Investigation of plasma resistance-mediating genes in *Escherichia coli*

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Abstract: Since cold atmospheric pressure plasmas can inactivate even multidrug-resistant bacteria, there is a special interest in understanding the mechanism underlying bacterial inactivation by plasma and in investigating possible resistance development. In this study, we showed that an upregulation of a single protein increases plasma resistance. Moreover, our findings regarding [Fe-S]-cluster containing proteins suggest that they limit survival and could present the bottleneck of surviving plasma-mediated stress.

Keywords: COST plasma jet, bacterial inactivation, plasma resistance, [Fe-S]-cluster, superoxide, hydrogen peroxide

The increasing number of multidrug-resistant bacteria leads to a growing interest in finding additive therapies that can be combined with antibiotics [1, 2]. For some applications, plasma can present such an additive, since it can inactivate even multidrug-resistant bacteria. In this study, the COST jet was used [3] to investigate potential intrinsic mechanisms of plasma resistance. We have screened a library of single-gene knockout *Escherichia coli* mutants for strains exhibiting increased plasma sensitivity. The collection constructed at KEIO university includes approximately 4000 strains, each missing one non-essential gene [4]. 87 mutants with increased plasma sensitivity were identified, indicating a potential function of these genes in mediating plasma resistance [5]. The increased plasma sensitivity of four strains (Δ*iscS,* Δ*mntH,* Δ*rep,* Δ*cysB*) was verified with an independent CFU-based assay and complementation experiments. The gene *iscS* encodes a cysteine desulfurase, which is involved in synthesis of [Fe-S]-clusters [6, 7]. CysB is the regulator of the cysteine regulon and activates the expression of cysteine-related genes in the absence of sulfur [8]. Rep is a DNA helicase [9, 10] and *mntH* encodes a proton-dependent transporter for divalent cations (mainly Mn^{2+}) [11]. Overexpression of *iscS*, *rep,* or *cysB* has led to survival rates higher than those of the wild type, indicating that plasma resistance can indeed increase, resulting in less sensitive strains (Fig. 1).

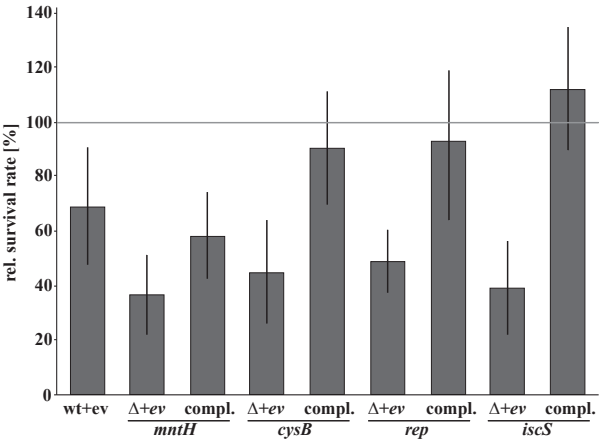

Fig. 1: Relative survival rates of *E. coli* wild type, Δ*mntH*, Δ*cysB*, Δ*rep*, and Δ*iscS* containing the empty vector or a plasmid for complementation after 30 s of plasma treatment. A low-density cell suspension of approximately 1600 CFU x ml^{-1} µl was exposed to the effluent of the COST jet. The survival rate of the wild type without plasmid was set to 100%. wt: wild type, ev: empty vector, compl.: complemented strain.

The overall data of the screening suggested [Fe-S]-clusters to be major targets of plasma, since 17 of the 87 strains initially identified as plasma-sensitive lacked a gene related to iron, sulfur, or [Fe-S]-cluster metabolism. In *E. coli* there are two systems for maturation of [Fe-S]-clusters: *isc* and *suf*. The *isc* system has a housekeeping function, while the *suf* system is active under stress conditions [12, 13]. Since [Fe-S]-proteins play a role in diverse biological processes (*e.g.* DNA repair or gene regulation) [12], an inactivation of [Fe-S]-clusters in the course of plasma treatment could lead to bacterial inactivation. Due to this fact, the plasma sensitivity of deletion mutants defective in the *isc* and *suf* operon was

determined. An increased plasma sensitivity for most of the deletion mutants was observed (Fig. 2).

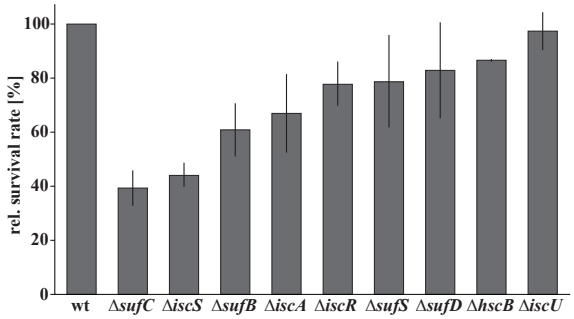

Fig. 2: Relative survival rates of the wild type and deletion mutants of the *isc* and *suf* operon after 30 s of plasma treatment. wt: wild type.

In vitro analysis of enzyme activities of the [Fe-S]-cluster free enzyme malate dehydrogenase and the [Fe-S]-enzymes aconitase, succinate dehydrogenase, and fumarase revealed that [Fe-S]-enzymes are more susceptible to plasma-induced inactivation than other enzymes. An incubation of the cells for 20 min after plasma treatment led to higher enzyme activities of the [Fe-S]-cluster containing enzymes, indicating a repair of damaged [Fe-S]-clusters. It is known that [Fe-S]-clusters are likely susceptible to O_2^- and H_2O_2. Therefore, we investigated whether SodA or KatE provide any benefit for [Fe-S]-clusters under plasma exposure. Overexpression of *sodA,* which protects against superoxide-induced damage, showed a positive effect on enzyme activities of succinate dehydrogenase and fumarase. Overexpression of *katE,* which protects against hydrogen peroxide, had a positive effect on enzyme activities of all [Fe-S]-cluster containing enzymes. Although many different radicals act on the cells during plasma treatment, the protection against hydrogen peroxide and superoxide seems to be critical for [Fe-S]-enzymes for surviving plasma since an overexpression of a combination of *sodA* and *katE* completely protected the [Fe-S]-enzymes from plasma-induced damage (Fig.3).

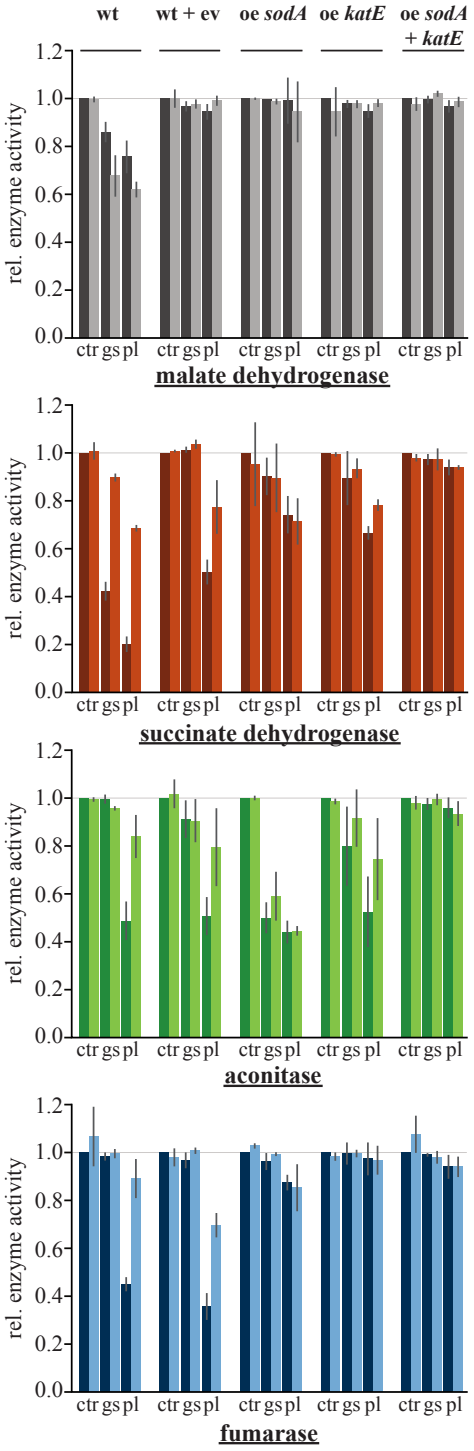

Fig. 3: Enzyme activities of the [Fe-S]-cluster-free enzyme malate dehydrogenase and [Fe-S]-enzymes aconitase, succinate dehydrogenase, and fumarase. Measurements were carried out without plasma treatment (ctr), after an exposure to helium/oxygen gas flow (gs) and after 1 min of plasma treatment (pl). Determination of enzyme activities was performed either directly after treatment (dark bars) or after incubation at 37°C for 20 min (light bars). wt: wild type, ev: empty vector, oe: overexpressing.

Conclusion

The KEIO collection, in which each non-essential gene of *E. coli* has been knocked out, was used to identify 87 plasma-sensitive deletion mutants. The increased plasma sensitivity of four deletion mutants was analysed in detail using a CFU-based assay. An overexpression of *iscS*, *rep*, and *cysB,* led to higher survival rates than observed for the wild type. Plasma resistance can thus be increased by elevated levels of a single protein. An upregulation of gene expression can emerge in bacteria, even at a higher rate due to the mutagenic effect of plasmas [14]. Our results indicate that plasma resistance can increase resulting in less sensitive strains. This may limit the clinical application of plasmas as antibacterial strategy.

Furthermore, the role of [Fe-S]-clusters in the course of bacterial inactivation by plasma was investigated. A comparison of enzyme activities after plasma treatment of [Fe-S]-enzymes and [Fe-S]-cluster free enzymes showed that [Fe-S]-enzymes are more susceptible to plasma than other enzymes. Moreover, an overexpression of *sodA* and *katE* completely protected the enzymes from plasma-induced damage, indicating that superoxide and hydrogen peroxide are presumably most important for [Fe-S]-cluster inactivation. Overall, our findings suggest that [Fe-S]-clusters could be the bottleneck in surviving plasma and that protective mechanisms (like overexpression of *sodA* and *katE*) mediate plasma resistance.

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