

Antibiotic potentiation and inhibition of cross-resistance in pathogens associated with cystic fibrosis

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Abstract

Critical Gram-negative pathogens, like *Pseudomonas*, *Stenotrophomonas* and *Burkholderia*, have become resistant to most antibiotics. Complex resistance profiles together with synergistic interactions between these organisms increase the likelihood of treatment failure in distinct infection settings, for example in the lungs of cystic fibrosis patients. Here, we discover that cell envelope protein homeostasis pathways underpin both antibiotic resistance and cross-protection in CF-associated bacteria. We find that inhibition of oxidative protein folding inactivates multiple species-specific resistance proteins. Using this strategy, we sensitize multi-drug resistant *Pseudomonas aeruginosa* to β -lactam antibiotics and demonstrate promise of new treatment avenues for the recalcitrant pathogen *Stenotrophomonas maltophilia*. The same approach also inhibits cross-protection between resistant *S. maltophilia* and susceptible *P. aeruginosa*, allowing eradication of both commonly co-occurring CF-associated organisms. Our results provide the basis for the development of next-generation strategies that target antibiotic resistance, while also impairing specific interbacterial interactions that enhance the severity of polymicrobial infections.

eLife assessment

This **important** study demonstrates that disrupting a common protein-folding system restores sensitivity to antibiotics in drug-resistant clinical bacterial pathogens. Although some additional controls would be welcome, the work is overall **convincing** in showing that targeting protein folding can be used to combat multi-drug resistant pathogens, both by potentiating the efficacy of existing drugs and by therapeutic use of small-molecule inhibitors. This study is significant and timely as it furnishes a new strategy that is relevant to microbiologists and clinicians interested in combating antimicrobial resistance.

Introduction

Antimicrobial resistance (AMR) is one of the most significant threats to health systems worldwide [1]. Since the end of the “golden age” of antibiotic discovery in the 1970’s, very few new antimicrobial agents have entered the clinic, and most of those that have gained approval are derivatives of existing antibiotic classes [2–4]. Meanwhile, resistance to useful antibiotics is continuously rising, resulting in more than 1.3 million deaths annually [5]. In addition to the undeniable surge of resistance, it is becoming apparent that intra- and inter-species interactions also play a role in AMR and its evolution [6], posing additional challenges during antibiotic treatment. This necessitates not only the development of novel antimicrobials and strategies that will expand the lifespan of existing antibiotics, but also the implementation of approaches that will address the polymicrobial nature of most infections.

Antibiotic resistance is most commonly evaluated by testing bacterial strains in monoculture. Nonetheless, the majority of clinical infections contain multiple species whose coexistence in complex pathobionts often limits our treatment options. This is of particular importance for recalcitrant infections such as the polymicrobial communities found in the lungs of cystic fibrosis (CF) patients. CF lung infections have become a paradigm for chronic infectious diseases that result in poor quality of life and early patient mortality [7]. Such infections are dominated by highly resistant opportunistic pathogens, including, but not limited to, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, species and strains belonging to the *Burkholderia* complex, and *Stenotrophomonas maltophilia* [8]. Most of these organisms carry an array of resistance mechanisms, like efflux pumps, atypical lipopolysaccharide structures, and β -lactamase enzymes. Their co-occurrence in the CF lung leads to treatment challenges since common clinical care options for one pathogen are not necessarily compatible with the antibiotic susceptibility profiles of other species that are present. For example, *P. aeruginosa* is the most prevalent organism in CF lung infections and its treatment, especially during pulmonary exacerbation episodes, relies heavily on β -lactam compounds [8]. Pulmonary exacerbations and severe disease states are also associated with the presence of *S. maltophilia* [8], a globally distributed opportunistic pathogen that causes serious nosocomial respiratory infections [9]. *S. maltophilia* is intrinsically resistant to almost all antibiotics, including β -lactams like penicillins, cephalosporins and carbapenems, as well as macrolides, fluoroquinolones, aminoglycosides, chloramphenicol, tetracyclines and colistin. As a result, the standard treatment option, i.e., broad-spectrum β -lactam antibiotic therapy, constitutes a severe risk for CF patients carrying both *P. aeruginosa* and *S. maltophilia* [10,11], creating an urgent need for antimicrobial approaches that will be effective in eliminating both pathogens.

The lack of suitably broad antibiotic regimes able to simultaneously eradicate all pathogens present in specific infection settings is not the only challenge when treating polymicrobial communities. Bacterial interactions can add to this problem by adversely affecting antibiotic drug sensitivity profiles [6]. In particular, some antibiotic resistance proteins, like β -lactamases, which decrease the quantities of active drug present, function akin to common goods, since their benefits are not limited to the pathogen that produces them but can be shared with the rest of the bacterial community. This means that their activity enables pathogen cross-resistance when multiple species are present [12,13]. For example, in the CF lung, highly drug-resistant *S. maltophilia* strains actively protect susceptible *P. aeruginosa* from β -lactam antibiotics [12], and ultimately facilitate the evolution of β -lactam resistance in *P. aeruginosa* [14]. The basis of such interactions could be exploited during the design of novel therapeutic strategies, since targeting appropriate resistance enzymes will not only render their producers susceptible to existing drugs but should also impair their capacity to protect co-occurring antibiotic-susceptible strains.

Protein homeostasis in the Gram-negative cell envelope, and in particular the formation of disulfide bonds by the thiol oxidase DsbA [16–20], is essential for the function of many resistance proteins [15]. Oxidative protein folding occurs post-translationally, after translocation of the nascent polypeptide to the periplasm through the general secretion (Sec) system [21]. There, disulfide bond formation assists the assembly of 40% of the cell-envelope proteome [22,23], promotes the biogenesis of virulence factors [24,25], controls the awakening of bacterial persister cells [26], and underpins the function of resistance determinants, including enzymes for which we do not currently have inhibitor compounds, such as metallo- β -lactamases [27]. Here, we target proteostasis through inhibition of disulfide bond formation, to combat highly resistant polymicrobial infections. Using this approach, we incapacitate species-specific resistance proteins in CF-associated bacteria and simultaneously abrogate protective effects between pathogens that coexist in these infections. Our results demonstrate that such strategies generate compatible treatment options for recalcitrant CF pathogens and, at the same time, eradicate interspecific interactions, which increase the likelihood of treatment failure in complex infection settings.

Results

Species-specific cysteine-containing β -lactamases depend on oxidative protein folding

B-lactamase activity

To investigate the potential of targeting disulfide bond formation as a strategy to overcome resistance mechanisms in challenging pathogens, we chose to primarily explore β -lactamases that are produced by bacteria intimately associated with CF lung infections. DsbA dependence has been previously shown for a few such enzymes [15], like the chromosomally-encoded class B3 metallo- β -lactamase L1-1 from *S. maltophilia* (Table S1), which contributes significantly to AMR in this organism [9], as well as β -lactamases from the GES and OXA families, which are broadly disseminated, but commonly found in *P. aeruginosa* [28,29]. Here, we selected six clinically important β -lactamases from different Ambler classes (classes A, B and D) that are exclusively encoded either by *P. aeruginosa* or by the *Burkholderia* complex. The *P. aeruginosa* enzymes (BEL-1, CARB-2, AIM-1, and OXA-50) are all phylogenetically distinct, while the *Burkholderia* β -lactamases (BPS-1m and BPS-6) belong to the same phylogenetic class (File S1). Between them, the selected enzymes are diverse; they have different numbers of cysteines, display varied hydrolytic activities, and while some are resident in the chromosomes of their host bacteria or show classical inhibitor susceptibility, others are associated with mobile genetic elements or cannot be inhibited (Table S1).

We expressed all six β -lactamases in the *Escherichia coli* K-12 strain MC1000 and its isogenic *dsbA* deletion mutant, and recorded β -lactam minimum inhibitory concentration (MIC) values for each enzyme in both strain backgrounds. We also performed a series of control experiments aiming to assess whether any obtained effect was due to the activity of DsbA rather than a result of a general inability of the *dsbA* mutant to resist antibiotic stress. We have previously shown that deletion of *dsbA* does not affect the aerobic growth of *E. coli* MC1000, or the permeability of its outer and inner membranes [15]. Furthermore, here we observed no changes in MIC values for the aminoglycoside antibiotic gentamicin, which is not degraded by β -lactamases, or between the parental *E. coli* strain and its *dsbA* mutant harboring only the empty vector (Fig. 1 and File S2A). In addition, *E. coli* strains expressing either of two disulfide-free enzymes, the class A β -lactamases L2-1 and LUT-1 from *S. maltophilia* and *Pseudomonas luteola*, respectively, did not exhibit decreased MICs in the absence of *dsbA* (Fig. 1 and File S2A). These proteins were selected because they both contain two or more cysteine residues, but lack disulfide bonds due to the fact

that they are transported to the periplasm, pre-folded, by the Twin-arginine translocation (Tat) pathway, rather than by the Sec system; in the case of L2-1, Tat-dependent transport has been experimentally confirmed [30], whilst LUT-1 contains a predicted Tat signal sequence (SignalP 5.0 [31] likelihood scores: Sec/SPI = 0.0572, Tat/SPI = 0.9312, Sec/SPII (lipoprotein) = 0.0087, other = 0.0029). In contrast to our control experiments, expression of all test enzymes in the *dsbA* mutant background resulted in markedly reduced (>2-fold) MICs for at least one β -lactam antibiotic (Fig. 1 and File S2A), compared to the MICs recorded in the wild-type *E. coli* strain, indicating that the presence of DsbA is important for the function of these resistance proteins. The specific interaction between DsbA and our selected test enzymes was further supported by the fact that complementation of *dsbA* restores MICs to wild-type values for the latest generation β -lactam that each β -lactamase can hydrolyze (Fig. S1).

Taken together, our data show that DsbA-mediated disulfide bond formation is important for the function of all tested, species-specific β -lactamases. Of these, the most affected enzymes (largest MIC value decreases; Fig. 1) are the class A extended-spectrum- β -lactamases (ESBLs) from *Burkholderia* (BPS-1m and BPS-6) and the class B3 metallo- β -lactamase AIM-1, which, like all other class B enzymes [32], is resistant to inhibition by classical β -lactamase inhibitor compounds (Table S1) [27].

B-lactamase abundance and folding

To gain insight into how impairment of disulfide bond formation impacts the production or activity of the tested enzymes (Fig. 1), we first performed immunoblotting for all phylogenetically distinct β -lactamases (AIM-1, BEL-1, OXA-50, CARB-2, and BPS-1m) to assess their protein levels in the presence and absence of *dsbA*. For four of the five tested β -lactamases (AIM-1, BEL-1, OXA-50, and CARB-2) deletion of *dsbA* resulted in drastically reduced protein levels compared to the levels of the control enzyme L2-1, which remained unaffected (Fig. 2A). This shows that without their disulfide bonds, these proteins are unstable and are ultimately degraded by other cell envelope proteostasis components [33]. This was further corroborated by the fact that lysates from *dsbA* mutants expressing these four enzymes showed significantly reduced hydrolytic activity towards the chromogenic β -lactamase substrate nitrocefin (Fig. 2B). In the case of BPS-1m, enzyme levels were unchanged in the absence of *dsbA* (Fig. 2A). However, without its disulfide bond, this protein was significantly less able to hydrolyze nitrocefin (Fig. 2B), suggesting a folding defect that results in loss of function. The latter is consistent with the reduced MICs conferred by BPS-1m (and its sister enzyme BPS-6) in the absence of *dsbA* (Fig. 1). The data presented so far (Fig. 1 and 2) demonstrate that oxidative protein folding is essential for the biogenesis and activity of an expanded set of clinically important β -lactamases (Fig. 3), including enzymes that currently lack inhibitor options.

Targeting oxidative protein folding inhibits both antibiotic resistance and interbacterial interactions in CF-associated pathogens

Sensitization of multidrug-resistant *P. aeruginosa* clinical isolates

The efficacy of commonly used treatment options against *P. aeruginosa* in CF lung infections, namely piperacillin-tazobactam and cephalosporin-avibactam combinations, as well as more advanced drugs like aztreonam or carbapenems [34,35], is increasingly threatened by an array of β -lactamases, encompassing both broadly disseminated enzymes and species-specific ones [34-36]. To determine whether the effects on β -lactam MICs observed in our inducible system (Fig. 1 and [15]) can be reproduced in the presence of other resistance determinants

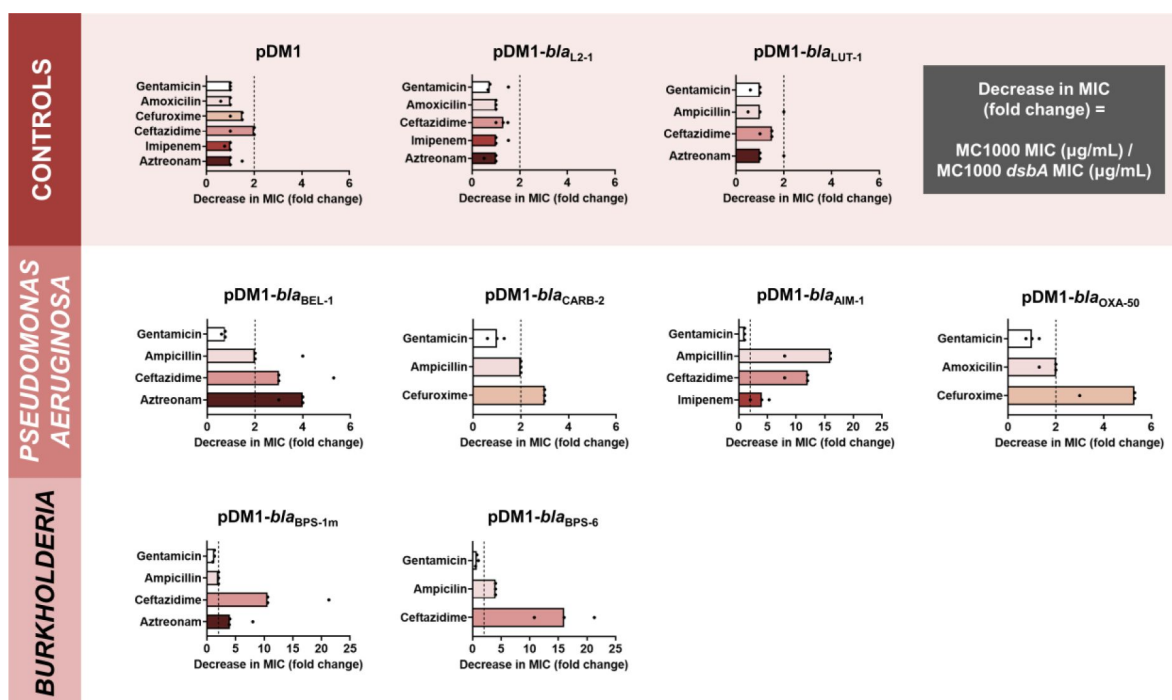


Figure 1.

The function of species-specific cysteine-containing β-lactamases from cystic-fibrosis-associated pathogens depends on DsbA-mediated oxidative protein folding.

β-lactam MIC values for *E. coli* MC1000 expressing diverse disulfide-bond-containing β-lactamases (Ambler classes A, B and D) are substantially reduced in the absence of DsbA (MIC fold changes: >2, fold change of 2 is indicated by the black dotted lines). No changes in MIC values are observed for the aminoglycoside antibiotic gentamicin (white bars) confirming that absence of DsbA does not compromise the general ability of this strain to resist antibiotic stress. No changes in MIC values are observed for strains harboring the empty vector control (pDM1) or those expressing the class A β-lactamases L2-1 and LUT-1, which contain two or more cysteines (Table S1), but no disulfide bonds (top row). Graphs show MIC fold changes for β-lactamase-expressing *E. coli* MC1000 and its *dsbA* mutant from three biological experiments each conducted as a single technical repeat; the MIC values used to generate this figure are presented in File S2A (rows 2-7 and 9-20).

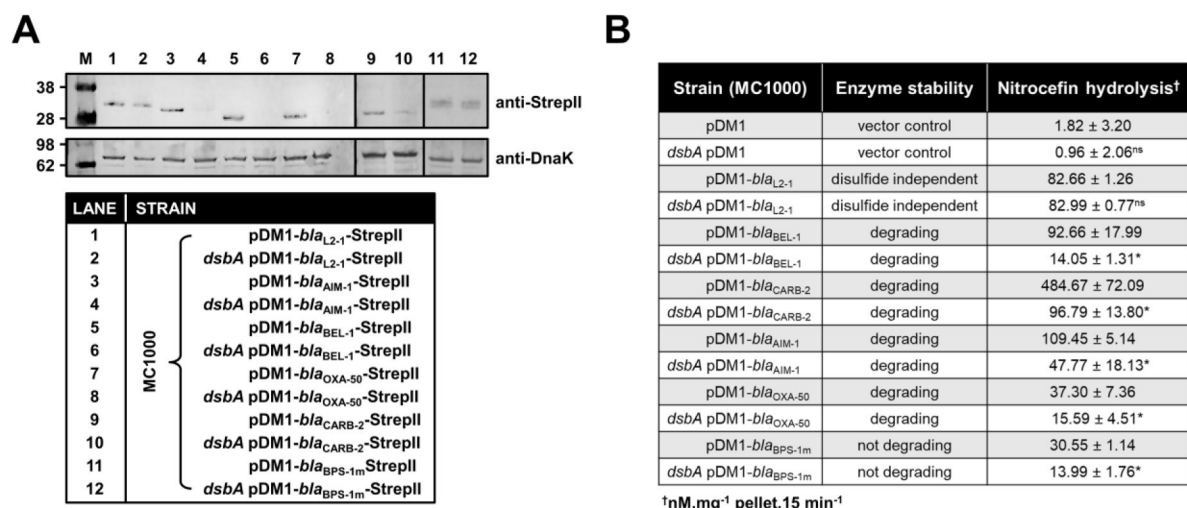


Figure 2.

Absence of DsbA results in degradation or misfolding of species-specific cysteine-containing β-lactamases.

(A) The protein levels of most tested disulfide-bond-containing Ambler class A, B, and D β-lactamases are drastically reduced when these enzymes are expressed in *E. coli* MC1000 *dsbA*; the amount of the control enzyme L2-1, containing three cysteines but no disulfide bonds, is unaffected. An exception to this is the class A enzyme BPS-1m for which no decrease in abundance is observed in the *dsbA* mutant (compare lanes 11 and 12). Protein levels of StrepII-tagged β-lactamases were assessed using a Strep-Tactin-AP conjugate or a Strep-Tactin-HRP conjugate. A representative blot from three biological experiments, each conducted as a single technical repeat, is shown; molecular weight markers (M) are on the left, DnaK was used as a loading control and solid black lines indicate where the membrane was cut. **(B)** The hydrolysis of the chromogenic β-lactam nitrocefin by cysteine-containing β-lactamases is impaired when these enzymes are expressed in *E. coli* MC1000 *dsbA*. The hydrolytic activities of strains harboring the empty vector or expressing the control enzyme L2-1 show no dependence on DsbA. The “Enzyme stability” column informs on the abundance of each enzyme when it is lacking its disulfide bond(s); this was informed from the immunoblotting experiments in panel (A). The “Nitrocefin hydrolysis” column shows the amount of nitrocefin hydrolyzed per mg of bacterial cell pellet in 15 minutes. n=3, table shows means ±SD, significance is indicated by * = p < 0.05, ns = non-significant.

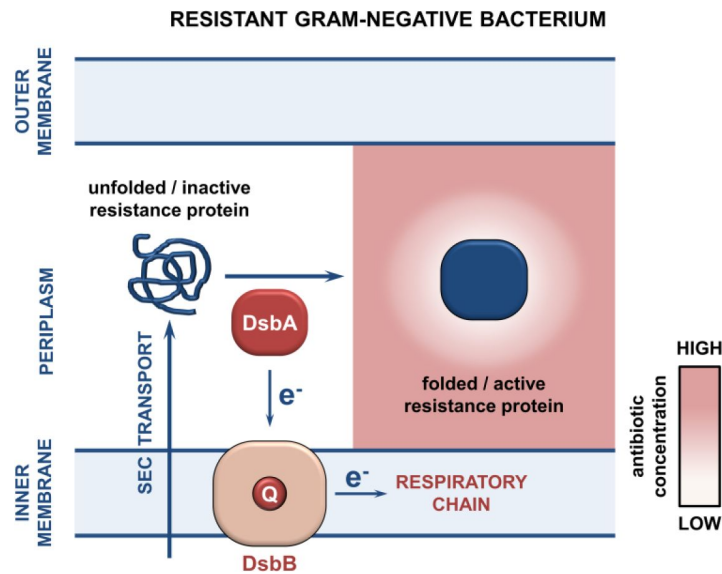


Figure 3.

The biogenesis of numerous cysteine-containing β -lactamase enzymes is dependent on disulfide bond formation.

Cysteine-containing β -lactamase enzymes, which are translocated to the periplasm through the Sec system, rely on DsbA-mediated disulfide bond formation for their stability and folding. We have previously shown that disulfide bond formation is essential for the activity of clinically important broad-spectrum enzymes, usually carried on plasmids [15]. Data presented here (Figures 1 and 2) demonstrate that oxidative protein folding is also critical for several species-specific β -lactamases from *P. aeruginosa* and *Burkholderia* spp. After translocation to the periplasm and DsbA-assisted folding, these enzymes are active and can hydrolyze β -lactam antibiotics, rendering bacteria resistant. However, in the absence of their disulfide bonds, DsbA-dependent β -lactamases either degrade or misfold, and thus can no longer confer resistance to β -lactam compounds. After each round of oxidative protein folding, DsbA is regenerated by the quinone (Q)-containing protein DsbB, which in turn transfers the reducing equivalents to the respiratory chain [46].

in a natural context with endogenous enzyme expression levels, we deleted the principal *dsbA* gene, *dsbA1* (pathogenic bacteria often encode multiple DsbA analogues [24,25]), in several multidrug-resistant (MDR) *P. aeruginosa* clinical strains (Table S2).

We first tested two clinical isolates (strains G4R7 and G6R7; Table S2) expressing the class B3 metallo- β -lactamase AIM-1, for which we recorded reduced activity in an *E. coli dsbA* background (Fig. 1 and 2). This enzyme confers high-level resistance to piperacillin-tazobactam and the third generation cephalosporin ceftazidime, both anti-pseudomonal β -lactams that are used in the treatment of critically ill patients [37]. Notably, while specific to the *P. aeruginosa* genome, *aim-1* is flanked by two ISCR15 elements suggesting that it remains mobilizable [37] (Table S1). MICs for piperacillin-tazobactam and ceftazidime were determined for both AIM-1-positive *P. aeruginosa* isolates and their *dsbA1* mutants (Fig. 4AB). Deletion of *dsbA1* from *P. aeruginosa* G4R7 resulted in a substantial decrease in its piperacillin-tazobactam MIC value by 192 μ g/mL and sensitization to ceftazidime (Fig. 4A), while the *dsbA1* mutant of *P. aeruginosa* G6R7 became susceptible to both antibiotic treatments (Fig. 4B). To further test our approach in an infection context, we performed *in vivo* survival assays using the wax moth model *Galleria mellonella* (Fig. 4C), an informative non-vertebrate system for the study of new antimicrobial approaches against *P. aeruginosa* [38]. Larvae were infected with *P. aeruginosa* G6R7 or its *dsbA1* mutant, and infections were treated once with piperacillin at a final concentration below the EUCAST breakpoint, as appropriate. No larvae survived beyond 20 hours post infection when infected with *P. aeruginosa* G6R7 or its *dsbA1* mutant without antibiotic treatment (Fig. 4C; blue and light blue survival curves). Despite this clinical strain being resistant to piperacillin *in vitro* (Fig. 4B), treatment with piperacillin *in vivo* increases larval survival (52.5% survival at 28 hours post infection) compared to the untreated conditions (Fig. 4C; blue and light blue survival curves) possibly due to the *in vivo* ceftazidime MIC values being discrepant to the value recorded *in vitro*. Nonetheless, treatment of *P. aeruginosa* G6R7 *dsbA1* with piperacillin resulted in a significant improvement in survival (77.5% survival), highlighting increased relative susceptibility compared to the treated wild-type condition, 28 hours post infection (Fig. 4C; compare the red and pink survival curves).

Next, we tested two *P. aeruginosa* clinical isolates (strains CDC #769 and CDC #773; Table S2) each expressing two class A enzymes from the GES family (GES-19/GES-26 or GES-19/GES-20), for which we have previously demonstrated DsbA dependence [15]. The GES family comprises 57 distinct ESBLs (File S1), which are globally disseminated and commonly found in *P. aeruginosa*, as well as other critical Gram-negative pathogens (for example *Klebsiella pneumoniae* and *Enterobacter cloacae*) [39]. Deletion of *dsbA1* in these clinical strains resulted in sensitization to piperacillin-tazobactam and aztreonam for *P. aeruginosa* CDC #769 (Fig. 4D), and to representative compounds of all classes of anti-pseudomonal β -lactam drugs (piperacillin-tazobactam, aztreonam, and ceftazidime) for *P. aeruginosa* CDC #773 (Fig. 4E). *P. aeruginosa* CDC #773 and its *dsbA1* mutant were further tested in a *G. mellonella* infection model using ceftazidime treatment (Fig. 4F). In this case, no larvae survived 24 hours post infection (Fig. 4F; blue, light blue and red survival curves), except for insects infected with *P. aeruginosa* CDC #773 *dsbA1* and treated with ceftazidime at a final concentration below the EUCAST breakpoint, whereby 96.7% survival was recorded (Fig. 4F; pink survival curves).

Our data on the sensitization of AIM- and GES-expressing *P. aeruginosa* clinical isolates to most commonly used anti-pseudomonal β -lactam drugs, combined with previous results on strains producing β -lactamases from the OXA family [15], show that our approach holds promise towards inactivating numerous clinically important *Pseudomonas*-specific enzymes. These include resistance determinants that cannot be currently targeted by classical β -lactamase inhibitor compounds (for example enzymes from the OXA and AIM families [27]) and, therefore, limit our treatment options.

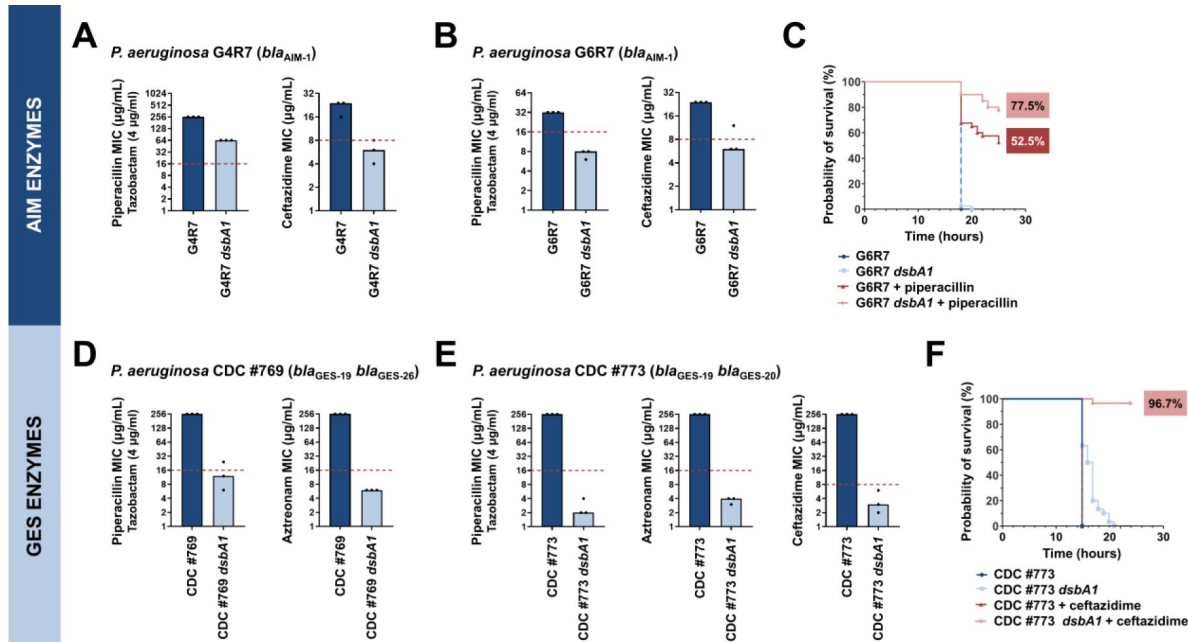


Figure 4.

Absence of the principal DsbA analogue (DsbA1) allows treatment of multidrug-resistant *Pseudomonas aeruginosa* clinical isolates with existing β-lactam antibiotics.

(A) Deletion of *dsbA1* in the AIM-1-expressing *P. aeruginosa* G4R7 clinical isolate sensitizes this strain to ceftazidime and results in reduction of the piperacillin/tazobactam MIC value by 192 μg/mL. (B) Deletion of *dsbA1* in the AIM-1-expressing *P. aeruginosa* G6R7 clinical isolate sensitizes this strain to piperacillin/tazobactam and ceftazidime. (C) 100% of the *G. mellonella* larvae infected with *P. aeruginosa* G6R7 (blue curve) or *P. aeruginosa* G6R7 *dsbA1* (light blue curve) die 18 hours post infection, while only 52.5% of larvae infected with *P. aeruginosa* G6R7 and treated with piperacillin (red curve) survive 28 hours post infection. Treatment of larvae infected with *P. aeruginosa* G6R7 *dsbA1* with piperacillin (pink curve) results in 77.5% survival, 28 hours post infection. The graph shows Kaplan-Meier survival curves of infected *G. mellonella* larvae after different treatment applications; horizontal lines represent the percentage of larvae surviving after application of each treatment at the indicated time point (a total of 40 larvae were used for each curve). Statistical analysis of this data was performed using a Mantel-Cox test; $n=40$; $p=0.3173$ (non-significance) (*P. aeruginosa* versus *P. aeruginosa dsbA1*), $p<0.0001$ (significance) (*P. aeruginosa* vs *P. aeruginosa* treated with piperacillin), $p<0.0001$ (significance) (*P. aeruginosa dsbA1* versus *P. aeruginosa* treated with piperacillin), $p=0.0147$ (significance) (*P. aeruginosa* treated with piperacillin versus *P. aeruginosa dsbA1* treated with piperacillin). (D) Deletion of *dsbA1* in the GES-19/GES-26-expressing *P. aeruginosa* CDC #769 clinical isolate sensitizes this strain to piperacillin/tazobactam and aztreonam. (E) Deletion of *dsbA1* in the GES-19/GES-20-expressing *P. aeruginosa* CDC #773 clinical isolate sensitizes this strain to piperacillin/tazobactam, aztreonam, and ceftazidime. (F) 100% of *G. mellonella* larvae infected with *P. aeruginosa* CDC #773 (blue curve), *P. aeruginosa* CDC #773 *dsbA1* (light blue curve) or larvae infected with *P. aeruginosa* CDC #773 and treated with ceftazidime (red curve) die 21 hours post infection. Treatment of larvae infected with *P. aeruginosa* CDC #773 *dsbA1* with ceftazidime (pink curve) results in 96.7% survival, 24 hours post infection. The graph shows Kaplan-Meier survival curves of infected *G. mellonella* larvae after different treatment applications; horizontal lines represent the percentage of larvae surviving after application of each treatment at the indicated time point (a total of 30 larvae were used for each curve). Statistical analysis of this data was performed using a Mantel-Cox test; $n=30$; $p<0.0001$ (significance) (*P. aeruginosa* versus *P. aeruginosa dsbA1*), $p>0.9999$ (non-significance) (*P. aeruginosa* vs *P. aeruginosa* treated with ceftazidime), $p<0.0001$ (significance) (*P. aeruginosa dsbA1* versus *P. aeruginosa* treated with ceftazidime), $p<0.0001$ (significance) (*P. aeruginosa* treated with ceftazidime versus *P. aeruginosa dsbA1* treated with ceftazidime). No changes in MIC values are observed for the aminoglycoside antibiotic gentamicin upon deletion of *dsbA1* from a representative clinical strain of *P. aeruginosa* (*P. aeruginosa* G6R7), confirming that disruption of disulfide bond formation does not compromise the general ability of this organism to resist antibiotic stress (Fig. S2A). For panels (A), (B), (D), and (E) the graphs show MIC values (μg/mL) from three biological experiments, each conducted as a single technical repeat; red dotted lines indicate the EUCAST clinical breakpoint for each antibiotic.

New treatment options for extremely-drug-resistant *S. maltophilia* clinical isolates

We have previously used our inducible *E. coli* K-12 experimental system to demonstrate that the function of the inhibitor-resistant class B3 metallo- β -lactamase L1-1 from *S. maltophilia* is dependent on DsbA [15]. By contrast, the second β -lactamase encoded on the chromosome of this species, L2-1, which we use as a negative control in this study (Fig. 1 and 2), is not DsbA dependent. The hydrolytic spectra of these β -lactamases are exquisitely complementary [9,40], making this bacterium entirely resistant to β -lactam compounds, which are commonly used for CF patients. Considering the contribution of *S. maltophilia* strains to treatment failure in CF lung infections [8,10,11] and the fact that L1 enzymes are the sole drivers of ceftazidime resistance, we wanted to investigate the DsbA dependency of L1-1 in its natural context to determine whether inhibition of oxidative protein folding potentiates the activity of complex cephalosporins.

We compromised disulfide bond formation in two clinical isolates of *S. maltophilia* (strains AMM and GUE; Table S2), by deleting the main *dsbA* gene cluster (directly adjacent *dsbA* and *dsbL* genes, with DsbL being a specialized DsbA analogue [25]) and recorded a drastic decrease of ceftazidime MIC values for both mutant strains (Fig. 5A,B). Since *S. maltophilia* cannot be treated with ceftazidime, there is no EUCAST breakpoint available for this organism. That said, for both tested *dsbA dsbL* mutant strains, the recorded ceftazidime MIC values were lower than the ceftazidime EUCAST breakpoint for the related major pathogen *P. aeruginosa* [41].

In addition to being resistant to β -lactams, *S. maltophilia* is usually intrinsically resistant to colistin, which precludes the use of yet another broad class of antibiotics. Bioinformatic analysis on 106 complete *Stenotrophomonas* genomes revealed that most strains of this organism carry two chromosomally-encoded MCR analogues that cluster with clinical MCR-5 and MCR-8 proteins (File S3). We have previously found the activity of all clinical MCR enzymes to be dependent on the presence of DsbA [15], thus we compared the colistin MIC value of the *S. maltophilia* AMM *dsbA dsbL* strain to that of its parent. We found that impairment of disulfide bond formation in this strain resulted in a decrease of its colistin MIC value from 32 μ g/mL to 0.5 μ g/mL (Fig. 5C). Once more, there is no colistin EUCAST breakpoint available for *S. maltophilia*, but a comparison with the colistin breakpoint for *P. aeruginosa* (4 μ g/mL) demonstrates the magnitude of the effects that we observe.

The DSB proteins have been shown to play a central role in bacterial virulence, and in this context, they have been proposed as promising targets against bacterial pathogenesis [24,25,42]. For this reason, several laboratory compounds against both DsbA [43,44] and its partner protein DsbB [45], which maintains DsbA in a catalytically active state [46] (Fig. 3), have been developed. We have successfully used one of these inhibitors, 4,5-dichloro-2-(2-chlorobenzyl)pyridazin-3-one, termed “compound 12” in [47], to achieve sensitization of clinical strains of Enterobacteria to β -lactam and colistin antibiotics [15]. Here, we used a derivative compound, 4,5-dibromo-2-(2-chlorobenzyl)pyridazin-3(2H)-one, termed “compound 36” in [47], which is an improved analog of compound 12 and has been shown to target several DsbB proteins from Gram-negative pathogens that share between 20-80% in protein identity. Compound 36 was previously shown to inhibit disulfide bond formation in *P. aeruginosa* [47]. Since *S. maltophilia* DsbB proteins share ~28% protein sequence identity with analogues from *P. aeruginosa*, we reasoned that this pathogen would be a good candidate for DSB system inhibition. Exposure of *S. maltophilia* AMM to the DSB inhibitor lowered its ceftazidime MIC value by 16 μ g/mL and decreased its colistin MIC value from 32 μ g/mL to 0.5 μ g/mL (Fig. 5D); the latter drop is commensurate with the results we obtained with the *S. maltophilia* AMM *dsbA dsbL* strain (Fig.

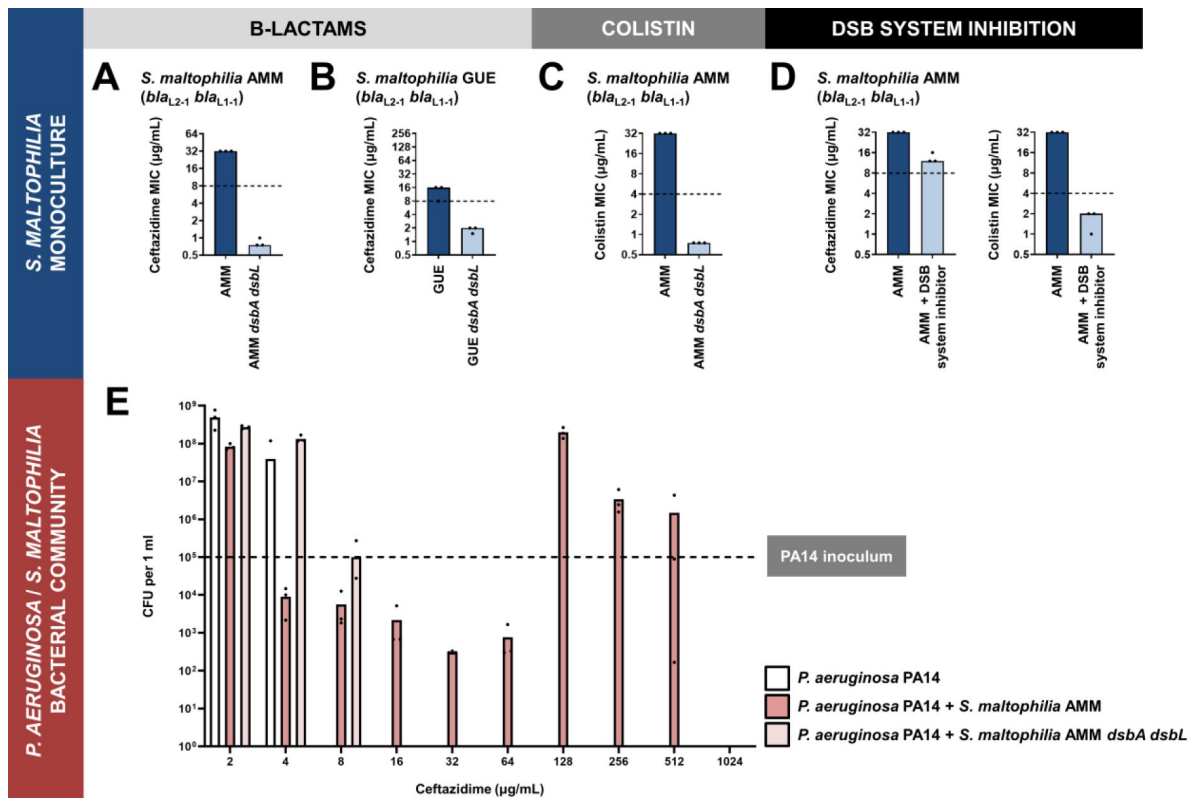


Figure 5.

(A-D) Impairment of disulfide bond formation allows the treatment of *Stenotrophomonas maltophilia* clinical strains with β -lactam and colistin antibiotics.

(A, B) Deletion of *dsbA dsbL* in the *S. maltophilia* AMM and *S. maltophilia* GUE clinical isolates results in drastic decrease of their ceftazidime MIC values. **(C)** Deletion of *dsbA dsbL* in the *S. maltophilia* AMM clinical strain results in drastic decrease of its colistin MIC value. **(D)** Use of a small-molecule inhibitor of DsbB against the *S. maltophilia* AMM clinical strain results in drastic decrease of its ceftazidime and colistin MIC values. No changes in MIC values are observed for the aminoglycoside antibiotic gentamicin upon deletion of *dsbA* and *dsbL* from a representative clinical strain of *S. maltophilia* (*S. maltophilia* AMM), confirming that disruption of disulfide bond formation does not compromise the general ability of this organism to resist antibiotic stress (Fig. S2B). For panels (A-D) graphs show MIC values ($\mu\text{g/mL}$) from three biological experiments; for β -lactam MIC assays each experiment was conducted as a single technical repeat, whereas for colistin MIC assays each experiment was conducted in technical triplicate. In the absence of EUCAST clinical breakpoints for *S. maltophilia*, the black dotted lines indicate the EUCAST clinical breakpoint for each antibiotic for the related pathogen *P. aeruginosa*. **(E) Protection of *P. aeruginosa* by *S. maltophilia* clinical strains is dependent on oxidative protein folding.** The susceptible *P. aeruginosa* strain PA14 can survive exposure to ceftazidime up to a maximum concentration of 4 $\mu\text{g/mL}$ when cultured in isolation (white bars). By contrast, if co-cultured in the presence of *S. maltophilia* AMM, which can hydrolyze ceftazidime through the action of its L1-1 β -lactamase enzyme, *P. aeruginosa* PA14 can survive and actively grow in concentrations of ceftazidime as high as 512 $\mu\text{g/mL}$ (dark pink bars). This protection is abolished if *P. aeruginosa* PA14 is co-cultured with *S. maltophilia* AMM *dsbA dsbL* (light pink bars), where L1-1 is inactive (as shown in Figure 5A and [15]). The graph shows *P. aeruginosa* PA14 colony forming unit counts (CFUs) for each condition; three biological replicates were conducted in technical triplicate, and mean CFU values are shown. The black dotted line indicates the *P. aeruginosa* PA14 inoculum. The mean CFU values used to generate this figure are presented in File S2B.

5C). Considering that this inhibitor has not been specifically optimized for *S. maltophilia* strains, the recorded drops in MIC values (Fig. 5D) are encouraging and suggest that the DSB system proteins are tractable targets against species-specific resistance determinants in this pathogen.

Currently, the best clinical strategy against *S. maltophilia* is to reduce the likelihood of infection [48] and, thus, novel treatment strategies against this organism are desperately needed. Overall, our results on targeting oxidative protein folding in this organism show promise for the generation of therapeutic avenues that are compatible with mainstream antibiotics (β -lactams and polymyxins), which are commonly used for the treatment of other co-occurring organisms in CF lung infections, like *P. aeruginosa*.

Inhibition of cross-resistance in *S. maltophilia* - *P. aeruginosa* mixed communities

The antibiotic resistance mechanisms of *S. maltophilia* impact the antibiotic tolerance profiles of other organisms that are found in the same infection environment. *S. maltophilia* hydrolyzes all β -lactam drugs through the action of its L1 and L2 β -lactamases [9,40] and, in doing so, protects other pathogens that are in principle, susceptible to treatment, such as *P. aeruginosa* [12]. This protection, in turn, allows active growth of otherwise treatable *P. aeruginosa* in the presence of complex β -lactams, like imipenem, and increases the rate of resistance evolution of *P. aeruginosa* against these antibiotics [14].

We wanted to investigate whether our approach would be useful in abrogating interspecies interactions that are relevant to CF infections. We posited that ceftazidime resistance in *S. maltophilia* is largely driven by L1-1, an enzyme that we can incapacitate by targeting disulfide bond formation [15] (Fig. 5A,B,D). As such, impairment of oxidative protein folding in *S. maltophilia* should allow treatment of this organism with ceftazidime, and at the same time eliminate any protective effects that benefit susceptible strains of co-occurring organisms. With ceftazidime being a standard anti-pseudomonal drug, and in view of the interactions reported between *P. aeruginosa* and *S. maltophilia* [12,14,49], we chose to test this hypothesis using *S. maltophilia* AMM and a *P. aeruginosa* strain that is sensitive to β -lactam antibiotics, *P. aeruginosa* PA14. We followed established co-culture protocols for these organisms [12] and monitored the survival and growth of *P. aeruginosa* under ceftazidime pressure in monoculture, or in the presence of *S. maltophilia* strains.

P. aeruginosa PA14 monoculture cannot grow in the presence of more than 4 μ g/mL of ceftazidime (Fig. 5E; white bars). However, the same strain can actively grow in concentrations of ceftazidime up to 512 μ g/mL in the presence of *S. maltophilia* AMM (Fig. 5E; dark pink bars), showing that the protective effects previously observed with imipenem [12] are applicable to other clinically relevant β -lactam antibiotics. Cross-resistance effects are most striking at concentrations of ceftazidime above 64 μ g/mL; for amounts between 16 and 64 μ g/mL, *P. aeruginosa* survives in the presence of *S. maltophilia*, but does not actively grow. This is in agreement with previous observations showing that the expression of L1-1 is highly induced by the presence of complex β -lactams [50]. In this case, increased expression of L1-1 in *S. maltophilia* grown in concentrations of ceftazidime equal or higher than 128 μ g/mL promotes ceftazidime hydrolysis and decrease of the active antibiotic concentration, in turn, shielding the susceptible *P. aeruginosa* strain. By contrast, protective effects are almost entirely absent when *P. aeruginosa* PA14 is co-cultured with *S. maltophilia* AMM *dsbA dsbL*, which cannot hydrolyze ceftazidime efficiently because L1-1 activity is impaired [15] (Fig. 5A,B,D). In fact, in these conditions *P. aeruginosa* PA14 only survives in concentrations of ceftazidime up to 8 μ g/mL (Fig. 5E; light pink bars), 64-fold lower than what it can endure the presence of *S. maltophilia* AMM (Fig. 5E; dark pink bars).

The data presented here show that targeting protein homeostasis pathways in specific recalcitrant pathogens has the potential to not only alter their own antibiotic resistance profiles (**Fig. 4** and **5A,B,C**), but also to influence their interactions with other bacteria (**Fig. 6**). This becomes especially important in infection settings where pathogen interactions affect treatment outcomes, and whereby their inhibition might facilitate treatment.

Discussion

Impairment of cell envelope protein homeostasis through interruption of disulfide bond formation holds promise as a broad-acting strategy against AMR in Gram-negative bacteria [15]. Here, we focus on the benefits of such an approach against pathogens encountered in challenging infection settings by studying organisms found in the CF lung. In particular, we show that incapacitation of oxidative protein folding compromises the function of diverse β -lactamases that are specific to CF-associated bacteria, like *P. aeruginosa* and *Burkholderia* complex (**Fig. 1**, **2** and **3**). Furthermore, we find that the effects we observe at the enzyme level are applicable to multiple MDR *P. aeruginosa* and extremely-drug-resistant *S. maltophilia* clinical strains, both *in vitro* (**Fig. 4A,B,D,E** and **5A,B**) and in an *in vivo* model of infection (**Fig. 4C,F**). Our findings, so far, concern β -lactamases encoded by Enterobacteria [15] or CF-associated organisms. Nonetheless, many other environmental bacteria are opportunistic human pathogens and encode β -lactamase genes that make them highly resistant to antibiotic treatment [9,51,52]. The ubiquitous nature of disulfide bond formation systems across Gram-negative species guarantees that the same approach can be expanded. To provide some proof on this front, we investigated two additional class B3 metallo- β -lactamases, POM-1 produced by *Pseudomonas otitidis* and SMB-1 encoded on the chromosome of *Serratia marcescens* (Table S1). We tested these enzymes in our inducible *E. coli* K-12 system and found that their activities are indeed DsbA dependent (Fig. S3A and S4), with SMB-1 degrading in the absence of DsbA and POM-1 suffering a folding defect (Fig. S3B,C). Since 57% of β -lactamase phylogenetic families that are found in pathogens and organisms capable of causing opportunistic infections contain members with two or more cysteines (File S1), we expect that thousands of enzymes rely on DsbA for their stability and function. Focusing solely on the β -lactamase families that we have investigated here and previously [15] (17 phylogenetic families), we estimate that upwards of 575 discrete proteins are DsbA dependent. This encompasses enzymes specific to pathogens with very limited treatment options, for example the *Burkholderia* complex (**Fig. 1**) and *S. maltophilia* (**Fig. 5A,B,D**), as well as 145 β -lactamases that cannot be inhibited by classical adjuvant approaches, like class B enzymes [27] from the AIM, L1, POM, and SMB families (**Fig. 1**, **4A,B,C** and S3).

Of the organisms studied in this work, *S. maltophilia* deserves further discussion because of its unique intrinsic resistance profile. The therapeutic options against this pathogen are currently limited to one antibiotic-adjuvant combination, trimethoprim-sulfamethoxazole, that is not always effective [53–56]. As a result, *S. maltophilia* infections have high case fatality rates [9]. We find that targeting disulfide bond formation in this species allows its treatment with complex cephalosporins, like ceftazidime, (**Fig. 5A,B,D**) and, at the same time, leads to colistin potentiation (**Fig. 5C,D**). This extends the usability of two invaluable broad-acting antibiotic classes against this challenging organism. Moreover, in CF, *S. maltophilia* is more abundant during pulmonary exacerbation episodes [8], whereby the use of β -lactams like ceftazidime or carbapenems is common practice [34,35]. Being fully resistant to such antibiotics, its presence causes many problems during treatment, including issues that arise from protective interactions between the *S. maltophilia* and other, usually susceptible, bacteria [8,12,14]. Use of β -lactam drugs puts CF patients at severe risk of *S. maltophilia* dominance in the lungs, which practically leaves them without any effective treatment routes [10,11]. At the same time, *S. maltophilia* has been shown to foster protection of sensitive *P. aeruginosa* strains [12], which can, in turn, promote evolution of resistance [14], impeding effective antibiotic treatment. Here, we demonstrate that by compromising L1-1 through impairing protein

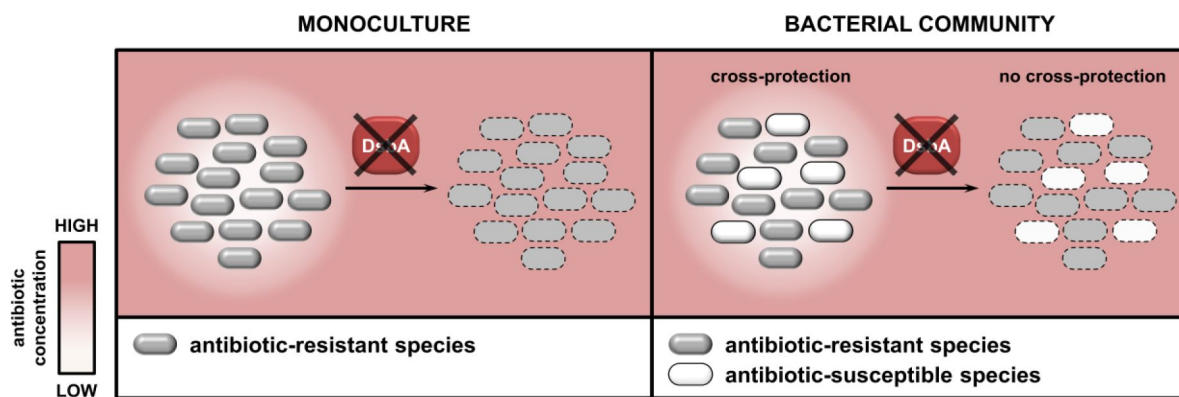


Figure 6.

Inhibition of oxidative protein folding prevents antibiotic resistance and inter-species interactions in CF-associated pathogens.

(Left) Abrogation of DsbA activity results in incapacitation of antibiotic resistance proteins and allows treatment of recalcitrant pathogens with existing antibiotics (the schematic depicts inhibition of β -lactam hydrolysis as an example).

(Right) In multispecies bacterial communities, protection of antibiotic susceptible strains by species that can degrade antibiotics is prevalent. Targeting disulfide bond formation impairs beneficial interactions that are reliant on the activity of DsbA-dependent resistance proteins (for example β -lactamase enzymes), thus allowing eradication of all species.

homeostasis in *S. maltophilia* (4A,B,D) [15], in addition to generating new treatment options (Fig. 5A-D), we abolish the capacity of this organism to protect other species (Fig. 5E). Since similar bacterial interactions are prevalent in most resistant infections [6], it can be expected that our approach will yield analogous results for other coexisting pathogens in the CF lung, for example *P. aeruginosa* and *S. aureus* [57,58] or *K. pneumoniae* and *Acinetobacter baumannii* [13], which also produce DsbA-dependent β -lactamases [15] (Fig. 6).

More generally, our findings serve as proof of principle of the added benefits of strategies that aim to incapacitate resistance determinants like β -lactamases. These proteins threaten the most widely prescribed class of antibiotics worldwide [59], and, at the same time, can promote cross-resistance between pathogens found in polymicrobial infections. It is therefore important to continue developing β -lactamase inhibitors, which, so far, have been one of the biggest successes in the battle against AMR [27,60]. That said, the deployment of broad-acting small molecules with the capacity to bind and effectively inhibit thousands of clinically important β -lactamases (7233 distinct documented enzymes (File S1) [61]) is challenging and, eventually, leads to the emergence of β -lactamase variants that are resistant to combination therapy. As such, development of additional alternative strategies that can broadly incapacitate these resistance proteins, ideally without the need to bind to their active sites, is critical. This has been shown to be possible through metal chelation for class B metallo- β -lactamases [62]. Adding to this, our previous work [15] and the results presented here lay the groundwork for exploiting accessible cell envelope proteostasis processes to generate new resistance breakers. Inhibiting such systems has untapped potential for the design of broad-acting next-generation therapeutics, which simultaneously compromise multiple resistance mechanisms [15], and also for the development of species- or infection-specific approaches that are well suited for the treatment of complex polymicrobial communities (Fig. 6).

Materials and methods

Reagents and bacterial growth conditions

Unless otherwise stated, chemicals and reagents were acquired from Sigma Aldrich or Fisher Scientific, growth media were purchased from Oxoid and antibiotics were obtained from Melford Laboratories. Lysogeny broth (LB) (10 g/L NaCl) and agar (1.5% w/v) were used for routine growth of all organisms at 37 °C with shaking at 220 RPM, as appropriate. Mueller-Hinton (MH) broth and agar (1.5% w/v) were used for Minimum Inhibitory Concentration (MIC) assays. Growth media were supplemented with the following, as required: 0.25 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG), 50 μ g/mL kanamycin, 33 μ g/mL chloramphenicol, 33 μ g/mL gentamicin, 12.5 μ g/mL tetracycline (for cloning purposes), 100-400 μ g/mL tetracycline (for the construction of *Pseudomonas aeruginosa* mutants), 50 μ g/mL streptomycin (for cloning purposes), 2000-5000 μ g/mL streptomycin and (for the construction of *Pseudomonas aeruginosa* mutants), and 6000 μ g/mL streptomycin (for the construction of *Stenotrophomonas maltophilia* mutants).

Construction of plasmids and bacterial strains

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Tables S2, S3 and S4, respectively. DNA manipulations were conducted using standard methods. KOD Hot Start DNA polymerase (Merck) was used for all PCR reactions according to the manufacturer's instructions, oligonucleotides were synthesized by Sigma Aldrich and restriction enzymes were purchased from New England Biolabs. All constructs were DNA sequenced and confirmed to be correct before use.

Genes for β -lactamase enzymes were amplified from genomic DNA extracted from clinical isolates (Table S5) with the exception of *bps-1*, *bps-6*, *carb-2*, *ftu-1* and *smb-1*, which were synthesized by GeneArt Gene Synthesis (ThermoFisher Scientific). β -lactamase genes were cloned into the IPTG-

inducible plasmid pDM1 using primers P1-P16. All StrepII-tag fusions of β -lactamase enzymes (constructed using primers P3, P5, P7, P9, P11, P13, P15, and P17-23) have a C-terminal StrepII tag (GSAWSHPQFEK).

P. aeruginosa dsbA1 mutants and *S. maltophilia dsbA dsbL* mutants were constructed by allelic exchange, as previously described [63]. Briefly, the *dsbA1* gene area of *P. aeruginosa* strains (including the *dsbA1* gene and 600 bp on either side of this gene) was amplified (primers P24/P25) and the obtained DNA was sequenced to allow for accurate primer design for the ensuing cloning step. The pKNG101-*dsbA1* plasmid was then used for deletion of the *dsbA1* gene in *P. aeruginosa* G4R7 and *P. aeruginosa* G4R7, as before [15]. For the deletion of *dsbA1* in *P. aeruginosa* CDC #769 and *P. aeruginosa* CDC #773, 500-bp DNA fragments upstream and downstream of the *dsbA1* gene were amplified using *P. aeruginosa* CDC #769 or *P. aeruginosa* CDC #773 genomic DNA (primers P28/P29 (upstream) and P30/P31 (downstream)). Fragments containing both regions were then obtained by overlapping PCR (primers P28/P31) and inserted into the XbaI/BamHI sites of pKNG102, resulting in plasmids pKNG102-*dsbA1*-769 and pKNG102-*dsbA1*-773. For *S. maltophilia* strains the *dsbA dsbL* gene area (including the *dsbA dsbL* genes and 1000 bp on either side of these genes) was amplified (primers P26/P27) and the obtained DNA was sequenced to allow for accurate primer design for the ensuing cloning step. Subsequently, 700-bp DNA fragments upstream and downstream of the *dsbA dsbL* genes were amplified using *S. maltophilia* AMM or *S. maltophilia* GUE genomic DNA (primers P32/P33 (upstream) and P34/P35 (downstream)). Fragments containing both of these regions were then obtained by overlapping PCR (primers P32/P35) and inserted into the XbaI/BamHI sites of pKNG101, resulting in plasmids pKNG101-*dsbA dsbL*-AMM and pKNG101-*dsbA dsbL*-GUE. The suicide vector pKNG101 [64] and its derivative pKNG102, are not replicative in *P. aeruginosa* or *S. maltophilia*; both vectors are maintained in *E. coli* CC118 λ pir and mobilized into *P. aeruginosa* and *S. maltophilia* strains by triparental and biparental conjugation, respectively.

P. aeruginosa PA14 was labelled with a gentamicin resistance marker using mini Tn7 delivery transposon vector adapted from Zobel et al. [65]. Briefly, *P. aeruginosa* PA14, *E. coli* CC118 λ pir pTn7-M (donor strain), *E. coli* DH5 α pir pTNS2 (strain expressing the TnsABC+D specific transposition pathway), and *E. coli* HB101 pRK600 (helper strain), were streaked on top of each other on an LB agar plate and incubated at 37 °C overnight. A patch of cells from the conjugation plate was then streaked for single colonies on Vogel Bonner Minimal medium supplemented with gentamicin and incubated at 37 °C overnight. Subsequently, colonies were streaked on LB agar supplemented with gentamicin and incubated at 37 °C overnight. Correct insertion of the transposon into the attTn7 site was confirmed via colony PCR and DNA sequencing.

Minimum inhibitory concentration (MIC) assays

Unless otherwise stated, antibiotic MIC assays were carried out in accordance with the EUCAST recommendations using ETEST strips (BioMérieux). Briefly, overnight cultures of each strain to be tested were standardized to OD₆₀₀ 0.063 in 0.85% NaCl (equivalent to McFarland standard 0.5) and distributed evenly across the surface of MH agar plates. E-test strips were placed on the surface of the plates, evenly spaced, and the plates were incubated for 18-24 hours at 37 °C. MICs were read according to the manufacturer's instructions. MICs were also determined using the Broth Microdilution (BMD) method for specific β -lactams, as required, and for colistin sulphate (Acros Organics). Briefly, a series of antibiotic concentrations was prepared by two-fold serial dilution in MH broth in a clear-bottomed 96-well microtiter plate (Corning). The strain to be tested was added to the wells at approximately 5 x 10⁴ colony forming units (CFU) per well and plates were incubated for 18-24 hours at 37 °C. The MIC was defined as the lowest antibiotic concentration with no visible bacterial growth in the wells. When used for MIC assays, tazobactam was included at a fixed concentration of 4 μ g/mL, in accordance with the EUCAST guidelines. All *S. maltophilia* MICs were performed in synthetic CF sputum medium (SCF) as described in [66], using E-test

strips (for β -lactam antibiotics) or the BMD method (for colistin). For *S. maltophilia* GUE, imipenem at a final concentration of 5 μ g/mL was added to the overnight cultures to induce β -lactamase production.

The covalent DsbB inhibitor 4,5-dibromo-2-(2-chlorobenzyl)pyridazin-3(2H)-one [47] was used to chemically impair the function of the DSB system in *S. maltophilia* strains. Inactivation of DsbB results in abrogation of DsbA function [67] only in media free of small-molecule oxidants [68]. Therefore, MIC assays involving chemical inhibition of the DSB system were performed using SCF media prepared as described in [66], except that L-cysteine was omitted. Either DMSO (vehicle control) or the covalent DsbB inhibitor 4,5-dibromo-2-(2-chlorobenzyl)pyridazin-3(2H)-one [47] (Bioduro-Sundia; 1 H-NMR and LCMS spectra are provided in File S4), at a final concentration of 50 μ M, were added to the cysteine-free SCF medium, as required.

SDS-PAGE analysis and immunoblotting

Samples for immunoblotting were prepared as follows. Strains to be tested were grown on LB agar plates as lawns in the same manner as for MIC assays described above. Bacteria were collected using an inoculating loop and resuspended in LB to OD₆₀₀ 2.0. The cell suspensions were centrifuged at 10,000 $\times g$ for 10 minutes and bacterial pellets were lysed by addition of BugBuster Master Mix (Merck Millipore) for 25 minutes at room temperature with gentle agitation. Subsequently, lysates were centrifuged at 10,000 $\times g$ for 10 minutes at 4 °C and the supernatant was added to 4 $\times$ Laemmli buffer. Samples were boiled for 5 minutes before separation by SDS-PAGE.

SDS-PAGE analysis was carried out using 10% BisTris NuPAGE gels (ThermoFisher Scientific) and MES/SDS running buffer prepared according to the manufacturer's instructions; pre-stained protein markers (SeeBlue Plus 2, ThermoFisher Scientific) were included. Proteins were transferred to Amersham Protran nitrocellulose membranes (0.45 μ m pore size, GE Life Sciences) using a Trans-Blot Turbo transfer system (Bio-Rad) before blocking in 3% w/v Bovine Serum Albumin (BSA)/TBS-T (0.1 % v/v Tween 20) or 5% w/v skimmed milk/TBS-T and addition of primary and secondary antibodies. The following primary antibodies were used in this study: Strep-Tactin-HRP conjugate (Iba Lifesciences) (dilution 1:3,000 in 3 w/v % BSA/TBS-T), Strep-Tactin-AP conjugate (Iba Lifesciences) (dilution 1:3,000 in 3 w/v % BSA/TBS-T), and mouse anti-DnaK 8E2/2 antibody (Enzo Life Sciences) (dilution 1:10,000 in 5% w/v skimmed milk/TBS-T). The following secondary antibodies were used in this study: goat anti-mouse IgG-AP conjugate (Sigma Aldrich) (dilution 1:6,000 in 5% w/v skimmed milk/TBS-T) and goat anti-mouse IgG-HRP conjugate (Sigma Aldrich) (dilution 1:6,000 in 5% w/v skimmed milk/TBS-T). Membranes were washed three times for 5 minutes with TBS-T prior to development. Development for AP conjugates was carried out using a SigmaFast BCIP/NBT tablet, while HRP conjugates were visualized with the Immobilon Crescendo chemiluminescent reagent (Merck) using a Gel Doc XR+ Imager (Bio-Rad).

β -lactam hydrolysis assay

β -lactam hydrolysis measurements were carried out using the chromogenic β -lactam nitrocefin (Abcam). Briefly, overnight cultures of strains to be tested were centrifuged, pellets were weighed and resuspended in 150 μ L of 100 mM sodium phosphate buffer (pH 7.0) per 1 mg of wet-cell pellet, and cells were lysed by sonication. Lysates were transferred into clear-bottomed 96-well microtiter plates (Corning) at volumes that corresponded to the following weights of bacterial cell pellets: strains harboring pDM1, pDM1-*bla*_{L2-1} and pDM1-*bla*_{OXA-50} (0.34 mg of cell pellet); strains harboring pDM1-*bla*_{BEL-1}, pDM1-*bla*_{AIM-1} and pDM1-*bla*_{SMB-1} (0.17 mg of cell pellet); strains harboring pDM1-*bla*_{POM-1} (0.07 mg of cell pellet); strains harboring pDM1-*bla*_{BPS-1m} (0.07 mg of cell pellet); strains harboring pDM1-*bla*_{CARB-2} (0.03 mg of cell pellet). In all cases, nitrocefin was added at a final concentration of 400 μ M and the final reaction volume was made up to 100 μ L using 100 mM sodium phosphate buffer (pH 7.0). Nitrocefin hydrolysis was monitored at 25 °C by

recording absorbance at 490 nm at 60-second intervals for 15 minutes using an Infinite M200 Pro microplate reader (Tecan). The amount of nitrocefin hydrolyzed by each lysate in 15 minutes was calculated using a standard curve generated by acid hydrolysis of nitrocefin standards.

Galleria mellonella survival assay

The wax moth model *G. mellonella* was used for *in vivo* survival assays [69]. Individual *G. mellonella* larvae were randomly allocated to experimental groups; no masking was used. Overnight cultures of all the strains to be tested were standardized to OD₆₀₀ 1.0, suspensions were centrifuged, and the pellets were washed three times in PBS and serially diluted. For experiments with *P. aeruginosa* G6R7, 10 µL of the 1:10,000 dilution of each bacterial suspension was injected into the last right abdominal proleg of 40 *G. mellonella* larvae per condition. One hour after infection, larvae were injected with 2.75 µL of piperacillin to a final concentration of 5 µg/mL in the last left abdominal proleg. For experiments with *P. aeruginosa* CDC #773 10 µL of the 1:1,000 dilution of each bacterial suspension was injected into the last right abdominal proleg of 30 *G. mellonella* larvae per condition. Immediately after the injection with the inoculum, the larvae were injected with 4.5 µL of ceftazidime to a final concentration of 6.5 µg/mL in the last left abdominal proleg. All larvae were incubated at 37 °C and their mortality was monitored for 30 hours. Death was recorded when larvae turned black due to melanization and did not respond to physical stimulation. For each experiment, an additional ten larvae were injected with PBS as negative control and experiments were discontinued and discounted if mortality was greater than 10% in the PBS control.

S. maltophilia - *P. aeruginosa* protection assay

The protection assay was based on the approach described in [12]. Briefly, 75 µL of double-strength SCF medium were transferred into clear-bottomed 96-well microtiter plates (VWR) and inoculated with *S. maltophilia* AMM or its *dsbA dsbL* mutant that had been grown in SCF medium at 37 °C overnight; *S. maltophilia* strains were inoculated at approximately 5×10^4 , as appropriate. Plates were incubated at 37 °C for 6 hours. Double-strength solutions of ceftazidime at decreasing concentrations were prepared by two-fold serial dilution in sterile ultra-pure H₂O, and were added to the wells, as required. *P. aeruginosa* PA14 attTn7::accC was immediately added to all the wells at approximately 5×10^4 CFUs, and the plates were incubated for 20 hours at 37 °C. To determine the presence of *P. aeruginosa* PA14 attTn7::accC, serial dilutions of the content of each well were performed in MH broth down to 10^{-7} , plated on MH agar supplemented with gentamicin (*S. maltophilia* AMM strains are sensitive to gentamicin, whereas *P. aeruginosa* PA14 attTn7::accC harbors a gentamicin resistance gene on its Tn7 site) and incubated at 37 °C overnight. CFUs were enumerated the following day.

Statistical analysis of experimental data

The total number of performed biological experiments and technical repeats are mentioned in the figure legend of each display item. Biological replication refers to completely independent repetition of an experiment using different biological and chemical materials. Technical replication refers to independent data recordings using the same biological sample. For MIC assays, all recorded values are displayed in the relevant graphs; for MIC assays where three or more biological experiments were performed, the bars indicate the median value, while for assays where two biological experiments were performed the bars indicate the most conservative of the two values (i.e., for increasing trends, the value representing the smallest increase and for decreasing trends, the value representing the smallest decrease). For all other assays, statistical analysis was performed in GraphPad Prism v8.3.1 using either an unpaired T-test with Welch's correction, or a Mantel-Cox logrank test, as appropriate. Statistical significance was defined as $p < 0.05$. Outliers were defined as any technical repeat >2 SD away from the average of the other

technical repeats within the same biological experiment. Such data were excluded and all remaining data were included in the analysis. Detailed information for each figure is provided below:

Figure 2B [↗](#)

unpaired T-test with Welch's correction; n=3; 3.417 degrees of freedom, t-value=0.3927, p=0.7178 (non-significance) (for pDM1 strains); 2.933 degrees of freedom, t-value=0.3296, p=0.7639 (non-significance) (for pDM1-*bla*_{L2-1} strains); 2.021 degrees of freedom, t-value=7.549, p=0.0166 (significance) (for pDM1-*bla*_{BEL-1} strains); 2.146 degrees of freedom, t-value=9.153, p=0.0093 (significance) (for pDM1-*bla*_{CARB-1} strains); 2.320 degrees of freedom, t-value=5.668, p=0.0210 (significance) (for pDM1-*bla*_{AIM-1} strains); 3.316 degrees of freedom, t-value=4.353, p=0.0182 (significance) (for pDM1-*bla*_{OXA-50} strains); 3.416 degrees of freedom, t-value=13.68, p=0.0004 (significance) (for pDM1-*bla*_{BPS-1m} strains).

Figure 4C [↗](#)

Mantel-Cox test; n=40; p=0.3173 (non-significance) (*P. aeruginosa* versus *P. aeruginosa dsbA1*), p<0.0001 (significance) (*P. aeruginosa* vs *P. aeruginosa* treated with piperacillin), p<0.0001 (significance) (*P. aeruginosa dsbA1* versus *P. aeruginosa* treated with piperacillin), p=0.0147 (significance) (*P. aeruginosa* treated with piperacillin versus *P. aeruginosa dsbA1* treated with piperacillin).

Figure 4F [↗](#)

Mantel-Cox test; n=30; p<0.0001 (significance) (*P. aeruginosa* versus *P. aeruginosa dsbA1*), p>0.9999 (non-significance) (*P. aeruginosa* vs *P. aeruginosa* treated with ceftazidime), p<0.0001 (significance) (*P. aeruginosa dsbA1* versus *P. aeruginosa* treated with ceftazidime), p<0.0001 (significance) (*P. aeruginosa* treated with ceftazidime versus *P. aeruginosa dsbA1* treated with ceftazidime).

Figure S3C

unpaired T-test with Welch's correction; n=3; 3.417 degrees of freedom, t-value=0.3927, p=0.7178 (non-significance) (for pDM1 strains); 2.933 degrees of freedom, t-value=0.3296, p=0.7639 (non-significance) (for pDM1-*bla*_{L2-1} strains); 3.998 degrees of freedom, t-value=4.100, p=0.0149 (significance) (for pDM1-*bla*_{POM-1} strains); 2.345 degrees of freedom, t-value=15.02, p=0.0022 (significance) (for pDM1-*bla*_{SMB-1} strains).

Bioinformatics

The following bioinformatics analyses were performed in this study. Short scripts and pipelines were written in Perl (version 5.18.2) and executed on macOS Sierra 10.12.5.

β-lactamase enzymes

All available protein sequences of β-lactamases were downloaded from <http://www.blddb.eu> [↗](#) [61 [↗](#)] (30 March 2023). Sequences were clustered using the ucluster software with a 90% identity threshold and the cluster_fast option (USEARCH v.7.0 [70 [↗](#)]); the centroid of each cluster was used as a cluster identifier for every sequence. All sequences were searched for the presence of cysteine residues using a Perl script. Proteins with two or more cysteines after the first 30 amino acids of their primary sequence were considered potential substrates of the DSB system for organisms where oxidative protein folding is carried out by DsbA and provided that translocation of the β-lactamase outside the cytoplasm is performed by the Sec system. The first 30 amino acids of each sequence were excluded to avoid considering cysteines that are part of the signal sequence mediating the translocation of these enzymes outside the cytoplasm. The results of the analysis can be found in File S1.

Stenotrophomonas MCR-like enzymes

Hidden Markov Models built with validated sequences of MCR-like and EptA-like proteins were used to identify MCR analogues in a total of 106 complete genomes of the *Stenotrophomonas* genus, downloaded from the NCBI repository (30 March 2023). The analysis was performed with *hmmsearch* (HMMER v.3.1b2) [71] and only hits with *e*-values < 1e-10 were considered. The 146 obtained sequences were aligned using MUSCLE [72] and a phylogenetic tree was built from the alignment using FastTree 2.1.7 with the *wag* substitution matrix and the *gamma* option [73]. The assignment of each MCR-like protein sequence to a specific phylogenetic group was carried out based on the best fitting *hmmsearch* model. The results of the analysis can be found in File S3.

Data availability

All data generated during this study that support the findings are included in the manuscript or the Supplementary Information. All materials are available from the corresponding author upon request.

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Author contributions

N.K., R.C.D.F. and D.A.I.M. designed the research. N.K. performed most of the experiments. P.B. and A.F. provided strains, genetic tools and advice on *P. aeruginosa* molecular biology. C.L. provided materials and advice on the chemical inhibition of the DSB system. D.G. performed *in silico* analyses and advised on several aspects of the project. L.E., E.M. and R.R.MC performed *G. mellonella* survival assays. N.K., R.C.D.F. and D.A.I.M. wrote the manuscript with input from all authors. D.A.I.M. directed the project.

Declaration of interests

The authors declare no competing interests.

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Editors

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Reviewer #1 (Public Review):

Summary:

In this work the authors provide evidence that impairment of cell envelope protein homeostasis through blocking the machinery for disulfide bond formation restores the efficacy of antibiotics including beta-lactam drugs and colistin against AMR in Gram-negative bacteria.

Strengths:

The authors employ a thorough approach to showcase the restoration of antibiotic sensitivity through inhibition of the DSB machinery, including the evaluation of various antibiotics on both normal and Dsb-deficient pathogenic bacteria (i.e. *Pseudomonas* and *Stenotrophomonas*). The authors corroborate these findings by employing Dsb inhibitors in addition to delta *dsbA* strains. The methodology is appropriate and includes measuring MICs as well as validating their observations in vivo using the Galleria model.

Weaknesses:

The study would benefit from presenting raw data in some cases, such as MIC values and SDS-PAGE gels, by clarifying the number of independent experiments used, as well as further clarification on statistical significance for some of the data.

Reviewer #2 (Public Review):

Summary:

This work by Kadeřábková et al. demonstrates the importance of a specific protein folding system to effectively folding β -lactamase proteins, which are responsible for resistance to β -lactam antibiotics, and shows that inhibition of this system sensitize multidrug-resistant pathogens to β -lactam treatment. In addition, the authors extend these observations to a two-species co-culture model where β -lactamases provided by one pathogen can protect another, sensitive pathogen from β -lactam treatment. In this model, disrupting the protein folding system also disrupted protection of the sensitive pathogen from antibiotic killing. Overall, the data presented provide a solid foundation for subsequent investigations and development of inhibitors for β -lactamases and other resistance determinants. This and similar strategies may have particular application to polymicrobial contexts, but the present state of knowledge regarding the existence and clinical effects of microbial interactions in disease, both specifically regarding *S. maltophilia* and *P. aeruginosa* as well as generally, is significantly overstated.

Strengths:

The authors use clear and reliable molecular biology strategies to show that β -lactamase proteins from *P. aeruginosa* and *Burkholderia* species, expressed in *E. coli* in the absence of the *dsbA* protein folding system, are variably less capable of resisting the effects of different β -lactam antibiotics compared to the *dsbA*-competent parent strain (Figure 1). The appropriate control is included in the supplemental materials to demonstrate that this effect is specifically dependent on *dsbA*, since complementing the mutant with an intact *dsbA* gene restores antibiotic resistance (Figure S1). The authors subsequently show that this lack of activity can be explained by significantly reduced protein levels and loss-of-function protein misfolding in the *dsbA* mutant background (Figure 2). These data support the importance of this protein folding mechanism in the activity of multiple clinically relevant β -lactamases.

Native bacterial species are used for subsequent experiments, and the authors provide important context for their antibiotic choices and concentrations by referencing the breakpoints that guide clinical practice. In Figure 4, the authors show that loss of the DsbA system in *P. aeruginosa* significantly sensitizes clinical isolates expressing different classes of β -lactamases to clinically relevant antibiotics. The appropriate control showing that the *dsbA1* mutation does not result in sensitivity to a non- β -lactam antibiotic is included in Figure S2. The authors further show, using an in vivo model for antibiotic treatment, that treatment of a *dsbA1* mutant results in moderate and near-complete survival of the infected organisms. The importance of this system in *S. maltophilia* is then investigated similarly (Figure 5), showing that a *dsbA dsbL* mutant is also sensitive to β -lactams and colistin, another antibiotic whose resistance mechanism is dependent on the DsbA protein folding system. Importantly, the authors show that a small-molecule inhibitor that disrupts the DsbA system, rather than genetic mutations, is also capable of sensitizing *S. maltophilia* to these antibiotics. It should be noted that while the sensitization is less pronounced, this molecule has not been optimized for *S. maltophilia* and would be expected to increase in efficacy once this is done. Together, the data support that interference with the DsbA system in native hosts can sensitize otherwise resistant pathogens to clinically relevant antibiotic therapy.

Finally, the authors investigate the effects of co-culturing *S. maltophilia* and *P. aeruginosa* (Figure 5E). These assays are performed in synthetic cystic fibrosis sputum medium (SCFM), which provides a nutritional context similar to that in CF but without the presence of more complex components such as mucin. The authors show that while *P. aeruginosa* alone is sensitive to the antibiotic, it can survive moderate concentrations in the presence of *S. maltophilia* and even grow in higher concentrations where *S. maltophilia* appears to overproduce its β -lactamases. However, this protection is lost in *S. maltophilia* without the DsbA protein folding system, showing that the protective effect depends on functional production of β -lactamase. The data support a protective role for DsbA-dependent β -lactamase under these co-culture conditions.

Weaknesses:

While Figure 5E demonstrates a protective effect of DsbA-dependent β -lactamase, the omission of CFU data for *S. maltophilia* makes it difficult to assess the applicability of the polymicrobial strategy. Since *S. maltophilia* is pre-cultured prior to the addition of *P. aeruginosa* and antibiotics, it is unclear whether the protective effect is dependent on high *S. maltophilia* CFU. It is also unclear what the fate of the *S. maltophilia dsbA dsbL* mutant is under these conditions. If DsbA-deficient *S. maltophilia* CFU is not impacted, then this treatment will result in the eradication of only one of the pathogens of interest. If the mutant is lost during treatment, then it is not clear whether the loss of protection is due specifically to the production of non-functional β -lactamase or simply the absence of *S. maltophilia*.

The alleged clinical relevance and immediate, theoretical application of this approach should be properly contextualized. At multiple junctures, the authors state or suggest that interactions between *S. maltophilia* and *P. aeruginosa* are known to occur in disease or have known clinical relevance related to treatment failure and disease states. For instance, the citations provided for *S. maltophilia* protection of *P. aeruginosa* in the CF lung environment both describe simplified laboratory experiments rather than clinical or in vivo observations. Similarly, the citations provided for both the role of *S. maltophilia* in treatment failure and CF disease severity do not support either claim. The role of *S. maltophilia* in CF is currently unsettled, with more recent work reporting conflicting results that support *S. maltophilia* as a marker, rather than cause, of severe disease. These citations also do not support the suggestion that *S. maltophilia* specifically contributes to treatment failure. While it is reasonable to pursue these ideas as a hypothesis or potential concern, there is no evidence provided that these specific interactions occur in vivo or that they have clinical relevance.

Reviewer #3 (Public Review):

Summary:

In the face of emerging antibiotic resistance and slow pace of drug discovery, strategies that can enhance the efficacy of existing clinically used antibiotics are highly sought after. In this manuscript, through genetic manipulation of a model bacterium (*Escherichia coli*) and clinically isolated and antibiotic resistant strains of concern (*Pseudomonas*, *Burkholderia*, *Stenotrophomonas*), an additional drug target to combat resistance and potentiate existing drugs is put forward. These observations were validated in both pure cultures, mixed bacterial cultures and in worm models. The drug target investigated in this study appears to be broadly relevant to the challenge posed by lactamases enzyme that render lactam antibiotics ineffective in the clinic. The compounds that target this enzyme are being developed already, some of which were tested in this study displaying promising results and potential for further optimization by medicinal chemists.

Strengths:

The work is well designed and well executed and targets an urgent area of research with the unprecedented increase in antibiotic resistance.

Weaknesses:

The impact of the work can be strengthened by demonstrating increased efficacy of antibiotics in mice models or wound models for *Pseudomonas* infections. Worm models are relevant, but still distant from investigations in animal models.