

Improvements to the GEM-PRO pipeline [43, 44] allowed for the refined selection of experimental protein structures for 42 iron and iron-sulfur binding enzymes. For these, selection of a representative structure was extended beyond sequence identity and structural resolution by considering cofactor-bound states if experimental structures were available in both apo (cofactor-unbound) and holo (cofactor-bound) forms. The integration of external data sources enabled the assessment of changes to experimentally determined damage points on proteins for carbonylation (24 proteins with 84 total experimentally carbonylated residues) and cysteine damage (94 proteins with 150 total experimentally damaged cysteines). These improvements result in a more rigorous reconstruction of the structural proteome and also enable future analyses to consider important protein subsequences (Fig. 1b), such as for changes within a certain radius of a metal-binding site or known sites of damage.

Strains with significant variance in ROStypes display enrichment of antioxidative properties in their proteomes

The protein sequences of 1764 strains of *E. coli* were gathered from public databases (see Methods). Simulations to predict the ROStype of available *E. coli* strains were successful for 695 strains, and resulted in a set of 16 strains that had a significantly higher basal ROS production rate (ROS^{hi} , $> 105\%$ measured K-12 MG1655 production rate [45]) and 26 that had a lower basal rate (ROS^{lo} , $< 95\%$ measured rate) (Fig. 2a). This cutoff was selected from a previous study that validated a number of gene deletions and their impact on measured endogenous ROS levels [41]. The production rate of H_2O_2 and O_2^- was highly correlated ($r = 0.98$) and thus, classification of a strain based on their ROStype refers to production rates of both H_2O_2 and O_2^- . A large number of strains (653) displayed non-varying levels (within $\pm 5\%$) compared to K-12 MG1655 ($\text{ROS}^{\text{K-12}}$) while the remaining strains (1069) failed to simulate growth under minimal media conditions, most often due to missing amino acid synthesis pathways that were

A) Predicted endogenous ROS levels (ROStype)



B) Metabolic subsystems of missing reactions leading to differences in predicted endogenous ROS levels

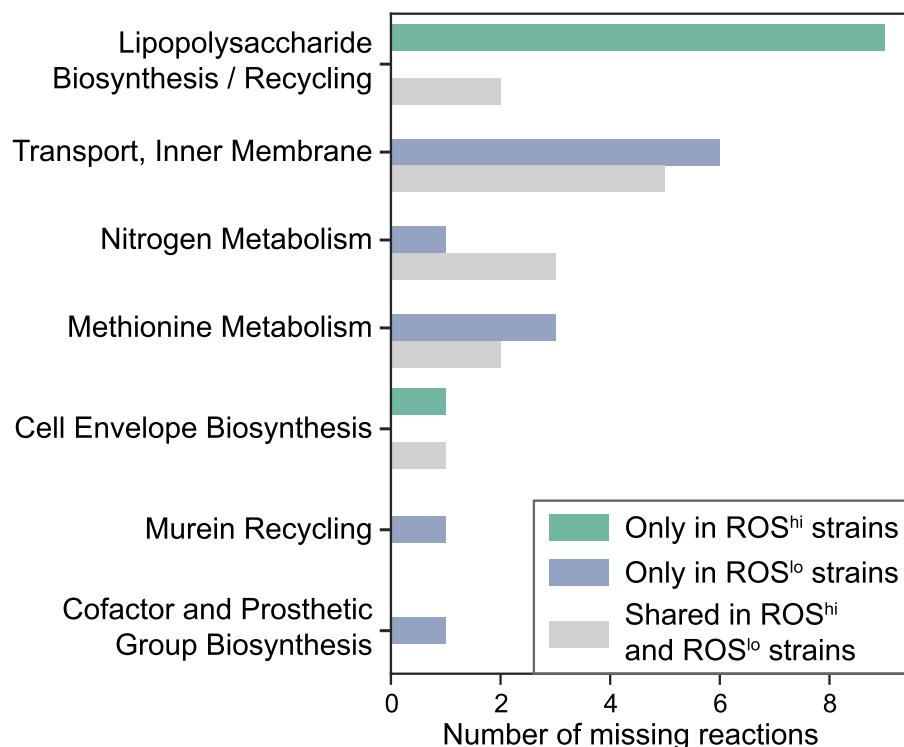
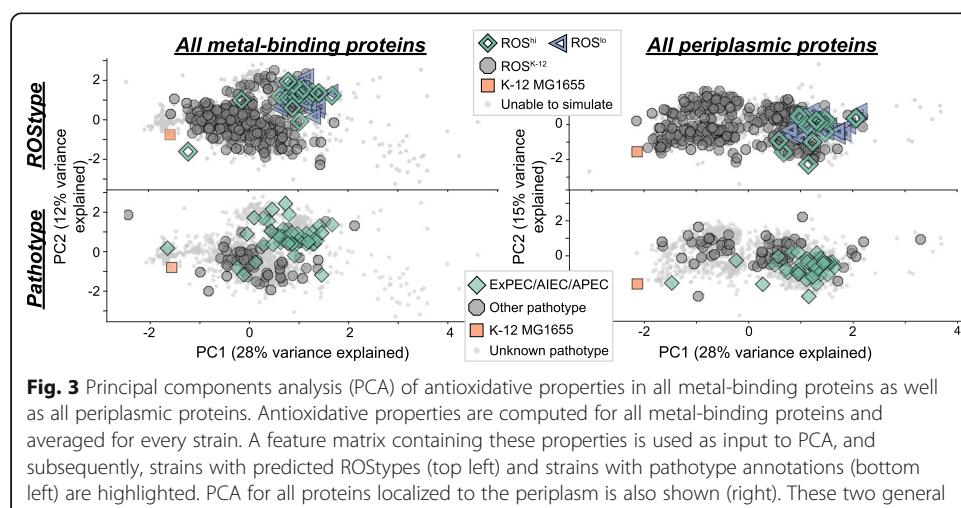
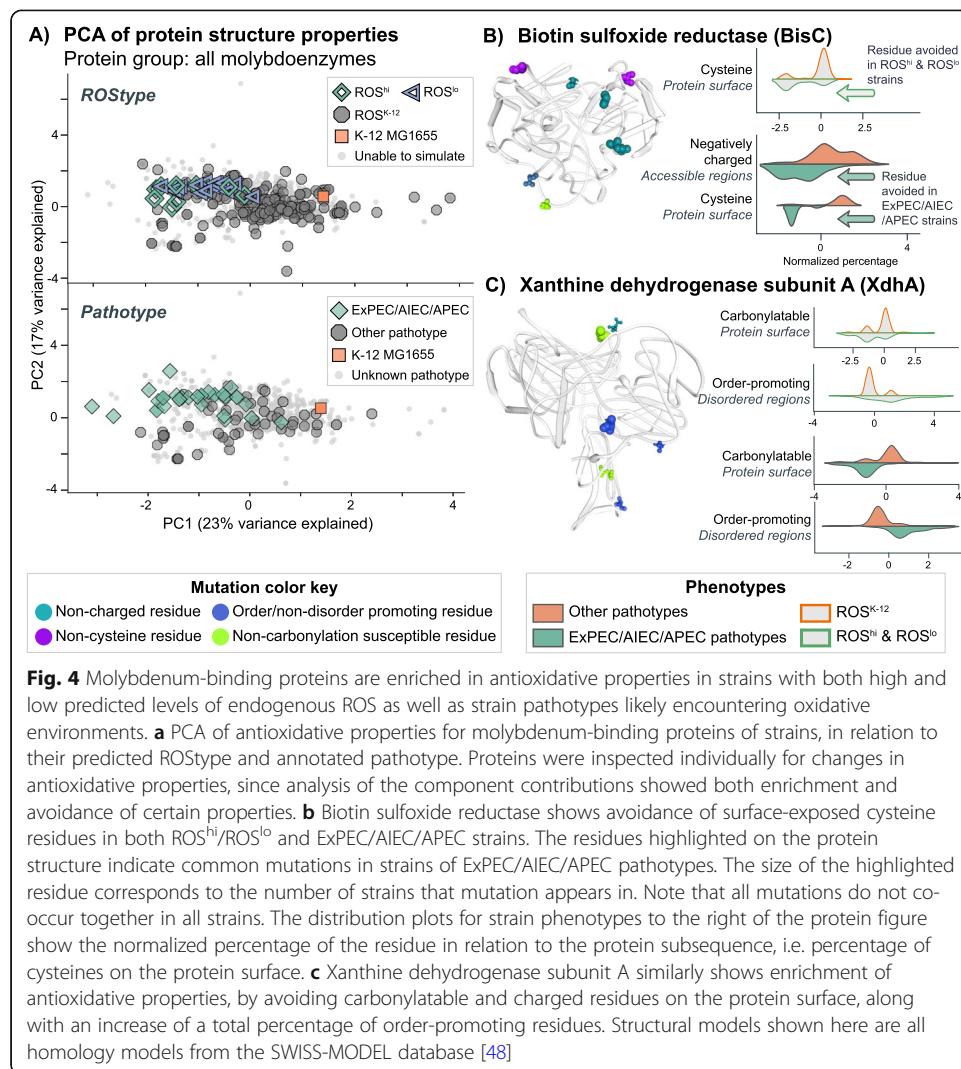


Fig. 2 Classifying strains by predicted endogenous ROS levels and missing reactions that contribute to the predicted phenotype. **a** Simulations of strain-specific metabolic models enable the prediction of endogenous ROS levels, or ROStype. A defined ROStype results from changes to pathway usage due to the deletion of certain reactions from missing genes. Strains are classified by their predicted endogenous ROS levels as “high” (ROS^{hi}) or “low” (ROS^{lo}) ROStypes if their predicted rates of production of hydrogen peroxide (H_2O_2) or superoxide (O_2^-) differ by more than 5% from the measured production rate in the K-12 MG1655 strain (orange dotted line). If predicted endogenous ROS levels do not differ by more than 5%, a strain is classified as similar to MG1655 ($\text{ROS}^{\text{K-12}}$). **b** Histogram of the metabolic subsystems of missing reactions that contribute to the ROStype. Reactions that are shared between strains with ROS^{hi} and ROS^{lo} predictions are denoted as shared missing reactions in gray

not mapped from orthologous genes. Gap-filling of the strain-specific metabolic models was not carried out.

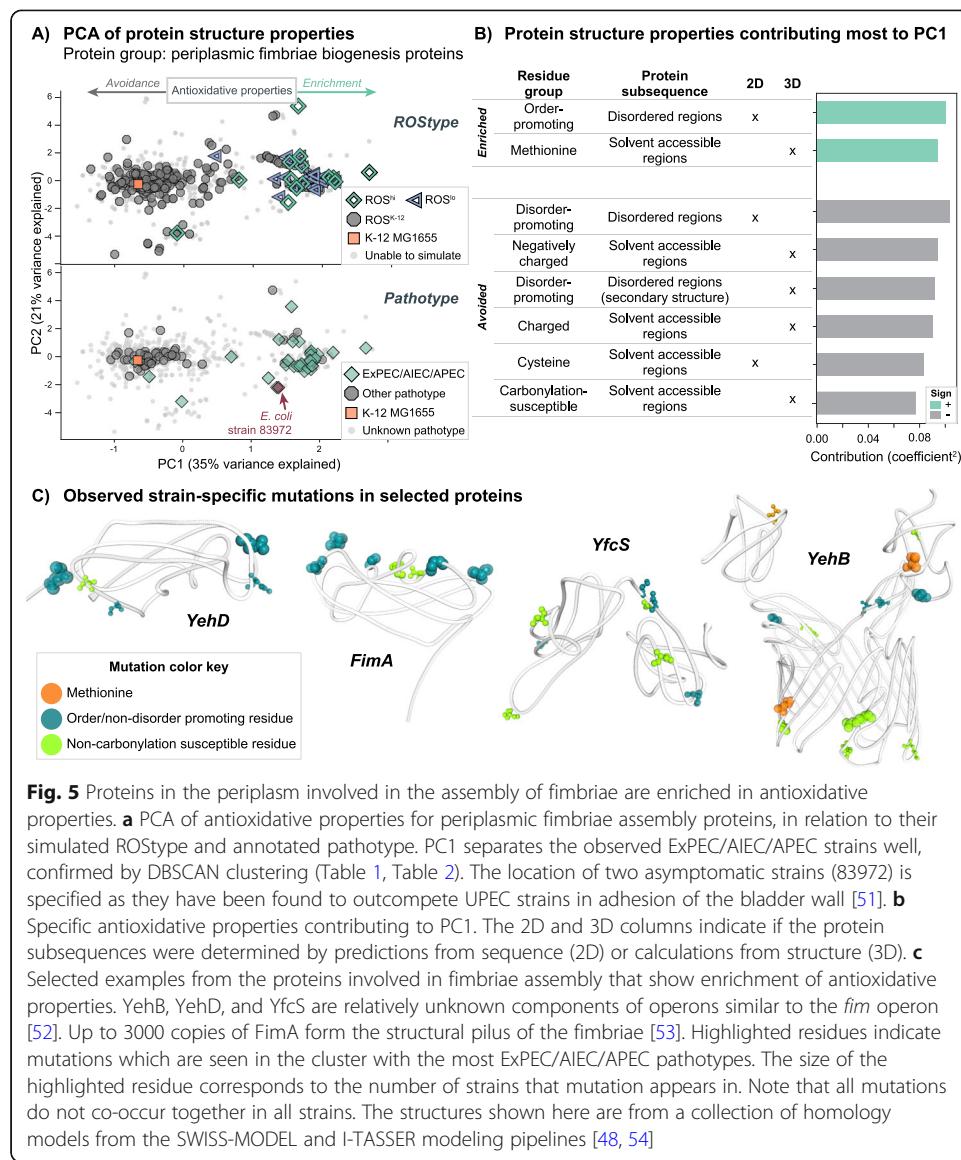
From the set of ROS^{hi} and ROS^{lo} strains, we inspected the gene deletions that resulted in the predicted phenotypes. Computationally, this could have been the case due to a common missing reaction from a set of sequenced strains that had a shared quirk, due to sequencing errors or other technical problems. Closer inspection of the missing reactions revealed no defined set of gene deletions that lead to a ROS^{hi} or ROS^{lo} phenotype, however, there are similarities between the two in terms of which reactions were eliminated. In both classes, the major subsystem of reactions missing due to gene deletions were involved in alternate carbon metabolism and lipopolysaccharide biosynthesis, consistent with other studies of the core and pan genome [46, 47]. Both strain sets shared missing reactions in methionine metabolism, nitrogen metabolism, and inner membrane transport pathways. ROS^{hi} strains were missing additional lipopolysaccharide (LPS) biosynthesis reactions, and the only non-LPS reaction unique to these strains was a deletion of UDP-galactopyranose mutase. ROS^{lo} strains on the other hand, had a variety of missing reactions in different subsystems (Fig. 2a). Interestingly, in most protein groups, the computed antioxidative properties did not significantly cluster apart the ROS^{hi} and ROS^{lo} strains in principal component analysis (PCA) (top panels of Fig. 3a, Fig. 4a, Fig. 5a). It was observed that these strains clustered together, but apart from strains with non-varying levels of endogenous ROS. To verify that these antioxidative features were indeed unable to be used to cluster ROS^{hi} and ROS^{lo} strains apart from each other, a random forest classifier was trained and features were tuned using leave-one-out cross validation. The classifier performed poorly ($AUC < 0.6$) using these features in the reported protein groups of this work, except for the metal-binding enzymes which had a modest AUC of 0.86. This indicates that there may indeed be some discriminating features in the metal-binding enzymes of ROS^{hi} versus ROS^{lo} strains, which is not unexpected as they are main targets of damage.





Known chemical mechanisms of oxidative damage to proteins enable the assessment of a strain's oxidative environment

Pathotype classifications were available for a subset of 116 from the overall set of 1764 strains, while growth/no growth phenotypes in a variety of media conditions were available for up to 650 strains depending on the condition [55]. PCA of the selected antioxidative properties successfully segregated strains with predicted ROStypes (i.e., ROS^{hi} and ROS^{lo} strains vs. ROS^{K-12}) and those with defined pathotypes (i.e., ExPEC/AIEC/APEC vs. other pathotypes) for a number of protein groups (Table 1, Table 2). These groups were ranked by cluster homogeneity following Density-Based Spatial Clustering of Applications with Noise (DBSCAN). Examples of proteins showing significant cluster homogeneity include the general protein groups of all metal-binding enzymes as well as all proteins localized to the periplasm (Fig. 3). Specifically, in these groups, the metal-binding enzymes that utilize a molybdenum cofactor and periplasmic enzymes involved in the assembly of fimbriae showed clear clustering. These specific groups are expanded upon below. Other protein groups with high homogeneity included other extracellular and



motility proteins, murein biosynthesis enzymes, and proteins involved in lipid or inorganic ion transport and metabolism (Table 1, Table 2).

We observed very little segregation of the panel of strains with available growth/no growth phenotypes in various media conditions [55], with our original hypothesis being that strains with growth phenotypes in oxidative environments would show enrichment of antioxidative properties in proteins important for metabolic function. A major reason for this observation was due to a much lower statistical power as many conditions contained a very small number of strains with “no growth” phenotypes compared to those with a “growth” phenotype.

Molybdoenzymes with promiscuous activity are enriched in antioxidative properties

Enzymes that utilize molybdenum as an inorganic cofactor in catalysis carry out a variety of redox reactions in *E. coli* [56] and in all eukaryotic organisms [57, 58]. The

Table 1 Homogenous clusters formed using DBSCAN on the first two principal components, following labeling with ROType

Protein group	Protein count	Cluster count	V-measure
COG I (Lipid transport and metabolism)	97	2	0.33
Metabolism - Murein recycling	40	3	0.30
Manganese-binding enzymes	33	2	0.29
Fimbriae assembly proteins	39	4	0.29
Metabolism - All metabolic proteins	1515	2	0.27
All metal-binding proteins	590	3	0.27
COG P (Inorganic ion transport and metabolism)	198	2	0.27
Metabolism - Inner membrane transport	299	2	0.26
All periplasmic proteins	315	3	0.14
Molybdenum-binding enzymes	14	4	0.14

For ROTypes, both high and low predictions were considered the same label in homogeneity measurements. If a ROType is unavailable for a strain, it was excluded from the homogeneity measurements. A V-measure is the harmonic mean of clustering homogeneity and completeness measures [49].

molybdoenzyme group created through the structural proteome reconstruction was composed of 14 enzymes containing molybdenum-binding sites in their associated UniProt entries (see Additional file 1: Table S5). PCA of their antioxidative properties displayed clear clustering of ROTypes and pathotypes (Fig. 4a). However, analysis of the antioxidative properties contributing to the first principal component revealed conflicting properties among the proteins contained within the group (see XdhD, below). Thus, we proceeded to inspect the properties of each of the 14 molybdoenzymes individually.

The subset of these enzymes that show enrichment of antioxidative properties in the pathotype and endogenous ROS clusters display dual functionalities in many cases. For example, the main function of biotin sulfoxide reductase (BisC) is in biotin salvage (i.e., to reduce the oxidized form of biotin), but it has also been shown to reduce oxidized free methionine [59]. BisC most significantly shows an avoidance of surface-exposed cysteines and negatively-charged residues ($p < 0.0001$, Mann-Whitney U test) (Fig. 4b). An avoidance of surface-exposed methionines was also observed. Trimethylamine-N-oxide (TMAO) reductase 2 (TorZ) reduces the compound TMAO, an alternative

Table 2 Homogenous clusters formed using DBSCAN on the first two principal components, following labeling with pathotype

Protein group	Protein count	Cluster count	V-measure
Fimbriae assembly proteins	24	2	0.47
Metabolism - All metabolic proteins	1515	2	0.44
COG Q (Secondary metabolites biosynthesis)	41	4	0.44
COG N (Cell motility)	27	3	0.41
Metabolism - Alternate carbon metabolism	231	3	0.39
Iron-sulfur-binding enzymes	118	2	0.38
Zinc-binding enzymes	125	3	0.37
All periplasmic proteins	315	2	0.37
All metal-binding proteins	590	3	0.33
Molybdenum-binding enzymes	14	2	0.31

For pathotypes, ExPEC/AIEC/APEC strains are grouped together as they likely encounter oxidative environments more frequently [50]. If a pathotype is unavailable for a strain, it was excluded from the homogeneity measurements.

electron acceptor for anaerobic growth [60, 61]. Additionally, TorZ carries out the same biotin sulfoxide reductase reaction as BisC [62], although at a lower catalytic rate. TorZ displayed properties in the same strain sets trending towards more ordered residues, and less surface-exposed carbonylatable and charged residues. Xanthine dehydrogenase subunit A (XdhA), which showed similar property enrichments as TorZ (Fig. 4c), is involved in purine catabolism and reduces NAD⁺ to NADH in the process [63]. Increases in xanthine levels are potentially indicative of higher rates of DNA damage, such as from ROS [64]. Out of the set of molybdoenzymes which avoided antioxidative properties, most are either known to only have a single function, or do not have their functions well characterized as of yet. As an example, a hypothesized xanthine oxidase (XdhD), displayed changes that shifted it to be more susceptible to damage, such as an enrichment of disordered and carbonylatable residues. These changes may be due to the fact that XdhD cannot carry out the dehydrogenase reaction that XdhA does, since it lacks the FAD-binding domain to catalyze it [63]. As such, being an oxidase, XdhD is involved in the generation of ROS and may not be desirable for use in high ROS environments.

Operons containing type 1 fimbriae biogenesis proteins are enriched in antioxidative properties

Fimbriae are special pili that are synthesized and transferred to the outer membrane of *E. coli*. They are involved in the attachment of the bacteria to their host environments [65]. The *fim* operon is the most well characterized system that assembles type 1 fimbriae [66]. A number of other similar operons are encoded within the K-12 MG1655 genome, but require specific environmental stimuli to be expressed [52]. PCA and subsequent DBSCAN clustering identified this set of proteins as creating highly homogenous clusters, again for both strains with predicted ROStypes and defined pathotypes (Fig. 5a).

One cluster contained a majority of the annotated ExPEC/AIEC/APEC strains (36 of 38), along with two asymptomatic (ABU) 83,972 strains and 8 strains with a variety of other pathotypes (see Additional file 1: Table S6). Another cluster was comprised by a majority of EHEC strains (37 of 65). The antioxidative properties contributing to the first principal component were largely consistent with many of the previously outlined properties hypothesized to contribute to oxidative stress resistance. Specifically, the rightmost strains on PC1 are enriched in order-promoting residues in disordered regions of their proteins, along with solvent accessible methionines. The leftmost strains on PC1 are characterized by amino acid features opposite to providing oxidative stress resistance, such as an increase in disorder-promoting residues in disordered regions, more solvent accessible cysteines, charged residues, and residues susceptible to irreversible carbonylation (Fig. 5b). The proteins that diverged in sequence the most from the orthologous K-12 sequence were those in the *yeh* and *yfc* operons (Fig. 5c). The function of these operons is relatively unknown but hypothesized to assemble other type 1 fimbriae due to their homology to *fim* components [52].

Discussion

Endogenous ROS levels suggest convergent evolution in oxidative stress resistance

The principal component analyses of the antioxidative properties of protein groups revealed that similar adaptive features are displayed by strains with both ROS^{hi} and ROS^{lo} ROStypes. This result was surprising as we had initially expected to see features in opposition to each other, such that ROS^{hi} strains would have adapted their proteomes to deal with this constant source of oxidative stress while ROS^{lo} strains would perhaps vary in other ways unique to their environment. However, it has been found that other organisms adapt to environments of high oxidative stress by lowering their endogenous ROS levels [67], but conversely, high endogenous ROS can allow for natural adaptive mutations to occur lending to a general increased tolerance to oxidative environments [68]. Thus, the relationship between genetic variation, endogenous ROS, and exogenous ROS is complex, but trends towards similar genetic adaptations as seen in our simulation results. A caveat that should be considered by the reader is that a 5% difference in endogenous ROS generation within our simulations only leads to a nanomolar increase in steady state ROS levels, which may not be substantial enough to warrant changes within the proteome. Furthermore, metadata for the gathered *E. coli* strain sequences was sparse with the only consistent annotation being the strain isolation site, which does not show any correlation to oxidative environments. The analysis of strains and their associated genome sequences could benefit greatly from richer and standardized annotations of observed phenotypes for large-scale studies. In regards to the shared antioxidative features seen in both these ROStypes, proteomics methods to quantify damage sites and create a “proteomic signature” of oxidative stress resistance represents a clear path forward to experimentally verify these predictions in the future [69].

Dual functionalities of molybdoenzymes potentially contribute to the oxidative damage repair response

The molybdoenzymes within *E. coli* generally catalyze unique redox reactions under aerobic conditions, such as biotin salvage, and also enable the usage of alternative electron acceptors in anaerobic conditions, such as nitrate [56]. Due to their redox capabilities, some molybdoenzymes can also act promiscuously to reverse oxidation events to other metabolites such as methionine [59]. Interestingly, the standard repair system for methionine sulfoxide - specifically in the stressful oxidative environment of the periplasm (MsrPQ) - utilizes molybdenum as its cofactor of choice and is able to reduce both stereoisomers of oxidized methionine [8]. The proposed stability of these molybdoenzymes under oxidative conditions suggests two factors: 1) that these enzymes would be upregulated in response to oxidative stress to reduce their main binding partners that are being oxidized by ROS, and 2) that they may be called upon to carry out their promiscuous repair functions on other oxidized metabolites.

Although the function of molybdoenzymes in anaerobic respiration seems unrelated to oxidative stress, there may be reasons for their use in aerobic conditions. Interestingly, a previous study indicated the metabolite trimethylamine-N-oxide (TMAO) to confer protein structural stability in vitro, by stabilizing charged residues, disordered regions, and preventing protein aggregation [70]. The identified TorZ enzyme in this analysis may then have a role in maintaining reduced TMAO in conditions of oxidative

stress. The usage of nitrate as an electron acceptor in anaerobic respiration generates reactive nitrogen species, which, similarly to ROS, damage cysteine and additionally tyrosine residues [71]. Manual analysis of TorZ and BisC structures displayed avoidance of surface-exposed tyrosines, suggesting that similar structural analysis could be carried out for other sites of protein damage.

Type 1 fimbriae biogenesis operons and their use in oxidative environments

The type 1 fimbriae biogenesis components are interesting to approach from the standpoint of oxidative damage resistance due to three reasons. First, the assembly of the fimbriae depends on an oxidation event to create a single disulfide bond on the components that make up the tip of the fimbriae, which ends up adhering to the environmental surface [72]. Second, there are many similar operons with homologous genes that encode for other fimbriae, supposedly for different environmental conditions [52]. Third, the expression of these components is sensitive to oxygen levels, being inactive under anaerobic conditions [73]. Fourth, recent work has identified this group of proteins as a more discriminatory typing assay to identify UPEC strains [74]. We grouped together ExPEC, AIEC, and APEC strains since their encountered environments are associated with high oxidative stress and because of their similarity in both phylogenetic origin and virulence factors [75–77].

The assembly of the fimbriae begins when a subunit is translocated into the periplasm and bound to a chaperone, which accompanies the subunit to the outer membrane “usher” (collectively known as the chaperone-usher pathway). This binding event depends on the subunit being oxidized by DsbA, a periplasmic enzyme that creates and maintains disulfide bonds [72]. The formation of disulfide bonds would be accelerated by an environment with higher levels of ROS, but would additionally demand the chaperone to have antioxidative properties if the fimbriae were to properly assemble. Additionally, previous studies have shown that most sequence variation on the extracellular fimbriae subunits do not decrease the specificity of adhesion to carbohydrates [78]. However, specific point mutations on the FimH adhesin do provide higher adhesion capabilities [79]. The results presented here suggest that variations are likely implicated in greater stability during translocation and when being presented on the exposed regions of the fimbriae. The existence of the other type 1 fimbriae operons points to a highly adaptable set of adhesins available to an *E. coli* cell. Finally, the finding that the expression of these operons responds to changes in oxygen levels [73] points to a likely link between their antioxidative properties and survival within an oxidative environment.

The two *E. coli* ABU 83972 strains in the cluster of ExPEC/AIEC/APEC strains have been shown to outcompete UPEC strains in colonization of the bladder [80]. The similar properties of their type I fimbriae biogenesis proteins may point towards a similar resistance to oxidative damage in the urinary tract, which is a stressful environment during urinary tract infection [81, 82]. We hypothesize that the greater stability of the biogenesis enzymes under these conditions potentially allow these nonpathogenic strains to assemble their fimbriae and colonize the bladder, outcompeting the UPEC strains. This finding suggests that further experimental work to characterize these

relatively unknown fimbriae operon components may elucidate adherence properties of *E. coli* strains.

Conclusion

In this study, we have applied two protocols in a structural systems biology approach to develop an understanding of the genotype-phenotype relationship. At the systems-level, we utilized strain-specific genome-scale metabolic models to predict levels of endogenous ROS, while also guiding orthologous gene mapping of strains for sequence-level variation analysis. With structural information, we mapped this variation to specific groups of proteins and their location within three-dimensional space. Specific protein groups were identified as differentiating in regards to their antioxidative properties. The analysis of 1764 sequenced strains confers strong evidence pointing towards further experimental study of the contributions of molybdenum-binding enzymes as well as fimbriae assembly proteins in the oxidative stress response. The workflow presented here also demonstrates a method for understanding important features of the structural proteome to enable modeling of different stress responses in silico. Looking forward, the exploration of natural variation in regards to enzymatic capabilities to confer a specific environmental advantage could be applied in the creation of fine-grained metabolic models taking into account the properties of the structural proteome. In the lab, this approach could also guide drug development pipelines by identifying susceptible targets as well as library design for directed evolution experiments in protein engineering.

Methods

Construction of the *E. coli* structural proteome

We applied an existing pipeline to create genome-scale models of metabolism with protein structures (GEM-PRO models) [43, 83] for the entire proteome of *E. coli* str. K-12 substr. MG1655 (reference proteome downloaded from UniProt [84] on March 20, 2018), using the current implementation contained in the ssbio Python package [44]. This pipeline results in the selection of a single representative three-dimensional tertiary protein structure, either from experimental data in the Protein Data Bank [85] or from homology models generated from the I-TASSER pipeline [54] and the SWISS-MODEL repository [48]. The representative structure is selected after a number of quality checks, which include 1) aligning the reference sequence to all available structures and ranking them based on their percent identity and sequence completeness (i.e. structures with missing portions outside the N- or C- termini are ranked lower than those with complete inner portions); 2) for experimental structures, ranking based on their experimentally determined resolution; and 3) for homology models, ranking them based on their provided quality scores (c-scores for I-TASSER models [54] and QMEAN scores for SWISS-MODEL models [86]), where I-TASSER models are preferentially chosen over SWISS-MODEL models. The list of available structures is first trimmed based on selected cutoffs for sequence identity and completeness (not missing more than 10% of the length of the sequence on both termini, no insertions or deletions, and over 60% sequence identity to the reference sequence), resolution ($< 3 \text{ \AA}$), and quality score (QMEAN >-4 or c-score > -1.5). Next, if experimental structures remain, the one with the highest sequence identity, completeness,

and resolution is selected. If there are no experimental structures that remain, the top ranking homology model is selected.

In addition to the methodology reported above, a number of improvements were implemented for this study and are slated for incorporation into the publicly available pipeline in ssbio. These improvements include 1) the selection of representative experimental structures with the consideration of bound substrates or cofactors [87]; 2) the definition of membrane spanning domains in transmembrane proteins by consolidating information as predicted by TMHMM [88] or OPM [89] and as annotated in UniProt; 3) the selection of quaternary structures of protein complexes by a breadth-first search matching algorithm to determine the structure with the highest quality and coverage of annotated subunits, either from biological assemblies in the PDB or from predicted complexes in the SWISS-MODEL repository.

For metal-binding proteins specifically, we implemented a more rigorous selection scheme due to their importance in oxidative stress tolerance. Annotated binding residues were retrieved from each protein's UniProt entry, and mapped to the correct residue numbering scheme in the structure file. The representative structure for the metal-binding protein was then based on the following four factors: 1) sequence identity, coverage, and resolution as described above; 2) presence of the annotated metal binding site in the solved structure; 3) presence of the metabolic model-annotated metal ion; and 4) presence or absence of any model-annotated cofactors other than the metal ion. The MetalPDB database [38] was used to help expedite this analysis, as they provide extracted metal-binding sites from the PDB structures that can be used to verify the presence of the metal ion along with the residues involved in binding. Per protein, these factors contributed to a weighted score enabling a custom rank-ordering of all available structures, leading to a final structure that best represents the enzyme and its state in the metabolic model.

Division of the proteome into groups

To delineate search spaces within the structural proteome we created groups of proteins first defined by their localization, and then defined by their functional assignment (Fig. 1a, Additional File 1: Table S1). Localization within a cell is defined by the categories: outer membrane, periplasm, inner membrane, and cytosol. This information was taken from a consensus of a previously generated genome-scale model of proteome synthesis [90], proteomics information from [91], the Echo-LOCATION database [92], the UniProt database, and finally predictions from TMHMM [88] if no other information was available. Secondary functional groups were either created with 1) the clusters of orthologous group (COG) categories [93]; 2) metabolic network subsystem as defined in iML1515; 3) metal-binding enzymes as annotated in UniProt; and 4) manual assignment in relation to ROS generation or repair. The manually curated list of proteins (see Additional file 1: Table S2) were collected based on their functional relation to oxidative stress levels in *E. coli*. These include proteins that are known generators of reactive oxygen species, are involved in the repair of oxidative damage to macromolecules, are involved in regulation of metal ion transport, etc. A table summarizing the protein groups can be found in Additional file 1: Table S1.

Protein property calculations and division into subsequences

The physicochemical properties of each representative protein's sequence and structure were calculated using a variety of tools and stored as per-residue annotations using ssbio. The properties used in this study include: 1) definitions of protein "sites" (i.e. binding, catalytic, or active sites) as annotated in UniProt, the Catalytic Site Atlas [94], and MetalPDB [38]; 2) regions of protein flexibility and disorder as retrieved from PDBFlex [95] or predicted from sequence with DisEMBL [96] and IUPred [97]; 3) parameters of solvent accessibility as calculated using FreeSASA [98] or predicted using SCRATCH [99]; 4) parameters of residue depth (a calculation of the distance in Angstroms of a residue from the surface of the protein [100]) as calculated using Biopython [101] and MSMS [102]; and 5) definitions of secondary structure using DSSP [103] or predicted using SCRATCH. We additionally incorporated the locations of known oxidizable cysteines from RedoxDB [40], known carbonylatable amino acids (R, K, P, T) from CarbonylDB [39], and predicted disulfide bridges using a distance-based metric (3 Å cutoff) in an extension of the PDB module in Biopython (<http://biopython.org/wiki/Struct>).

The aforementioned properties were then used to delineate search spaces within single proteins into what we refer to as "protein subsequences" (Fig. 1b). In all cases, there exists at least one of the following methods to compute their definition: 1) "2D subsequences", which are simply a 3D structural feature predicted from the amino acid sequence (such as running SCRATCH for predicting secondary structure); 2) "2.5D subsequences", that only apply to sites of a protein that have an annotated site feature in a sequence database such as UniProt and can be mapped to a 3D structure, but for which there exists no structural evidence of the binding (an example of this would be an annotated metal-binding site that is annotated in UniProt, but no metal ion is present in the experimental structure); and finally 3) "3D subsequences" for which clear structural evidence is available (such as a calculated secondary structure).

To give an illustrative example for one subsequence, let us outline the procedure for "surface" residues. If a 3D structure was available for a protein, residue depth and solvent accessibility algorithms are run on this structure. If a 3D structure is not available, we fall back to defining a 2D subsequence by running a solvent accessibility predictor algorithm (SCRATCH) on the protein sequence. Next, a surface residue is defined as one with a relative solvent accessibility of above 25%, and if a 3D structure was available, an additional constraint of residue depth of less than 2.5 Å. These cutoffs were then applied to all residues in a protein sequence and those that meet the cutoff then form a defined surface subsequence (this corresponds to Additional file 1: Table S3, on the first row). Other properties that were utilized to form protein subsequence definitions are: disordered regions, transmembrane domains, solvent-exposed residues surrounding a sphere of a defined radius around a metal-binding site, catalytic site, DNA-binding site, or any other annotated generic site, and residues within ROS-sensitive sites as found in RedoxDB or CarbonylDB. For a summary table describing all cutoffs used, prediction methods, or structural calculations for these features, please see Additional file 1: Table S3.

Gathering strains, phenotypes, and pathotypes

The proteomes of *E. coli* strains in this study were gathered from three separate sources: 1) the Ecoref strain panel [55]; 2) the iML1515 metabolic network

reconstruction resource [42]; and 3) a manually curated set of adherent-invasive *E. coli* (AIEC) strains. The Ecoref strain panel is a published panel of 696 strains that were tested for growth/no growth under a number of media conditions. The sequenced genomes, resulting protein sequences, and pathotype annotations were downloaded from the publicly accessible database (<https://evocellnet.github.io/ecoref/download/>). Out of the 214 media conditions, 24 of which include a chemical that induces oxidative stress in some manner were selected for this study (see Additional file 1: Table S7). Out of the 696 strains, 676 were selected due to the presence of phenotypic data under the selected media conditions. From the iML1515 resource, the proteomes and pathotype information of 1045 strains of *E. coli* were downloaded from the PATRIC database [104]. The number differs from the original reported number of strains in the resource due to database updates and removal of poorly annotated genomes. The strains obtained from Ecoref and PATRIC also contained pathotype information for 116 of the strains. Finally, the manually curated set of 23 AIEC strains was obtained through literature review and downloading of the individual genomes from various publications [105–111]. Two pathotype groups was created by 1) extra-intestinal pathogenic (ExPEC), adherent-invasive (AIEC), and avian pathogenic (APEC) strains (which have been shown to be similar to ExPEC strains, see [112, 113]) and 2) all other pathotypes. This grouping was chosen to discriminate pathotypes by the oxidative environments they may encounter.

Simulation of strain-specific endogenous ROS levels

The construction of strain-specific metabolic models follows a previously established protocol [114]. Briefly, this involves creating a presence/absence orthology matrix of proteins in a strain following orthology detection through bidirectional-best BLAST hits (BBH) at an 80% sequence identity cutoff. The orthology matrix is then used to trim reactions in a metabolic model given a protein's usage in a reaction. Instead of trimming the original metabolic model of *E. coli* K-12 MG1655, the iML1515-ROS model was used as the base model. Based on a previously developed model [41], iML1515-ROS includes 298 reactions that have the potential to produce H_2O_2 and O_2^- [42]. Thus, the strain-specific ROS models predicts changes in endogenous ROS production levels based on the deviation from the measured MG1655 ROS production rates.

Ensemble models (sampling different stoichiometric coefficients of the ROS generating reactions) of each strain were then simulated to sample total endogenous production of ROS. Simulations were conducted under glucose minimal media per [41]. The mean H_2O_2 and O_2^- production rates were normalized to the growth rate, thus assigning per-strain predictions of $mmol\ H_2O_2\ gDW^{-1}$ and $O_2^- gDW^{-1}$. The deviations of endogenous ROS production from the “wild-type” MG1655 strain were classified as “high” or “low” if they deviated above or below 5% of the measured ROS production levels, and “non-varying” if otherwise [45]. This cutoff was chosen as it was previously found that increasing endogenous ROS production rates above this level resulted in a higher likelihood of cell death after treatment with antibiotics, indicating that ROS detoxification methods are compromised [41].

Characterization of strain-specific changes

The orthology matrix used to generate strain-specific models was used to align orthologous strain protein sequences to the K-12 proteins, using default parameters in the

Needleman-Wunsch pairwise alignment tool in the EMBOSS package [115]. All orthologous sequences were loaded into their related Protein objects using the ssbio Python package, and alignments were executed in parallel using the Apache Spark Python API (PySpark, <https://spark.apache.org>). From this, strain-by-feature matrices describing the proteomic features of all strains and the averaged antioxidative properties were generated (see Additional file 1: Table S8, for a description of the types of averaged properties calculated per subsequence). To deal with missing data in the case of portions of protein sequences that have been truncated (either due to technical reasons such as genome sequence or annotation errors, or true deletions of a strain's sequence compared to MG1655), we only compared sequences where the aligned length was greater than or equal to 80% of the original K-12 sequence length. In the case of proteins absent from certain strains in protein groups, missing values were imputed using mean imputation for percentages of physicochemical properties.

The strain-by-feature matrices are created for principal component analysis (PCA) as follows. For all combinations of protein groups and subsequences, amino acid ratios were calculated relative to the subsequence length. Ratios of certain groupings of amino acids were also calculated, such as from the number of positively charged, bulky, or disorder promoting residues. These groupings were again chosen due to previous hypotheses that enrichment or avoidance of these changes may be associated with resistance to oxidative damage [7, 9, 26, 31, 116]. All groupings are defined in Additional file 1: Table S9.

As an example, let us take the protein group of cytoplasmic, metal-binding enzymes, retrieved from UniProt and defined in row 11 of Additional file 1: Table S1. The subsequences of all of their metal-binding sites are next defined (Additional file 1: Table S3, row 8). Next, important changes within these binding sites are manually defined (Additional file 1: Table S8, rows 12–18). These changes are stored as percentages of the full subsequence length, in order to summarize changes in bulk. If a strain has an overall avoidance of positively charged residues in proximity to the metal-binding site, this is reflected as a lower overall percentage stored for this strain's protein. Finally, for the group of metal-binding enzymes, the percentages are averaged together and normalized for sequence length. The strain-by-feature matrix thus contains the strain identifiers as the columns, and summarized features of their metal-binding enzymes as the rows.

Statistical analysis of protein groups

The data set gathered here could potentially be run through unsupervised, semi-supervised, or supervised learning algorithms due to the existence of labels (ROStype or pathotype) for only a portion of the strains. Unsupervised learning was carried out due to the desire to utilize the entire set of observations (strains) along with their features, to understand if variability existed in the dataset without biasing for the assigned labels, which could potentially be incorrect due to their nature: ROStype being solely a simulated prediction and pathotype coming from multiple disparate annotation databases.

For each protein group, subsequence, and associated strains, features were first standardized to be centered around a zero mean with unit variance, using the preprocessing module from the Python package scikit-learn [117]. Features were then filtered for

PCA following the hierarchical clustering by rank correlation coefficient method as presented in [26, 118]. Briefly, this clusters the features together based on Spearman rank correlation, and cuts off similar features over a coefficient of 0.9, keeping the feature closest to the center of the cluster as representative. Since the availability of features varied from prediction from sequence and calculation from structure, this allowed for the filtering out of redundant features when both predictions and calculations were available. To further filter down the feature set before PCA, we created manual filters for features specific to a protein group. For example, if the group to inspect was a set of metal-binding proteins, only then would features specific to the metal-binding site (such as the percentage of cysteines in proximity to site) be included in the feature matrix. A table describing these group specific features is included in Additional file 1: Table S4. PCA was then carried out to understand if specific features contributed to the differentiation of the following: 1) growth/no growth phenotypes in different media conditions; 2) strains with predicted ROStypes higher or lower than “wild-type” (K-12) endogenous ROS levels; and 3) annotated strain pathotypes. To do so, observation labels associated with these phenotypes were assigned back to the transformed data. Next, we wanted to identify protein groups which showed the largest separation between phenotypes. Clustering of the transformed data points on the principal components (PC1 and PC2) was carried out using Density-Based Spatial Clustering of Applications with Noise (DBSCAN) [119]. The “performance” of the clustering relative to the labeled phenotypes acted as a proxy to judge which protein groups showed the best clustering. Performance was measured and ranked using the V-measure [49] which reports the harmonic mean of homogeneity (if a cluster contains only one label type) and completeness (if a label type is assigned to the same cluster) and ranges from 0 to 1, with a value of 1 denoting good clustering.

Training random forest classifiers

To understand if the identified proteome properties can be used to distinguish between ROS^{hi} and ROS^{lo} ROStypes, we trained random forest classifiers using the R package randomForest (version 4.6–14) [120, 121]. Proteomic features were centered and scaled before training. The number of features that were sampled at each split (i.e., the hyper-parameter mtry) was tuned using leave-one-out cross-validation by selecting the highest area under the ROC curve that resulted from ten equidistant integers between two and the respective number of features. This cross-validation procedure was implemented in the caret package version 6.0–82 [122].

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12859-020-3505-y>.

Additional file 1.

Abbreviations

ROS: Reactive oxygen species; GEM-PRO: Genome-scale metabolic model with protein structures; iML1515-ROS: Metabolic model of *E. coli* with additional ROS generating reactions; ROStype: Classification of strain by predicted basal endogenous ROS levels using iML1515-ROS; ROShi: Strain with predicted higher levels (+ 5%) of endogenous ROS relative to K-12 MG1655; ROSlo: Strain with predicted lower levels (- 5%) of endogenous ROS relative to K-12 MG1655; RKPT: Arginine, lysine, proline, threonine - amino acids which are carbonylatable; COG: Clusters of orthologous group; PCA: Principal component analysis; DBSCAN: Density-based spatial clustering of applications with noise; ROC: Receiver operating characteristic curve; AUC: Area under curve in a ROC curve; ExPEC: Extra-intestinal

pathogenic *Escherichia coli*; AIEC: Adherent-invasive *Escherichia coli*; APEC: Avian pathogenic *Escherichia coli*; EHEC: Enterohemorrhagic *Escherichia coli*; UPEC: Uropathogenic *Escherichia coli*; TMAO: Trimethylamine-N-oxide

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Authors' contributions

NM, JMM, LY and BOP designed research; NM, JMM performed research; NM, EC, and DH contributed new analytic tools; JMM and XF contributed strain sequences, NM and JMM analyzed data; and NM and BOP wrote the paper. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the results of this article are included within the article and the tables within Additional file 1. The methods used to analyze the data are part of a publicly available Python package at <https://github.com/SBRG/ssbio>. Additional code used to run the analysis is available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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