

Research



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Plasma-sensitive *Escherichia coli* mutants reveal plasma resistance mechanisms

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Non-thermal atmospheric pressure plasmas are investigated as augmenting therapy to combat bacterial infections. The strong antibacterial effects of plasmas are attributed to the complex mixture of reactive species, (V)UV radiation and electric fields. The experience with antibiotics is that upon their introduction as medicines, resistance occurs in pathogens and spreads. To assess the possibility of bacterial resistance developing against plasma, we investigated intrinsic protective mechanisms that allow *Escherichia coli* to survive plasma stress. We performed a genome-wide screening of single-gene knockout mutants of *E. coli* and identified 87 mutants that are hypersensitive to the effluent of a microscale atmospheric pressure plasma jet. For selected genes (*cysB*, *mntH*, *rep* and *iscS*) we showed in complementation studies that plasma resistance can be restored and increased above wild-type levels upon over-expression. To identify plasma-derived components that the 87 genes confer resistance against, mutants were tested for hypersensitivity against individual stressors (hydrogen peroxide, superoxide, hydroxyl radicals, ozone, HOCl, peroxyxynitrite, NO•, nitrite, nitrate, HNO₃, acid stress, diamide, heat stress and detergents). k-means++ clustering revealed that most genes protect from hydrogen peroxide, superoxide and/or nitric oxide. In conclusion, individual bacterial genes confer resistance against plasma providing insights into the antibacterial mechanisms of plasma.

1. Introduction

The steady discovery of multi-drug-resistant bacteria and cancers is a serious threat in modern medicine [1]. Therefore, development of new strategies, which can act alongside antibiotics and anti-cancer agents, is of growing interest. One of those therapies is the application of cold atmospheric pressure plasmas [2–4]. Several devices for plasma generation are already in clinical use for treatment of wounds or cancer, such as the kINPen MED plasma jet of neoplas (Greifswald, Germany) [5], the PlasmaDerm dielectric barrier discharge of Cinogy (Duderstadt, Germany) [6] or the MicroPlaSter or SteriPlas devices of Adtec Healthcare (Hounslow, UK) for treatment with a plasma effluent [7]. Plasma jets and plasma effluent devices use a defined gas mixture, which is ionized by application of an electric field forming the gas plasma. The plasma effluent is then blown out of a jet nozzle directly onto the patient's wound [8]. The effluents consist of a variety of reactive species, mostly oxygen and nitrogen-based such as atomic oxygen (O•), superoxide (O₂⁻), ozone (O₃), excited oxygen and nitrogen, and/or nitric oxides (N_xO_y). Upon interaction with an aqueous sample even more reactive species are formed (•OH, H₂O₂ and peroxyxynitrite) [9]. Several mechanisms have been described for bacterial inactivation by plasma including the oxidation or irreversible modification of essential enzymes [10] and the perturbation of the cell envelope, e.g. by lipid peroxidation [11]. Further, due to UV radiation and DNA-damaging species

(e.g. hydroxyl radicals) plasma also introduces modifications to DNA causing strand breaks and mutations [12,13].

Several transcriptomic [14–16], proteomic [17,18] and metabolomic studies [19] on prokaryotic as well as eukaryotic cells were performed to gain system-wide insights into the effects of plasma on cells, the cellular response to plasma treatment and cellular defences against plasma-derived reactive species. Inspired by these studies that demonstrated upregulation of cellular stress responses as a means of physiological adaptation, we performed a genome-wide screening in *Escherichia coli* for plasma-protective genes that confer plasma resistance to the *E. coli* wild-type. Although different definitions of the term ‘resistance’ can be found in the antibiotic research literature depending on the field of research (e.g. epidemiology or clinical perspective [20]) all definitions have in common that resistance is based on genetic alterations caused by mutations or horizontal gene transfer, and thus a heritable trait [21]. In contrast to resistance, the term ‘tolerance’ refers to the transient state of a subpopulation of genetically identical bacteria that allows this subpopulation to survive antibiotic treatment better than their growing peers. Tolerance is thus not based on a genetic difference, but the result of a different cellular state. One example is the dormancy of cells that refers to a state of reduced metabolism that makes the dormant subpopulation less vulnerable to antibiotics than the fast-growing part of the population [20,21]. In the present study, we address the phenomenon of plasma resistance, since *E. coli* strains with different genetics were analysed (deletion mutants or strains over-expressing certain genes). Typically, vegetatively growing bacteria survive only short plasma exposures. We show here, that mutants lacking certain protective genes are hypersensitive and survive even shorter plasma exposures. This difference in plasma sensitivity indicates that there are genes that, by means of the encoded protein products, confer a basic level of plasma resistance to wild-type cells. The identification of plasma-protective genes helps identify targets of plasma in cells contributing to our understanding of plasma-mediated bacterial inactivation. The characterization of inherent protection mechanisms may also provide insights into genetic changes that lead to bacteria surviving longer plasma exposures, giving rise to plasma-resistant strains in the future.

2. Results

2.1. Screening for plasma-sensitive mutants

A collection of *E. coli* single-gene knockout mutants (KEIO collection) [22] was screened to identify genes and proteins that protect wild-type cells when exposed to non-thermal plasma. This strain collection of 3985 strains each lacking a single non-essential gene is comprehensive. It covers the entire *E. coli* genome. The screening was performed with a microscale atmospheric pressure plasma jet (μ APPJ) operated with He/O₂ as feed gas [8]. Similar devices are already used as medical devices to treat patients [21]. Screening was performed by growing the mutant strains in 96-well microtitre plates in LB broth overnight and then spotting 2 μ l of culture onto LB agar plates with a replicator. Spotted cells were exposed to the effluent of the plasma jet for 100 s and incubated at 37°C for 16 h. When the wild-type was spotted and exposed, colonies were observed after the 16 h incubation. To identify knockout strains with increased plasma

sensitivity, each strain was spotted and exposed three times in independent experiments and the results were scored. Mutants accrued one point for each experiment in which no colonies were detected after the 16 h incubation. Score values of 2 indicated that the knockout mutant did not survive plasma treatment in two out of three biological replicates (electronic supplementary material, figure S1). Mutants with a score of 2 or higher were termed ‘plasma sensitive’ and analysed further.

Of the 3985 mutants tested, 87 (equalling 2.2%) exhibited an increased plasma sensitivity (table 1). Information on the encoded proteins was obtained from the NCBI and UniProt databases [23]. Protein properties of the set of 87 were compared to all *E. coli* proteins with regard to subcellular protein localization, metal cofactors, amino acid representation and functional category to identify potential patterns in the data.

The 87 proteins were analysed with regard to their subcellular localization to see if e.g. extracellular proteins were over-represented in the screening (figure 1a). While the majority of the 87 proteins are located in the cytoplasm, compared to all proteins of *E. coli*, the set of 87 is enriched in proteins with extracellular localization, i.e. proteins located in the outer membrane or in the periplasm. For instance, in this screening FlgB and FlgG (flagellar basal-body rod proteins) [24] as well as FimC and FimD (type 1 fimbriae formation) [25] came up. Based on our current knowledge of the proteins, their role in protecting the cells against plasma is not obvious. Possibly, extracellular proteins act as unspecific scavengers of reactive species at the cell surface.

Transition metals are redox-active and many enzymes, such as catalases or superoxide dismutases contain metal cofactors that are needed to complete the catalytic cycle [26,27]. We therefore compared the set of 87 proteins to all *E. coli* proteins with regard to metal cofactor content (figure 1b). We found that iron-containing proteins were over-represented (37% in our dataset compared to 26% in all proteins encoded in the *E. coli* genome), but no over-representation was observed for other metal ions or the overall presence of metal cofactors.

It has been observed previously that some amino acids are more susceptible to plasma-induced modifications than others [28]. Cysteine for instance is one of the most vulnerable amino acids as it is easily oxidized to sulfenic or sulfonic acid [10,27–29]. We investigated the amino acid composition of the proteins in the set of 87 proteins and compared it to the average for *E. coli* proteins. However, on the whole, no amino acid was over-represented significantly, neither those with comparably reactive groups like cysteine, nor those with aromatic rings like tryptophan or histidine (figure 1c). The average cysteine content of *E. coli* proteins is 1.3% (figure 1c). And while most of the 87 plasma-protective proteins have a similar cysteine content (on average 1.4%), YdhX contains 7.2% cysteines (16 cysteine residues of 222 amino acids total). It ranks among the top 30 *E. coli* proteins with regard to relative cysteine content, making YdhX a very cysteine-rich protein. YdhX is a predicted [4Fe-4S]-ferredoxin-type protein with yet unknown function located in the outer membrane facing the periplasm [29]. Interestingly, in the screening proteins directly involved in asparagine (AsnB), cysteine (CysB) and tryptophan (TrpB) biosynthesis were identified as protective, suggesting that these amino acids might be of special importance as free amino acids and/or as sulfur donors for repair mechanisms after plasma treatment.

Table 1. Plasma-protective genes. Strains of the KE10 collection were spotted onto LB agar and exposed to the effluent of the μ APPJ for 100 s. The jet was driven with He and a 0.6% O₂ admixture. Genes were listed as protective when the respective knockout mutant did not form colonies after plasma exposure and a 16 h incubation in at least two out of three experiments. Annotations are based on NCBI database.

#	gene	protein	NCBI ID	#	gene	protein	NCBI ID
1	<i>abgT</i>	p-aminobenzoyl glutamate:H ⁺ symporter	945912	29	<i>holC</i>	DNA polymerase III, χ subunit	948787
2	<i>ada</i>	DNA-binding transcriptional regulator	946710	30	<i>holD</i>	DNA polymerase III, ψ subunit	948890
3	<i>apaH</i>	diadenosine tetraphosphatase	944770	31	<i>ihfA</i>	integration host factor, α subunit	945472
4	<i>asnB</i>	asparagine synthetase B	945281	32	<i>ihfB</i>	integration host factor, β subunit	945533
5	<i>atoA</i>	β subunit, acetate CoA transferase	946719	33	<i>iscS</i>	cysteine desulfurase	947004
6	<i>atoE</i>	short chain fatty acid transporter	946721	34	<i>iscU</i>	scaffold protein for iron-sulfur cluster assembly	947002
7	<i>bamb</i>	outer membrane protein assembly complex	946982	35	<i>katE</i>	catalase II	946234
8	<i>bcsC</i>	cellulose biosynthesis protein	948047	36	<i>lpd</i>	lipoamide dehydrogenase	944854
9	<i>carA</i>	carbamoyl phosphate synthetase	949025	37	<i>lsiD</i>	autoinducer-2 ABC transporter	946264
10	<i>cpxA</i>	sensory histidine kinase	948405	38	<i>merC</i>	O-succinylbenzoate synthase	946734
11	<i>csgG</i>	curli secretion channel	945619	39	<i>mgIB</i>	D-galactose/D-galactoside ABC transporter	949041
12	<i>cysB</i>	transcriptional regulator	945771	40	<i>mngA</i>	2-O- α -mannosyl-D-glycerate PTS permease	945355
13	<i>degP</i>	serine protease Do	947139	41	<i>mntH</i>	Mn ²⁺ /Fe ²⁺ :H ⁺ symporter	946899
14	<i>dksA</i>	RNA polymerase-binding transcriptional factor	944850	42	<i>moaC</i>	cyclic pyranopterin monophosphate synthase	945397
15	<i>dnaT</i>	primosomal protein	948813	43	<i>nagC</i>	DNA-binding transcriptional dual regulator	945285
16	<i>ecnB</i>	bacteriolytic entericidin B lipoprotein	2847737	44	<i>napC</i>	periplasmic nitrate reductase	946706
17	<i>essQ</i>	Qin prophage, lysis protein	946093	45	<i>nlpE</i>	outer membrane lipoprotein involved in surface sensing	946782
18	<i>fimC</i>	periplasmic chaperone for type 1 fimbriae	948843	46	<i>appD</i>	murein tripeptide ABC transporter	945802
19	<i>fimD</i>	export and assembly of type 1 fimbriae	948844	47	<i>oxyR</i>	OxyR DNA-binding transcriptional dual regulator	948462
20	<i>figB</i>	flagellar basal-body rod protein FigB	945678	48	<i>pepQ</i>	XAA-Pro dipeptidase	948335
21	<i>figG</i>	flagellar basal-body rod protein FigG	945647	49	<i>pgm</i>	phosphoglucosyltransferase	945271
22	<i>ftn</i>	ferritin iron storage protein	946410	50	<i>ppp</i>	polynucleotide phosphorylase	947672
23	<i>fucA</i>	L-fucose-phosphate aldolase	947282	51	<i>poxA</i>	EF-P-lysine lysyltransferase	7156434
24	<i>gspL</i>	putative protein secretion protein for export	947842	52	<i>prfA</i>	primosome factor N'	948426
25	<i>guaA</i>	GMP synthetase	947334	53	<i>purH</i>	AICAR transformylase/IMP cyclohydrolase	948503
26	<i>hdeB</i>	acid stress chaperone	948026	54	<i>rscC</i>	sensory histidine kinase	948993
27	<i>hicA</i>	toxin of the HicA-HicB-HicC toxin-antitoxin system	945989	55	<i>recA</i>	DNA strand exchange and recombination protein	947170
28	<i>hlpA</i>	chaperone protein	944861	56	<i>rep</i>	DNA helicase	948292

(Continued.)

Table 1. (Continued.)

#	gene	protein	NCBI ID	#	gene	protein	NCBI ID
57	<i>rimM</i>	ribosome maturation protein	947101	73	<i>ydhQ</i>	conserved protein	944851
58	<i>mt</i>	RNase T	946159	74	<i>ydhX</i>	predicted 4Fe-4S ferredoxin-type protein	947308
59	<i>rpmJ</i>	50S ribosomal subunit protein L36	947805	75	<i>ydiL</i>	conserved protein	946181
60	<i>rsxA</i>	integral membrane protein of SoxR-reducing complex	946148	76	<i>ydjA</i>	predicted oxidoreductase	945964
61	<i>rsxC</i>	member of SoxR-reducing complex	946137	77	<i>yeiA</i>	NADH-dependent dihydropyrimidine dehydrogenase subunit	949037
62	<i>sufB</i>	SufBCD iron – sulfur cluster scaffold protein	945753	78	<i>yelG</i>	S-formylglutathione hydrolase/S-lactoylglutathione hydrolase	949045
63	<i>thyA</i>	thymidylate synthase	949035	79	<i>yfaU</i>	2-keto-3-deoxy-L-rhamnose aldolase	948054
64	<i>trpB</i>	tryptophan synthase, β subunit	945768	80	<i>ygiR</i>	predicted NAD(P)-binding dehydrogenase	947600
65	<i>tufA</i>	elongation factor Tu	947838	81	<i>yifL</i>	predicted lipoprotein	1450304
66	<i>wzxE</i>	lipid III flippase	948294	82	<i>yigA</i>	conserved protein	948359
67	<i>yaiT</i>	putative protein	4056040	83	<i>ymgG</i>	putative protein	945728
68	<i>ybcS</i>	DLP12 prophage, lysozyme	7156271	84	<i>yndK</i>	predicted transposase	4056028
69	<i>ybdO</i>	predicted DNA-binding transcriptional regulator	945216	85	<i>yoiL</i>	flavin transferase	946711
70	<i>yqU</i>	β -phosphoglucomutase	945891	86	<i>yqfA</i>	predicted oxidoreductase	947381
71	<i>ydeS</i>	predicted fimbrial-like adhesin protein	946047	87	<i>zapA</i>	cell division factor	947404
72	<i>ydfH</i>	DNA-binding transcriptional regulator	7158646				

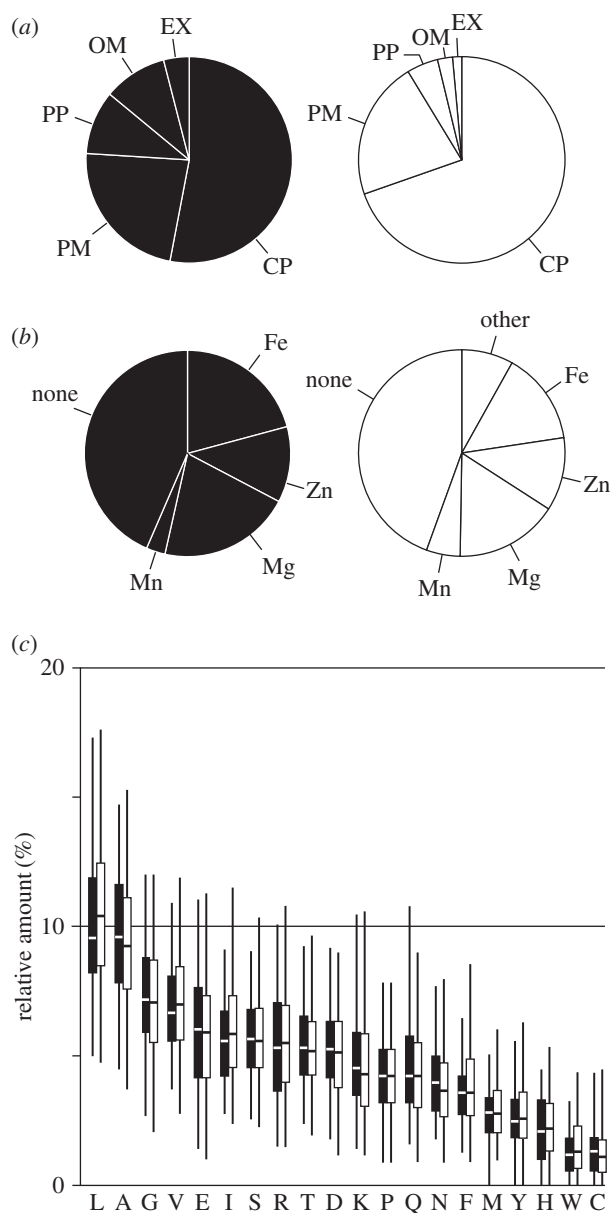


Figure 1. Properties of the 87 identified proteins compared to the entirety of *E. coli* proteins. (a) Subcellular localization of the set of 87 plasma-protective proteins (black) compared to all proteins (white). CP, cytoplasm; EX, extracellular; OM, outer membrane; PM, plasma membrane; PP, periplasm. (b) Metal cofactors of the 87 proteins (black) compared to all proteins (white). (c) Relative content of each amino acid per protein averaged over the set of 87 proteins (black bars) or over all *E. coli* proteins (white bars). Horizontal lines mark the median. The whiskers cover 95% of all data points. For all comparisons (a–c), the UniProt database was used as reference [23].

To identify pathways and functional categories that might be over-represented, the 87 genes were grouped based on GO terminology (electronic supplementary material, figure S2) [30]. Protein-related pathways were found to be most strongly represented. This includes protein synthesis, translocation, protection and degradation.

2.2. Quantitative analyses

The screening of the KEIO collection was based on a qualitative assay that only distinguishes between growth and no growth after plasma exposure. To determine the influence of a general growth defect that the knockout mutants might have, the 87 mutants were tested for plasma sensitivity in a quantitative

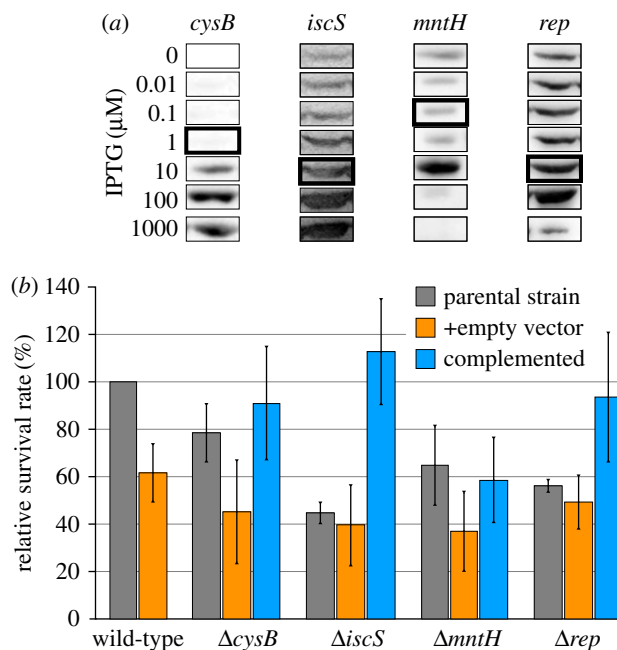


Figure 2. Protein levels and plasma sensitivity of complemented deletion strains. (a) Western blot analysis with anti-His₅ antibody was performed to measure protein levels at different IPTG concentrations. The IPTG concentrations of the highlighted expression levels were chosen for the plasma sensitivity assay. (b) Complementation was performed by inducing with 0.1 μM IPTG (*mntH*), 1 μM (*cysB*) or 10 μM (*iscS*, *rep*). Strains harbouring the empty vector (pCA24N) served as control. Relative survival rates were calculated by dividing the CFU of plasma-treated samples by the CFU of the gas-treated control and by subsequently normalizing to the wild-type (set to 100%). Experiments were performed three times independently. Averages and standard deviations are shown. (Online version in colour.)

assay that takes into account plasma-independent growth defects. Cell suspensions containing approximately 500 colony forming units (CFU) in 30 μl LB broth were exposed for 30 s to the μAPPJ operated as described above or left untreated. The cells were then plated onto agar and CFU counted after overnight incubation. With this quantitative approach, the plasma sensitivity of about 20 mutants was confirmed to be significantly higher compared to the wild-type (electronic supplementary material, figure S3). The strain ΔiscS lacking the cysteine desulfurase of one of the two iron-sulfur cluster biosynthesis operons [31] exhibited the lowest survival rate after plasma treatment (22%) compared with the wild-type (60%). The second most sensitive strain was Δrep with 29% survival rate. Rep is an ATP-dependent DNA helicase and a component of the replisome [32]. It has been shown to be crucial for the resumption of replication after UV radiation-induced replication arrest [33]. Since the plasma jet emits UV radiation [8], the role of Rep in plasma resistance seems evident.

The knockout mutants of the KEIO collection were made by substituting the open reading frames with a kanamycin resistance cassette [22]. To ensure that the observed plasma sensitivity was indeed a consequence of the gene replacement, exemplarily four deletion mutants with significantly increased plasma sensitivity (ΔcysB , ΔiscS , ΔmntH and Δrep) were complemented with the deleted gene encoded episomally on the plasmid pCA24N (figure 2). The plasmids were taken from the ASKA collection, a comprehensive plasmid library for

over-expression of *E. coli* genes [34]. Controls with the empty vector (not expressing an *E. coli* gene) were performed as well to control for effects of plasmid replication and maintenance (selection pressure by chloramphenicol). Induction was performed with IPTG concentrations ranging from 0.01 to 1000 μM . The production of the plasmid-encoded proteins was analysed by western blot analysis (figure 2a). Furthermore, growth of the strains at each IPTG concentration was recorded (electronic supplementary material, figure S4). For each complemented strain, an IPTG concentration was chosen for plasma sensitivity assays based on protein expression levels and growth rates of the mutants (electronic supplementary material, figure S4). The survival rates of all complemented strains were significantly higher compared with the corresponding deletion strains harbouring the empty vector as control (figure 2b). For ΔcysB , ΔiscS and Δrep complementation caused the survival rate to increase above that of the wild-type harbouring pCA24N, which indicates that over-production of these proteins can increase the plasma resistance of bacteria. For ΔmntH the effect of complementation was less pronounced.

2.3. Stressors mimicking individual plasma-derived stress factors

Plasma is a cocktail mostly consisting of short-lived reactive species, like excited states of helium, oxygen, and nitrogen, atomic oxygen, or superoxide. When in contact with water (in air or in aqueous samples), these species recombine giving rise to a different set of reactive molecules, e.g. peroxyxynitrite or hydroxyl radicals. Formation of hypochlorous acid (HOCl) is also possible, when atomic oxygen reacts with chloride ions in the liquid [9].

For some of the identified proteins, the participation in plasma resistance can be explained, like for the helicase Rep described above, the proteolytic enzyme DegP (periplasmic endoprotease), which participates in degrading damaged proteins [35], AtoA/E, which engage in short chain fatty acid degradation [36] and could thus play a role in protecting cells from lipid oxidation, or the hydrogen peroxide detoxifying KatE (catalase E) [26]. For many others, like the above-mentioned FlgB/G, the roles remain to be elucidated. To gain first insights into which plasma components the 87 genes protect the cells from, the mutants were tested for their sensitivity to conditions mimicking single species generated by plasma, plasma air and plasma-liquid interaction. Some molecules like H_2O_2 , nitrite and nitrate were added to the medium directly, while stressors like paraquat, nitroprusside and copper ions combined with hydrogen peroxide (promoting Fenton reaction) were used to generate O_2^- , $\text{NO}\bullet$ and $\bullet\text{OH}$, respectively. Further, acid stress (generated by HCl), heat stress or membrane stress (induced by SDS, CTAB, saponine or Triton X-100) were tested. With the exception of nitrate, the mutants were incubated with each stressor at a defined concentration, at which the growth of the wild-type was reduced to about 60% compared with an untreated control. Since none of the tested nitrate concentrations (0.1–45 mM) inhibited the growth of the wild-type, 10 mM nitrate were used in the assay (higher concentrations are not expected to be generated during treatment with the μAPPJ [37]). The growth of the mutants was measured after a 16 h incubation with each stressor, and normalized first to an untreated culture and subsequently to the wild-type (figure 3). The greater the growth defect, the higher the sensitivity of the mutant to that particular

stress condition. Already without addition of external stressors, five strains showed a growth defect in LB medium (ΔcysB , ΔfimC , ΔguaA , ΔiscS and ΔpriA), and eight other mutants exhibited reduced growth in M9 minimal medium (ΔapaH , ΔdksA , ΔnlpE , Δpnp , ΔtrpB , ΔtufA , ΔydiL and ΔyigA) (figure 3a). These results confirm that general growth restrictions are indeed occurring and need to be considered when comparing plasma resistance of the knockout strains.

Strains exhibiting a growth defect of 20% or higher when exposed to a stressor were termed ‘sensitive’ towards that stressor (figure 3b). To compare the importance of the stressors, for each stressor the number of sensitive mutants was plotted against the average growth defect of these mutants (figure 4a). We used a ‘severity score’ based on the Euclidean distance (0, no impact; 118 maximal impact on all mutants; figure 4b). According to the severity score, hydrogen peroxide had the highest impact causing a growth defect of 70% in 21 mutants (severity score of 54). Superoxide was another important stressor, affecting the growth of 43 mutants, with a 29% inhibition on average (severity score 44). These results indicate that wild-type bacteria have a number of genes that protect them from two of the main plasma-derived reactive oxygen species.

The tested nitrogen salts impacted only weakly on the growth of few of the *E. coli* mutants resulting in severity scores below 5 and indicating that they are probably not important in bacterial resistance against plasma. The addition of 4 mM HNO_3 decreased the pH of the medium to 5.5 by dissociation into H^+ and NO_3^- , but even at that low pH, nitrate had little effect on the mutants. Peroxyxynitrite, a strong oxygen- and nitrogen-based oxidant causing e.g. lipid peroxidation, had a severity score of 16 (figure 4b). Of all reactive nitrogen species tested, nitric oxide generated by the decay of sodium nitroprusside affected the highest number of mutants (30 mutants, severity score 32).

The four strains that were selected for complementation studies (ΔcysB , ΔiscS , ΔmntH and Δrep) and tested for curing the plasma-sensitive phenotype (figure 2), were also tested for curing stressor sensitivity (figure 5a). The strain ΔiscS pCA24N (empty vector) exhibited an increased H_2O_2 sensitivity. No growth was detectable at 0.6 mM H_2O_2 , whereas the wild-type harbouring the empty vector still showed growth at 1 mM H_2O_2 . Complementation of ΔiscS partially restored the wild-type phenotype and the complemented mutant grew at 0.8 mM. The complementation further cured the sensitivity against superoxide, SDS, and an acidic milieu (electronic supplementary material, figure S5A). The complementation of the ΔcysB mutant did not restore H_2O_2 (figure 5a) or peroxyxynitrite resistance to wild-type levels (electronic supplementary material, figure S5B). The complementation of Δrep increased the H_2O_2 resistance beyond wild-type levels such that growth was detected at 2 mM H_2O_2 (figure 5a). Similarly, the complementation of ΔmntH enabled the cells to grow at elevated superoxide levels (electronic supplementary material, figure S5C).

2.4. Clustering

In the first screening, several genes organized in operons or belonging to the same metabolic pathways were identified, like *atoA/E*, *fimC/D*, *flgB/G*, *holC/D*, *ihfA/B*, *iscS/U/sufB* and *rsxA/C*. A clustering based on the k-mean++ algorithm was performed to reveal whether mutants belonging to the same pathways exhibit similar profiles with regard to stressor sensitivity as determined in the second screening approach (figure 6). The stressors with the lowest severity scores (nitrite,

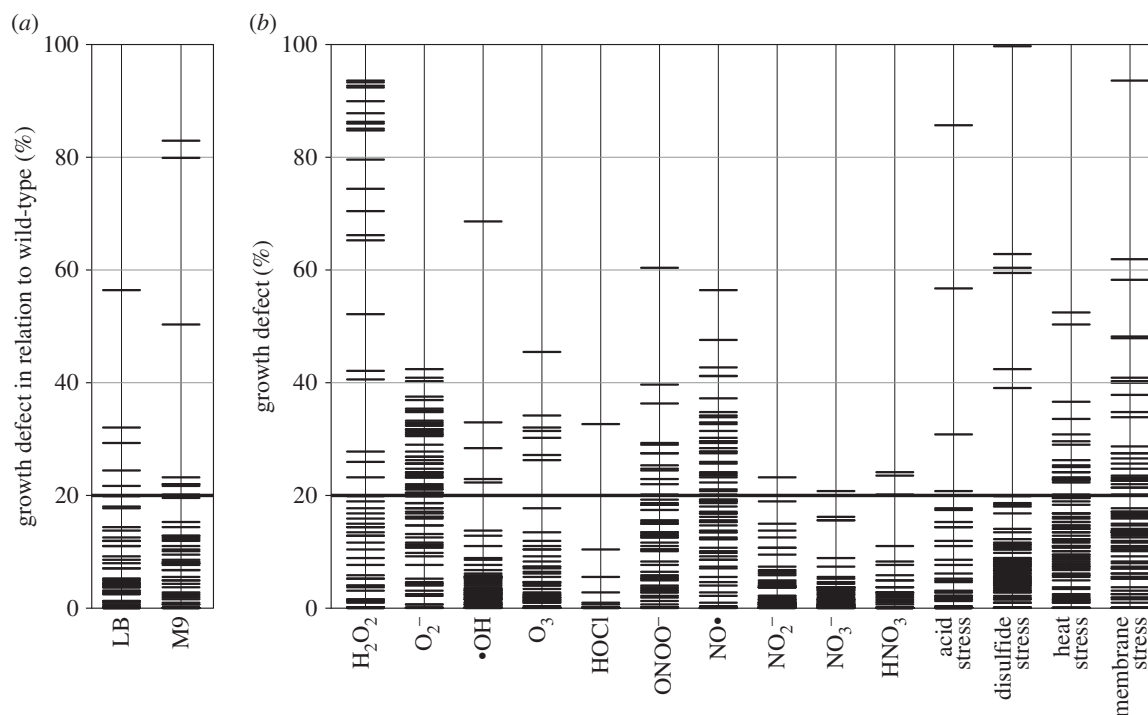


Figure 3. Effects of stressors on the growth of 87 plasma-sensitive strains. (a) To determine growth defects of the mutants in LB and M9 medium at 37°C, the OD after a 16 h incubation in the respective medium was set in relation to that of the wild-type, for which the growth defect was set to 0%. (b) To determine growth defects of the strains in the presence of stressors, cells were incubated in LB medium containing the indicated plasma-mimicking components. Acid stress was caused by HCl, disulfide stress by diamide, heat stress by overnight incubation at 40.5°C, and membrane stress by addition of different detergents. The OD of the deletion strains after 16 h incubation was set in relation to the corresponding unstressed control and subsequently in relation to the wild-type (set to 0% growth defect). A growth defect greater than 20% was regarded significant. All data represent three independent biological replicates.

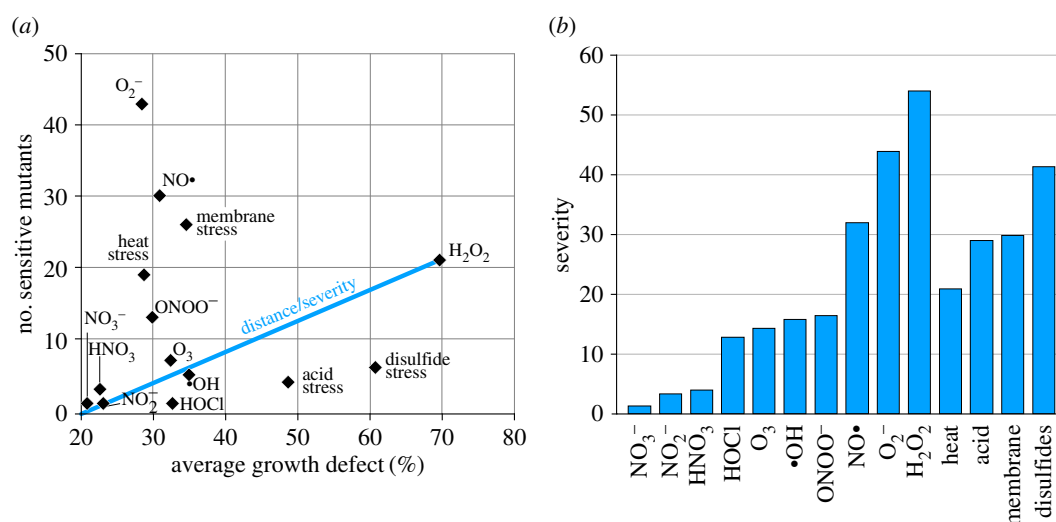


Figure 4. Comparison of the importance of individual stressors. (a) The number of knockout mutants with a growth defect greater 20% were plotted against the average growth defect of those strains. To illustrate the calculation of the severity score, the distance of the stressor H₂O₂ to the point of origin is shown as an example. (b) Severity score based on the Euclidean distance of each stressor from the point of origin. (Online version in colour.)

nitrate, nitric acid and HOCl) were excluded from this calculation to lower the number of dimensions and to thereby increase the reliability of the clustering. The strains sensitive to all stressors ($\Delta holC$, $\Delta lpdA$ and $\Delta rimM$) or none of the stressors ($\Delta asnB$, $\Delta csqG$, $\Delta hicA$, $\Delta napC$, $\Delta oppD$, $\Delta yfaU$ and $\Delta zapA$) were excluded (figure 6a). Eight mutants exhibited a unique stressor profile and were not grouped (figure 6b). The remaining 72 mutants were sorted into 12 clusters (figure 6c).

As expected, some mutants impaired in the same operon or pathway grouped together in the same cluster. The two strains $\Delta iscS$ and $\Delta iscU$ forming cluster #5 are defective in one of the two iron–sulfur cluster biosynthesis pathways and have very

similar stressor profiles (sensitive against H₂O₂, O₂⁻, NO•, acidic pH and membrane stress). $\Delta sufB$, another strain lacking a gene of the iron–sulfur cluster biosynthesis network, had a unique stressor profile. Two strains $\Delta ihfA$ and $\Delta ihfB$ missing genes encoding different subunits of histone-like proteins grouped to cluster #2 and #1, respectively. Both are sensitive to superoxide and nitric oxide, but the $\Delta ihfA$ strain, like the other strains in cluster #2, was also susceptible to general membrane stress. Both the genes *cpxA* and *degP* are part of the heat shock response [38] and the knockout strains both cluster in cluster #8.

The hallmark of cluster #10 is a significant sensitivity against superoxide. The strain $\Delta mntH$ previously used in

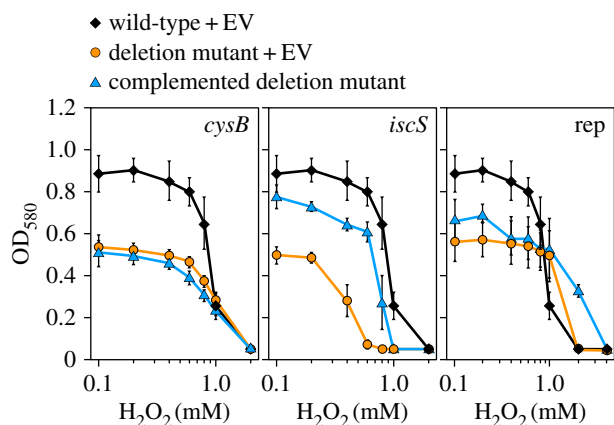


Figure 5. Stressor sensitivity of complemented strains. (a) The OD after 16 h incubation at varying concentrations of H₂O₂ is shown for wild-type and deletion strains harbouring the empty vector (EV) pCA24N and for the corresponding complemented strains. Complementation was performed by induction with 1 μ M IPTG (*cysB*) or 10 μ M (*iscS*, *rep*), respectively. The data represent three independent experiments. Averages and standard deviations are shown. (Online version in colour.)

complementation experiments (figures 2 and 4c) is part of this cluster. We investigated whether superoxide is indeed the plasma component causing the Δ *mntH* strain to be plasma-sensitive. To counteract superoxide stress, *sodA* (encoding a cytoplasmic superoxide dismutase) was over-expressed from the plasmid pCA24N::*sodA* in the Δ *mntH* background (figure 7). Induction of *sodA* with 10 μ M IPTG completely restored the plasma resistance of the Δ *mntH* mutant (compare WT with empty vector, Δ *mntH* with empty vector and Δ *mntH* with p*SodA*) emphasizing that indeed superoxide is the plasma-generated species responsible for the sensitivity of the Δ *mntH* mutant.

3. Discussion

The reaction of different eukaryotic and prokaryotic cells to plasma treatment was already investigated on different levels using system-wide approaches like transcriptomic [14–16], proteomic [17,18] and metabolomic analyses [19]. Here, we focused on the genetic level. Using a genome-wide screening approach, we identified genes that contribute to the capability of *E. coli* to survive short exposures to the effluent of a plasma jet operated with a He/O₂ mixture as feed gas.

3.1. What do the plasma-sensitive mutants reveal about plasma targets?

The first screening of the KEIO collection of approximately 4000 *E. coli* single-gene knockout mutants [20] for sensitivity to treatment with the μ APPJ identified 87 genes that mediate protection against plasma. Among these are genes indicative of DNA damage such as *ada* encoding a transcriptional regulator with protective function against DNA-alkylating agents [39], or *recA* [40] with a number of functions related to DNA repair and recombination, most prominently the SOS response allowing error-prone DNA repair when high fidelity repair systems are overwhelmed. Two other genes do not encode proteins considered classical stress proteins but are also connected functionally to DNA. The phosphoglucomutase Pgm is responsible for the breakdown of glycogen

to maltose and galactose [41]. Besides this role in supplying energy, Pgm was found to indirectly modulate DNA topology and favour supercoiled structures in a high-throughput screening for DNA topology-manipulating proteins in *E. coli* [42]. A similar role was attributed to DksA [42], an RNA polymerase-binding transcription factor, which in addition participates in the repair of double-strand DNA breaks [43]. While DNA-damaging effects of plasma have been observed and are actively being studied by different groups, the influence of the degree of supercoiling on plasma sensitivity has not yet been considered in great depth.

Several genes encoding components of cellular appendices were identified as protective: proteins of the flagellar apparatus (FlgB, FlgG), fimbriae formation (BamB, FimC and FimD) and curli assembly (CsgG). These structures are responsible for motility [24], adhesion to surfaces and biofilm formation [44], respectively, and are thus needed under different growth conditions. Rather than by functional commonality, the different cell appendices might mediate protection from plasma components in an unspecific manner, e.g. by acting as scavengers of reactive species on the cell surface preventing their entering the cell.

Iron-containing proteins were over-represented among the 87 proteins. In particular, *mntH* is a manganese and iron transporter induced upon iron limitation [45] and at least seven of the identified genes are directly or indirectly involved in the biosynthesis (*iscS*, *iscU* and *sufB*), repair (*ftn*), or regulation (*ihfA*, *ihfB* and *oxyR*) of iron–sulfur clusters [45]. Iron–sulfur clusters are important cofactors in many cellular pathways and processes, i.e. in the respiratory electron transport chain and the TCA cycle [46,47]. However, iron–sulfur clusters are known to be highly sensitive to oxidation by several ROS, especially superoxide [48]. Taken together, the over-representation of iron-containing metalloproteins, the identification of the iron–sulfur cluster-related genes, the high severity score of superoxide and the protective effects of *sodA* over-expression in a Δ *mntA* mutant indicate that the disruption of iron–sulfur clusters poses a severe challenge to cells exposed to plasma. As the oxidative inactivation of those clusters has been described to be very fast (rate constants in the order of 10^8 – 10^9 M⁻¹ s⁻¹) [49–51], targeting of these structures might be among the first survival-limiting challenges occurring during plasma treatment.

3.2. What do the plasma-sensitive mutants reveal about plasma components?

The effluent of the μ APPJ is composed of a versatile mixture of reactive oxygen species. At the relevant distance of the jet from the sample, atomic oxygen (approx. 10^{14} cm⁻³), ozone (approx. 10^{15} cm⁻³) and superoxide (10^{11} cm⁻³) have been identified as dominant species [8]. Atomic oxygen has recently been shown to enter the liquid phase [49]. For the generation of H₂O₂ or NO•, humidity or impurities from ambient air are necessary, respectively [50]. All those species target a variety of molecules in cells. In the presence of water, superoxide and nitric oxide readily recombine to peroxynitrite [51], which then reacts directly with biomolecules like metalloproteins [52] and thiols [53]. Depending on the pH and CO₂ availability, peroxynitrite can decay to •NO₂ and •CO₃⁻ or •NO₂ and •OH, causing nitrosylations and oxygenations of proteins, DNA and lipids [54]. Owing to this diverse range of reactions, peroxynitrite is often described as more harmful to cells than

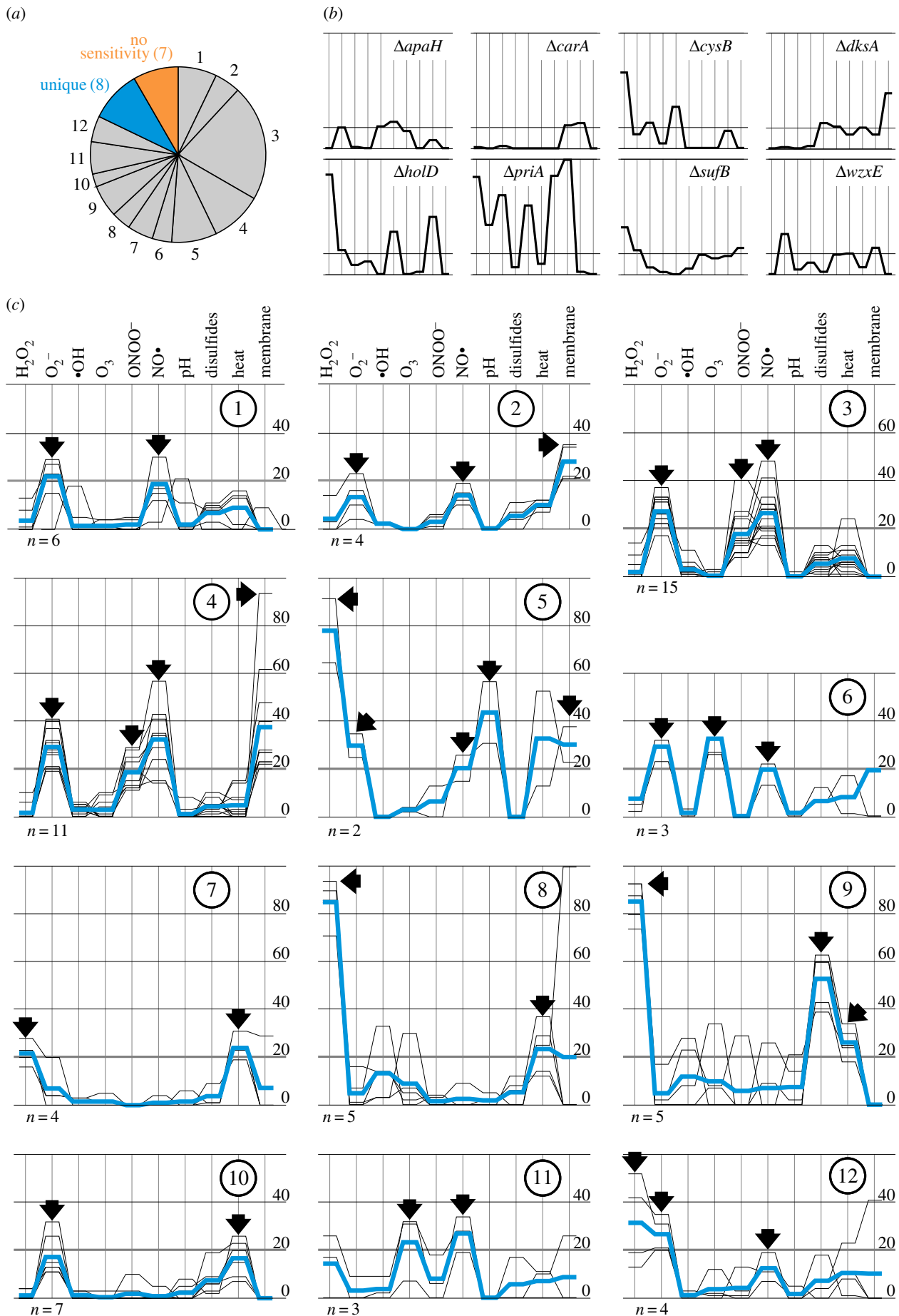


Figure 6. The mutants were clustered based on their sensitivity against plasma-relevant stressors. (a) Mutants are grouped into 12 clusters, strains with unique profiles and strains not sensitive to any of the tested stressors. (b) Stressor profiles of the strains with unique stressor profiles. The order of the stressors is the same as in (c). (c) The 12 clusters (numbered 1–12) were generated using the k-mean++ clustering algorithm. Arrows mark the stressors characteristic for each cluster; *n* indicates the number of strains in the cluster and blue lines represent the average stressor profile of the cluster members. (Online version in colour.)

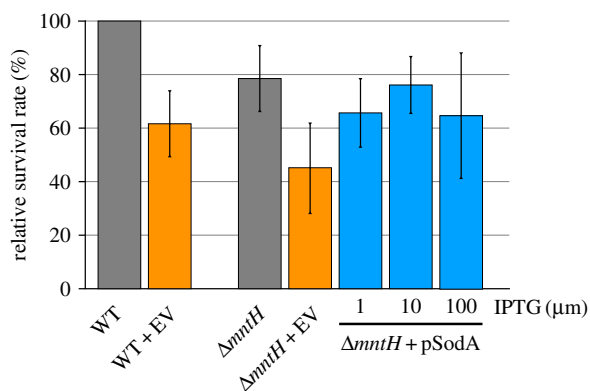


Figure 7. Plasma sensitivity of the wild-type and a $\Delta mntH$ mutant harbouring either the empty vector (EV) or the plasmid pCA24N::sodA (pSodA) was investigated by determining survival rates relative to the wild-type after 30 s plasma treatment. *sodA* was induced with increasing amounts of IPTG. The data represent three independent experiments. Averages and standard deviations are shown. (Online version in colour.)

either superoxide or nitric oxide alone [51,55]. In recent studies, the antibacterial properties of liquids exposed to plasma were even attributed mainly to peroxyntirite [56,57].

In the presented stressor profiling, H_2O_2 , O_2^- and $NO\bullet$ were the stressors with the highest severity scores (figure 4b), indicating that when the respective protective genes are missing, the cells become highly sensitive to plasma exposure. Among the plasma-protective proteins were for instance the stress-inducible catalase KatE [26] and the hydrogen peroxide-sensing transcriptional regulator OxyR [58], which regulates a number of genes protecting from hydrogen peroxide stress. The clustering of the mutants based on stressor profiles resulted in seven clusters (#1–6, #12) with sensitivity towards superoxide and nitric oxide, encompassing 45 of the 72 strains. Both reactive species target similar molecules in cells, like metallo- and iron–sulfur cluster proteins [52] or DNA [48,59–61] that are discussed as plasma targets above. Somewhat surprisingly, the stressor profiling indicated that reactive nitrogen species like $NO\bullet$ play an important role in bacterial inactivation. Given that nitrogen was not admixed in the process gas, reactive nitrogen species can stem from impurities of the feed gases or secondary reactions with ambient air-derived nitrogen. Nevertheless, both sources of nitrogen have been shown to play only minor roles regarding the device and distance to the sample used here [62,63]. Further reactive nitrogen species might form by interaction of plasma-generated species with the liquid phase, like the medium or cellular components. When using plasmas with higher levels of reactive nitrogen species, we would expect to observe an increased importance of reactive nitrogen protective mechanisms, i.e. more mutants lacking genes encoding reactive nitrogen detoxifying genes. Such a plasma could be generated, for example, by increasing the distance of the μ APPJ to the sample, as the admixture of ambient air increases from almost zero at 4 mm to about 20% with 2 cm distance [62].

In our screening, more genes protecting against O_2^- and $NO\bullet$ were identified than genes protecting from peroxyntirite. This could reflect that the bacterial defence is focused on detoxifying the two precursors rather than the recombination product peroxyntirite. It could further reflect an adaptation of *E. coli* to interacting with the mammalian immune system, which exposes *E. coli* to superoxide and nitric oxide, which

act as precursors of other reactive oxygen and nitrogen species including peroxyntirite [51]. However, it might also simply be a reflection of the number of genes that *E. coli* harbours that protect against individual stressors and the type of stressors that were available for profiling. Many plasma-generated species and components as well as recombination products were not tested as individual stressors, like atomic oxygen, singlet oxygen, or UV radiation, although cellular targets of those species are described manifold [64,65]. Furthermore, plasma jets like the one applied here use noble gases for ionization. Winter *et al.* observed a significant impact of argon gas treatment (without plasma ignition) on the proteome of *Bacillus subtilis* [17]. The effect of the helium/oxygen gas used in this study was not investigated. Nonetheless, our screening revealed that for inherent plasma resistance, *E. coli* relies heavily on mechanisms of detoxification of species stemming from the effluent, namely H_2O_2 , O_2^- and $NO\bullet$.

3.3. Implications of inherent plasma resistance for medical applications

Plasmas are complex and dynamic mixtures exerting antibacterial properties through reactive species, (V)UV emission, and some by means of electric fields. The joint action of multiple stressors acting on multiple molecular targets simultaneously has led to the possibility of emergence of plasma resistance being broadly discounted [66,67]. Yet, plasma resistance has already been described at least once [68]. In the present study, we identified a set of genes that when missing leave the bacterial cell less well protected from plasma, thus conferring a certain level of resistance to the wild-type. The complementation and over-expression studies, e.g. of the strains $\Delta iscS$ pCA24N::iscS and $\Delta cysB$ pCA24N::cysB indicate that some of the genes can increase the level of plasma resistance beyond the levels of the wild-type with empty vector simply through elevated levels of a single protein (figure 2). Such an elevation in gene expression can easily occur in natural mutants e.g. by point mutations in the promoter region [69,70]. Additionally, plasmas have been shown to be mutagenic [10,13,71], thus they may increase the frequency at which such mutants occur. It is therefore conceivable that strains over-producing protective proteins evolve under non-laboratory conditions and that their evolution is expedited by plasma exposure both through increased mutation rates and selective pressure. The duration of plasma exposure tolerated by prokaryotic and eukaryotic cells are within the same order of magnitude ranging from seconds to a few minutes depending on the device and treatment conditions [72,73]. An increase in plasma resistance of bacteria by a factor of 3–10 may therefore already limit the clinical application of plasmas as antibacterial strategy.

4. Experimental procedure

4.1. Plasma sources

Plasma was generated by a well-described microscale atmospheric pressure plasma jet (μ APPJ) [8]. Helium (1.4 slm, 5.0 purity) with an admixture of oxygen (0.6%, 0.0084 slm, 4.8 purity) was used as the feed gas. Plasma was driven with 13.56 MHz and 230 V_{RMS}. Samples were placed at 4 mm distance to the nozzle of the jet.

To generate ozone as a stressor (see below), a dielectric barrier discharge plasma device (Cinogy, Duderstadt, Germany) was used [6,74]. The copper electrode had a diameter of 20 mm and was driven with a pulsing frequency of 300 Hz with 13.5 kV peak amplitude.

4.2. Strains, media and chemicals

The KEIO collection of single-gene knockout mutants was used [22]. All strains were cultivated in LB medium at 37°C under aerobic conditions. Knockout mutants were maintained in the presence of 50 µg ml⁻¹ kanamycin. The strains were stored in 96-well microtitre plates filled with Hogness modified freezing medium at -80°C [75]. Before usage for experiments, cells were freshly stamped into new microtitre plates filled with LB medium and incubated overnight.

All chemicals used for media, as additives, stressors or for synthesis were reagent grade and purchased from Sigma Aldrich.

4.3. Screening of the KEIO collection

From each well of a microtitre plate containing different strains 2 µl were dropped onto an LB agar plate using a 96-well replicator. After a 2 h incubation at 37°C, the spots were treated with the effluent of the µAPPJ for 100 s. Afterwards, the plates were incubated at 37°C overnight. The experiment was repeated three times independently. For each mutant, the number of experiments in which no cells grew after overnight incubation was summed up to give a score. Mutants with a score of 2 or higher were classified as plasma sensitive.

4.4. Plasma sensitivity

To quantify the plasma sensitivity of the 87 mutants, the optical density of overnight cultures was determined, adjusted to OD₅₈₀ = 0.03, and then further diluted 1:500 in LB medium to give approximately 16 000 CFU ml⁻¹. To prevent the cell suspension from spreading on the support due to the gas flow coming from the jet, 30 µl (containing approx. 500 CFU) of this low-density bacterial suspension were soaked into filter paper (1 cm², Whatman 3 MM) which was then exposed to the effluent of the µAPPJ for 30 s with or without (gas control) igniting the plasma. Directly after treatment, the filter paper was immersed in 1 ml NaCl (0.9% (w/v)) and incubated at 37°C for 20 min to detach the cells from the filter and bring them into suspension. Afterwards, the filter was removed and the suspension plated and incubated overnight to determine CFU counts. The CFU counts of the gas controls were set to 100% to calculate plasma survival rates for each strain. This experiment was performed at least three times independently for each mutant.

To study the effects of complementation, the survival rate of the mutants was normalized to that of the wild-type (set to 100%) for each of the three replicates prior to calculating the average.

4.5. Complementation

The mutants of the KEIO collection were transformed with the plasmid of the ASKA collection [34] that harbours the respective gene under the control of an IPTG-inducible promoter for complementation of the knockout. Chloramphenicol (50 µg ml⁻¹) was added to the culture medium to maintain the plasmid. For any of the following experiments, the strains

were inoculated 1:100 (v/v) from an overnight culture and incubated with IPTG ranging from 0.01 to 1000 µM for 120 min. Afterwards, the OD₅₈₀ was determined and the cultures used as described.

4.6. SDS-PAGE and western blot

For detection and semi-quantitative analysis of the episomally encoded proteins, SDS-PAGE and subsequent western blot analysis according to standard protocols were performed [76]. Like for the plasma sensitivity assay, 1 ml of the bacterial culture was harvested and resuspended in 75 µl denaturing loading buffer. After incubation at 95°C for 10 min and removal of cell debris by centrifugation (10 min, 12 000g), 5 µl of each sample were applied to SDS-PAGE. Detection of the His₆-tagged proteins was performed with an anti-His₆ antibody (Qiagen, Hilden, Germany).

4.7. Synthesis of peroxyntirite

Peroxyntirite was synthesized freshly from isopentyl nitrite and hydrogen peroxide as described by Uppu & Pryor [77]. The concentration of peroxyntirite was determined photometrically by Beer-Lambert Law using $\epsilon_{302} = 1670 \text{ M}^{-1} \text{ cm}^{-1}$ and adjusted to 100 mM with potassium phosphate buffer (100 mM, pH 7) directly prior to use.

4.8. Screening against defined stressors

The plasma-sensitive mutants were treated with the following stressors: hydrogen peroxide (2 mM), paraquat (0.5 mM), products of the Fenton reaction (0.5 mM CuCl₂ and 0.8 mM H₂O₂ in LB buffered with 100 mM potassium phosphate, pH 6), hypochlorous acid (3 mM), peroxyntirite (5 mM in LB buffered with 100 mM potassium phosphate, pH 7), nitric acid (4 mM), sodium nitrate (10 mM), sodium nitrite (4 mM), sodium nitroprusside (4 mM), heat shock (40.5°C) and acidic medium (LB pH 4.5 adjusted with HCl). Membrane stress was induced by incubation with sodium dodecyl sulfate (SDS, 1 mM), cetrimonium bromide (CTAB, 0.02 mM), Triton X-100 (3% (v/v)) or saponin (2% (w/v)). For each mutant, the results for the highest-impact membrane stressor were used to describe the sensitivity to membrane stress. Except for ozone, treatment with the stressors took place in microtitre plates. To this end, LB medium containing the stressor was inoculated by stamping from overnight pre-cultures. For treatment with ozone, a 96-well replicator was briefly immersed in an overnight pre-culture. Droplets were then allowed to dry on the stamp for 15 min. Afterwards, the stamp was placed in a glass beaker with approximately 2 mm distance to the bottom. Ozone was generated by the DBD device described above [6] positioned next to the stamp. After operating the DBD for 30 min, the cells were suspended in fresh LB medium. Concentrations/doses of all the stressors were determined to be non-lethal for the wild-type but reducing its growth to about 60%. Growth of the cultures was recorded photometrically at 580 nm with a plate reader (EnSpire, PerkinElmer) after 16 h of incubation at 37°C, or 40.5°C (heat stress). The sensitivity was calculated according to formula 1. A mutant was classified as stressor sensitive, when the relative growth defect was greater than

20% compared with the parental strain.

$$\text{growth defect [\%]} = 100 - 100 \times \text{OD}_{\text{wt, LB}} \times \text{OD}_{\text{wt, stressed}}^{-1} \\ \times (\text{OD}_{\text{mutant, LB}} \times \text{OD}_{\text{mutant, stressed}}^{-1})^{-1}.$$

4.9. Clustering of stressor profiles

The stressor profiles of the mutants were grouped by performing the k-mean++ algorithm (MatLab, R2016a) with the cosine distance for calculating the similarity between two profiles. To avoid local minima, the algorithm was repeated 10 000 times searching for the lowest sum of distances. As the k-mean++ algorithm needs a predefined number of clusters,

this procedure was performed assuming 2–20 clusters using the silhouette coefficient for identification of the best fitting number of clusters.

Data accessibility. The data have been uploaded as electronic supplementary material.

Authors' contributions. M.K. carried out experiments, data analysis, designed the study and wrote the manuscript; F.J., T.D. and B.S. carried out experiments; J.B. provided the plasma source and its characterization; J.W.L. designed the study; J.E.B. designed the study and wrote the manuscript. All authors gave final approval for publication.

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