Interspecies Comparison of the Bacterial Response to Allicin Reveals Species-Specific Defense Strategies

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Allicin, a broad-spectrum antimicrobial agent from garlic, disrupts thiol and redox homeostasis, proteostasis, and cell membrane integrity. Since medicine demands antimicrobials with so far unexploited mechanisms, allicin is a promising lead structure. While progress is being made in unraveling its mode of action, little is known on bacterial adaptation strategies. Some isolates of Pseudomonas aeruginosa and Escherichia coli withstand exposure to high allicin concentrations due to as yet unknown mechanisms. To elucidate resistance and sensitivity-conferring cellular processes, the acute proteomic responses of a resistant P. aeruginosa strain and the sensitive species Bacillus subtilis are compared to the published proteomic response of E. coli to allicin treatment. The cellular defense strategies share functional features: proteins involved in translation and maintenance of protein quality, redox homeostasis, and cell envelope modification are upregulated. In both Gram-negative species, protein synthesis of the majority of proteins is downregulated while the Gram-positive B. subtilis responded by upregulation of multiple regulons. A comparison of the B. subtilis proteomic response to a library of responses to antibiotic treatment reveals 30 proteins specifically upregulated by allicin. Upregulated oxidative stress proteins are shared with nitrofurantoin and diamide. Microscopy-based assays further indicate that in B. subtilis cell wall integrity is impaired.

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1. Introduction

Garlic (Allium sativum) is a plant used both as food and as ingredient in traditional medicine for centuries by various peoples around the globe. It is mentioned in historic medical records as a potent therapeutic for cardiovascular diseases, cancer, digestive disorders, and microbial infections (reviewed in ref. [1]). In the early 20th century, inhalation of vapor from garlic oil was reported as a highly effective treatment for tuberculosis.[2,3] In 1944, Cavallito et al. discovered that garlic’s antimicrobial activity is attributed to the reactive sulfur species diallylthiosulfinate named allicin.[4] This compound is released by garlic upon tissue damage that results in the mixing of the cytosolic, non-proteinogenic amino acid alliin and the enzyme alliinlyase, which is stored in vacuoles. Allylsulfenic acid is released from alliin and two molecules condense, resulting in the generation of the plant defense substance allicin.[6] Allicin profoundly impairs the microbial cellular redox and thiol homeostasis by introducing S-allylmercapto adducts through reaction with accessible thiols, preferably those forming thiolate ions, including cysteine residues in proteins.[4,6] Recently, several antimicrobial effects related to allicin-induced thiol depletion have been described. Proteins carrying catalytic cysteines are prone to thiol oxidation and thiol modification. Thus, the loss of function of essential metabolic enzymes, protein misfolding, and protein aggregation have been connected to allicin treatment.[7,8] Allicin was further reported to disturb quorum sensing systems and to eradicate bacterial biofilms.[9,10] Furthermore, allicin-induced permeabilization of plant cell membranes, tonoplasts, and artificial lipid-bilayers, caused by transient pore formation, was reported.[11] This global impact of allicin on physiology results in a broad spectrum of antimicrobial activity against fungi, bacteria, and protozoa, but also affects human cell physiology in a dose-dependent manner.[12] In times of multidrug-resistant pathogens spreading worldwide, the unexploited multifaceted mode of action renders allicin and allicin-derivatives promising candidates for combating currently untreatable pathogens.[10,13,14]

While progress is being made in understanding its mode of action, little is known about the bacterial strategies to cope
with allicin stress. A recent proteome-wide study gave detailed insights into the initial stress response of E. coli to allicin exposure.\(^7\) Numerous chaperones as well as components of the oxidative stress response, maintenance of redox homeostasis, and ROS detoxification were upregulated. However, different isolates of the important nosocomial pathogen P. aeruginosa differ widely in their ability to withstand exposure to allicin. Some isolates of P. aeruginosa can naturally withstand very high concentrations of allicin by raising the question as to whether there are additional cellular strategies that confer resistance.

To gain insights into cell protective strategies, we monitored the acute proteomic response to allicin treatment of an allicin-resistant Gram-negative P. aeruginosa isolate and compared it to that of the allicin-sensitive Gram-positive B. subtilis, as well as to the data published on E. coli.\(^7\) Initial changes in translation rates and proteome composition were analyzed by pulse-labeling newly synthesized proteins with l\(^{15}\)S-methionine within the first few minutes after antibiotic challenge. Radioactive pulselabeling of newly synthesized proteins further provides an exquisitely sensitive method for detecting changes in the allocation of cellular protein synthesis capacity. Early changes in the proteome can provide insights into the direct impact of antibiotic treatment on protein synthesis and protein integrity\(^2\) as well as into strategies to cope with antibiotic stress.\(^4\) From the response one can therefore infer which processes or pathways are impaired or which structures are targeted.\(^17\) Hence, proteomic responses mirror the antibiotic insult and often provide insights into modes of action. Cytosolic protein extracts of pulse-labeled cells were separated by 2D-PAGE and relative synthesis rates were determined based on autoradiographs of the gels. Upregulated proteins were identified by MS. In addition to investigating species-specific adaptation strategies, we further compared the allicin proteome profile of B. subtilis to an extensive library of antibiotic responses to identify allicin-specific and shared marker proteins.

2. Experimental Section

2.1. Synthesis of Allicin

Allicin was synthesized following an optimized protocol for diallyl-disulfide (DADS) oxidation by H\(_2\)O\(_2\) published by Lawson and Wang.\(^19\) In brief, distilled DADS was mixed with methanol. Performic acid (H\(_2\)O\(_2\) and formic acid, mixed in a 3:5 ratio) was added slowly. The reaction was quenched after 15 min with water. All reactions were carried out on ice. The mixture was extracted with dichloromethane and the solvent removed. The product was dissolved in a 2:1 mixture of n-hexane and ethyl acetate and purified using LC. After removal of solvents, a clear and oily substance was obtained that was diluted to 10 mg mL\(^{-1}\) in A. dest. for growth experiments.

2.2. Growth Conditions

P. aeruginosa PA01 was grown aerobically in chemically defined M9 medium (38.09 mM Na\(_2\)HPO\(_4\), 12.79 mM KH\(_2\)PO\(_4\), 8.56 mM NaCl, 18.69 mM NH\(_4\)Cl, 22 mM glucose, 100 mM CaCl\(_2\), 2 mM MgSO\(_4\), 29.65 mM thiamium dichloride, 46.26 mM H\(_3\)BO\(_3\), 0.01 mM MnCl\(_2\), 0.77 mM ZnSO\(_4\), 1.61 mM Na\(_2\)MoO\(_4\), 0.49 mM CuSO\(_4\), 0.17 mM Co(NO\(_3\))\(_2\)) on a rotary shaker at 200 rpm at 37 °C. B. subtilis 168 (ΔtrpC2) was grown aerobically at 37 °C as described previously in chemically defined Belitzky minimal medium (BMM) (15 mM (NH\(_4\))\(_2\)SO\(_4\), 8 mM MgSO\(_4\), 27 mM KCl, 7 mM sodium citrate, 500 mM Tris, 200 mM KH\(_2\)PO\(_4\), 200 mM CaCl\(_2\), 1 mM FeSO\(_4\), 10 mM MnSO\(_4\), 4.5 mM glutamic acid, 780 mM L-tryptophan, 0.2% glucose).\(^20\) In the cited work on E. coli, E. coli K12 MG1655 was grown aerobically in chemically defined MOPS medium (Teknova MOPS minimal medium kit; 1× MOPS mixture, 2% (w/v) glucose, 1.32 mM K\(_2\)HPO\(_4\), 1 µg mL\(^{-1}\) thiamine) at 37 °C.\(^7\)

2.3. Determination of the Minimal Inhibitory Concentration

Minimal inhibitory concentrations (MICs) were determined for all three strains in minimal media using a test tube assay as described previously.\(^21\) Briefly, 2 mL minimal medium were inoculated with 5 × 10\(^5\) cells mL\(^{-1}\) and exposed to serial dilutions of allicin. After an incubation for 16–24 h, the lowest concentration that inhibited visible growth was recorded as MIC. The MIC against B. subtilis in complex medium (Mueller Hinton medium) was determined in a microdilution assay according to clinical laboratory standard initiative guidelines as described previously for E. coli and P. aeruginosa.\(^7\)

2.4. Proteome Analysis

All bacterial cultures were grown in minimal medium inoculated with cells from overnight cultures still growing logarithmically. The cultures were incubated in a water bath (orbital shaking at
200 rpm) at 37 °C. *B. subtilis* cultures were split into subcultures directly prior to addition of alliin. Optical density was recorded at 500 nm. *P. aeruginosa* cultures were split into subcultures a few minutes after inoculation. Optical density was recorded at 578 nm. In mid-logarithmic growth phase at an OD of 0.4, alliin was added in sublethal physiologically effective concentrations (PEC) that reduced growth rates to 50–70% of control conditions. Ten minutes after alliin addition, 1.8 MBq l−[35S]-methionine (Hartmann Analytic, Braunschweig, Germany) were added. Pulse labeling was stopped after 5 min by addition of chloramphenicol and cold methionine to final concentrations of 100 and 150 µg mL−1, respectively. Cells were harvested, washed, and disrupted by ultra-sonication. 35S-Incorporation rates were determined by scintillation counting. For 2D-PAGE analysis published protocols were followed.[21] In brief, 55 µg of protein for analytical and 300 µg of protein for preparative gels were loaded onto 24 cm immobilized pH gradient strips pH 4–7 (GE Healthcare, Uppsala, Sweden) by passive rehydration for 18 h. After equilibration, proteins were separated in the second dimension on 12.5% SDS-PAGE gels. Dried gels were exposed to phosphorscreens, which were scanned at 633 nm. Relative protein synthesis rates were determined for each protein spot by subjecting autoradiographs to spot densitometry using Decodon Delta 2D 4.1 image analysis software and normalizing against the total signal on the autoradiograph.[21] Allicin-response-associated proteins that had at least twofold increased relative synthesis rates in each one of three biological replicates (they were termed alliin marker proteins) were cut from non-radioactive ruthenium (II)-Tris(4,7-diphenyl-1,10-phenantrolin)-disulfonate (RuBPs)[22] stained gels and prepared for mass spectrometrical analysis by tryptic in-gel digest as described previously.[23]

### 2.5. Mass Spectrometry

For protein identification, tryptic digests were analyzed by a nanoACQUITY-UPLC-coupled Synapt G2-S-HDMS mass spectrometer (Waters, Milford, Massachusetts, USA) equipped with the NanoLockSpray-Source (Waters) and a time-of-flight detector. Samples were desalted using a nanoACQUITY UPLC Symmetry C18 Trap column (pore size, 100 Å; particle size, 5 µm; length, 20 mm; Waters) with a flow rate of 10 µL min−1 in 0.5% buffer B (0.1% formic acid in acetonitrile). Samples were separated using a nanoACQUITY-UPLC Peptide BEH C18 column (pore size, 130 Å; particle size, 1.7 µm; length, 150 mm; Waters) with a flow rate of 0.35 µL min−1 at 40 °C. Gradient elution was performed as follows: initial, 0.5% buffer B; 22 min, 50% buffer B; 23 min, 99% buffer B; 26 min, 99% buffer B; 27 min, 0.5% buffer B; 30 min, 0.5% buffer B. Spectra were recorded in positive resolution mode with the following settings: capillary voltage, 1.7 kV; cone voltage, 40 V; source temperature, 100 °C; cone gas flow rate, 50 L h−1; desolvation gas flow rate, 500 L h−1; desolvation temperature, 150 °C. Continuous MS2 spectra were recorded in a mass range of 50–1200 m/z with a scan time of 1 s. Collision energy was ramped from 14 to 45 eV. Serving as mass reference, leucine-encephalin was injected with a capillary voltage of 3 kV every 60 s. The mass spectra were processed with ProteinLynx Global Server (Waters, version 2.5.2). Processing parameters were adjusted as follows: chromatographic peak width, automatic; MS TOF resolution, automatic; lock mass for charge 1, 556.2771 m/z; lock mass window, 0.25 Da; low energy threshold, 100 counts; elevated energy threshold, 30 counts; and intensity threshold, 750 counts. For protein identification, databases containing 5562 proteins of *P. aeruginosa* PA01 (NCBI reference sequence: NC_002516.2, added manually: trypsin, keratin) and 4180 proteins of *B. subtilis* (NCBI reference sequence: NC_000964.3, added manually: trypsin, keratin) were searched. The following settings were used: peptide tolerance, automatic; fragment tolerance, automatic; minimum fragment ion matches per peptide, 2; minimum fragment ion matches per protein, 4; maximum protein mass, 300,000 Da; primary digest reagent, trypsin; secondary digest reagent, none; missed cleavages, 1; fixed modifications, carbamidomethyl C; variable modifications, deamidation N, deamidation Q, oxidation M; and false positive rate, 4. When multiple proteins were detected in a protein digest, all identified proteins were reported. It is not possible to distinguish the contribution of each protein to the 35S signal.

The MS data have been deposited into the PRIDE partner repository of the ProteomeXchange Consortium[24] with the dataset identifier PXD012622.

### 2.6. Pathway Representation in Proteomic Responses

Allicin-response-associated proteins were assigned to their cellular function and pathways based on pseudomonas genome database,[25] Uniprot,[26] and Kyoto encyclopedia of genes and genomes[27–29] database entries. Representation of each function or pathway was calculated based on the fraction of marker proteins assigned to it. The data were displayed in a heatmap[30] using R 3.5.2 in combination with the ComplexHeatmap package in version 3.8. This led to the development of a heatmap with the score calculation (“scale” argument). The z scores were transformed to a scale of grey shades. The datasets of the three species were arranged into a tree by calculating the overall similarity of pathway regulation using the Euclidean distance for the complete-linkage clustering method.

### 2.7. Microscopy

*B. subtilis* 168 was grown to an OD600 of 0.35 in BMM at 37 °C under steady agitation and transferred into 2 mL Eppendorf tubes. The samples were further processed for microscopic analysis as described below. Cells were examined with an Olympus BX51 microscope using a U-UCD8 condenser and an UPlanSApo 100XO objective (Olympus, Hamburg, Germany). Pictures were taken using a QImaging Retiga-SRV camera and VisiView cell imaging software (Visitron Systems GmbH, Puchheim, Germany).

#### 2.7.1. Assessment of Cell Wall Integrity

Cell wall integrity was assessed using the “bubble assay” as described by Schneider et al.[32] and modified by Wenzel et al.[33]
In brief, 200 µL of a B. subtilis culture were transferred into 2 mL Eppendorf tubes and exposed to 14 µg mL\(^{-1}\) allicin or 1.5 µg mL\(^{-1}\) nisin for 15 min. The cells were fixed in 1:3 acetate/methanol and 0.5 µL transferred onto glass slides covered with 1% low-melting agarose. Cell membrane extrusions indicated cell wall damage.

2.7.2. Testing for Dissipation of the Membrane Potential

The GFP-MinD localization assay was described as an in vivo assay to assess the dissipation of the membrane potential upon stress. It was used following published protocols.\(^{[34]}\) In brief, B. subtilis 1981 GFP-MinD was grown in glucose-supplemented BMM, harvested, and washed in xylose-supplemented BMM to remove the remaining glucose. Xylose-supplemented BMM was then inoculated to an OD\(_{500}\) of 0.1 and incubated until the culture reached an OD\(_{500}\) of 0.35. Aliquots of 500 µL were stressed with 14 µg mL\(^{-1}\) allicin or 1.5 µg mL\(^{-1}\) nisin for 15 min. Overall 0.5 µL of the aliquots were transferred onto glass slides covered with 1% low-melting agarose. GFP-MinD delocalization indicated membrane depolarization.

2.7.3. Testing for Pore Formation

The assay was performed using the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, Carlsbad, USA) as described previously.\(^{[36]}\) All cells stained green when SYTO 9 was used, while the red-fluorescing propidium iodide selectively stained cells with an impaired cell membrane barrier due to formation of pores through which the dye can pass. Membrane impaired cells appeared orange to red due to the overlay of both color channels.

3. Results

3.1. Bacterial Susceptibility to Allicin in Minimal Medium

Müller et al. and Reiter et al. showed that in complex medium allicin exhibits broad-spectrum activity against bacteria (Acinetobacter baumannii, Staphylococcus aureus, E. coli) and fungi (Candida albicans) but lacks efficacy against some isolates of P. aeruginosa.\(^{[7,10]}\) Since allicin unspecifically reacts with free thiols including thiols of media components, antimicrobial activity is expected to be dependent on the medium composition. Following the workflow of Müller et al., we studied allicin susceptibility of P. aeruginosa and B. subtilis using the standard microdilution MIC test (treatment of 5 \(\times\) 10\(^5\) cells mL\(^{-1}\) overnight), the impact on growth of cultures treated in early logarithmic phase for determination of the physiologically effective concentration, and the proteomic response to allicin in chemically defined, thiol-free medium. The MIC of allicin against E. coli in minimal medium was previously determined to be 23 µg mL\(^{-1}\).\(^{[7]}\) For P. aeruginosa and B. subtilis the MICs were 16 and 12 µg mL\(^{-1}\), respectively. All MICs were thus substantially lower than in complex medium (B. subtilis, 128 µg mL\(^{-1}\); P. aeruginosa, > 512 µg mL\(^{-1}\); E. coli, 64 µg mL\(^{-1}\)).\(^{[7]}\)

3.2. Allicin Exposure Globally Affects Protein Synthesis Rates

To investigate the acute stress response to allicin, we challenged B. subtilis and P. aeruginosa with physiologically effective doses that reduced logarithmic growth of a culture treated at an OD of 0.4 by 30–50% compared to the untreated control. This dosing has proven to reliably elicit responses on the proteome level.\(^{[17,21]}\) During logarithmic growth, B. subtilis was the most sensitive of the tested species. A total of 14 µg mL\(^{-1}\) (90 µM) allicin was sufficient to reduce growth of B. subtilis. A total of 128 µg mL\(^{-1}\) (790 µM) of allicin was required for an equal reduction of growth of P. aeruginosa (Figure 1A). While P. aeruginosa growth was only temporarily inhibited, the same concentration of allicin led to a long-lasting growth arrest in E. coli. Newly synthesized proteins were pulse labeled for 5 min starting 10 min after allicin addition. l-[\(^{35}\)S]-methionine incorporation rates were determined for the samples generated for the proteome experiment (three biological replicates) to assess the cellular translation capacity using scintillation counting and protein concentration measurements (Bradford assay). As reported by Müller et al., allicin exposure decreased protein synthesis rates by 56% in E. coli.\(^{[7]}\) Translation capacities of P. aeruginosa and B. subtilis were decreased by 16% and 12%, respectively (Figure 1B).

To quantify protein synthesis rates on a proteome scale, the pulse-labeled cytosolic protein fraction was subjected to 2D-PAGE followed by autoradiography (Figure 1C) (see Supporting Information Appendix and Figure S1 for representative autoradiographs of gel images). Using untreated cells as controls for each protein spot, we calculated the changes in relative synthesis rates based on spot densitometry. Allicin treatment caused major proteomic changes in all three species (Figure 1C). In B. subtilis, the relative protein synthesis rates of 23% of all protein spots were increased, and those of 28% decreased by at least a factor of 2, respectively. In both Gram-negative species, even larger proportions of proteins showed at least a twofold reduced relative synthesis rate: 43% in E. coli and 64% in P. aeruginosa. In P. aeruginosa, only 7% of the protein spots had at least twofold higher relative intensity than under control conditions. While in E. coli, the strong growth inhibition and reduction in l-[\(^{35}\)S]-methionine incorporation are well reflected in the downregulation of a large portion of proteins, it is striking that in P. aeruginosa despite the even greater number of down-regulated proteins, allicin treatment caused only a minor reduction in the overall translation capacity. Apparently, in this species, cellular resources were efficiently redirected into the synthesis of only few proteins counteracting allicin stress.

3.3. Comparative Analysis of Proteomic Responses Revealed Species-Specific Adaptation Strategies

To unravel the cellular stress responses of B. subtilis and P. aeruginosa, allicin-response-associated proteins were identified by MS/MS. To this end, protein spots with at least twofold increased relative intensity that were detectable in the fluorescence stained preparative gels were subjected to tryptic in-gel digestion and LC–MS/MS analysis (Figure 2A; see Supporting Information Appendix and Tables S1–S3 for detailed information on
Figure 1. Impact of allicin treatment on bacterial growth, translation capacity, and protein regulation in *E. coli*, *P. aeruginosa*, and *B. subtilis*. A) Bacterial subcultures were stressed with physiologically effective, sublethal doses of allicin in the logarithmic growth phase (arrow marks the timepoint of allicin addition). Ten minutes after allicin addition, l-[35S]-methionine was added to pulse-label newly synthesized proteins for 5 min. The experiment was performed three times independently. Representative data are shown. B) Mean relative protein synthesis rates were calculated based on l-[35S]-methionine incorporation rates of treated and untreated cells during the 5 min pulse in the three replicate experiments. C) Graphical representation of 2D gel-based proteomic data. Protein spots were ranked according to their relative intensity ratios (mean log2-fold change) between allicin-treated and untreated cells and statistical significance (−log10 p-value). Cutoffs were set at average fold changes in relative signal intensity of 0.5 and 2 across three biological replicates. Original experimental data for *E. coli* was taken from ref. [7] and reanalyzed.

Identified proteins. We schematically displayed the allicin-response-associated proteins for each species colored by cellular function in a model to facilitate an interspecies comparison of the stress responses (Figure 2B). The scaled distribution of identified allicin-response-associated proteins was calculated for all associated cellular pathways (again color-coded by function) and an overall similarity of regulated pathways calculated using complete-linkage clustering as described in Section 2 (Figure 2C).

Upregulation of chaperones and proteases was a universal response among the tested species. However, *B. subtilis* responded to allicin stress by upregulation of multiple regulons, in both Gram-negative species translation of many proteins was downregulated, resembling a stringent response-like shutdown and leaving only a few upregulated allicin-response-associated proteins representing few functional groups (see Supporting Information Appendix and Tables S1–S3 for information on regulons). An overview of all allicin-response-associated proteins and their functions is presented for each of the species (Figure 2B). In *E. coli*, chaperones (GroESL, IbpAB, DnaK) and structural constituents of the ribosome (RplL, RplS, RpsM) were highly represented in the proteomic response (see Supporting Information Appendix and Table S1 for information on proteins). The response also encompassed upregulation of proteins involved in redox homeostasis (TrxA, TrxC), ROS detoxification (AhpC), and cell envelope associated processes. Müller et al. concluded that antimicrobial activity of allicin is attributed to disturbance of redox homeostasis and introduction of S-allylmercapto modifications in essential proteins causing protein aggregation and folding stress.

Other proteins participate in cell division (ZapB), motility (FliC), or are structural constituents of the outer membrane (Lpp, OmpA; Figure 2B). OmpA is one of the main outer membrane proteins in *E. coli*) that confers structural integrity to the cell envelope. It is involved in conjugation and virulence, but also acts as porin. It cooperates with the most abundant protein in *E. coli*, the outer membrane lipoprotein Lpp, which was also upregulated. Lpp maintains structural and functional integrity of the cell envelope by physically tethering the outer membrane to the peptidoglycan layer through an N-terminal N-acyl-diacylglycerol residue and C-terminal lysine residue. In addition, H-NS (histone-like nucleoid structuring protein) was upregulated, a major constituent of the bacterial nucleoid that acts as a global regulator of gene expression by condensing DNA and trapping RNA polymerase in the initiation complex. H-NS was further shown to participate in regulating transcription of rRNA and growth phase-dependent shutdown of translation.

Interestingly, the upregulated ribosomal proteins are involved in tuning translation fidelity. RpsL (L19) and RpsM (S13) form bridges between the 50S and 30S ribosomal subunits that are involved in EF-G·GTP-dependent subunit translocation. RplL (L7/L12) is part of the ribosomal stalk, which is a finger-like protrusion on the large ribosomal subunit that constitutes one of the main interaction sites for GTPase factors involved in protein synthesis. The rate of protein synthesis in response to varying growth conditions is modulated by L12 dimerization.
Figure 2. Impact of allicin on the *P. aeruginosa*, *E. coli*, and *B. subtilis* proteomes. A) The acute proteomic response of *B. subtilis* 168 and *P. aeruginosa* PA01 to treatment with 14 and 128 µg mL⁻¹ allicin, respectively, were analyzed by pulse labeling and subsequent 2D-PAGE. Synthesis rates of l-[³⁵S]-methionine pulse-labeled cytosolic proteins in untreated (green) and allicin-treated (red) cells were compared based on autoradiography. Marker proteins that were at least twofold upregulated in each of the three biological replicates were excised from preparative gels and identified by LC–MS/MS (Supporting Information Appendix and Tables S1–S3). Proteins identified in multiple spots are indicated by superscript figures. B) Marker proteins for the *P. aeruginosa* and *B. subtilis* response as well as for the previously published *E. coli* response were sorted according to cellular function. C) Pathway representation was calculated using the scaled distribution of marker proteins between cellular pathways. Grey shades represent the weighted proportion of marker proteins in relation to all other cellular processes. Based on that, dendrograms were generated using complete-linkage clustering. The same color code was used in (B) and (C) to highlight functions discussed in the text.
In *P. aeruginosa*, the translation capacity was predominantly allocated to synthesis of chaperones mediating protein folding (GroESL, DnaK, GrpE, and HtpG; Figure 2B; see Supporting Information Appendix and Table S2 for information on proteins). Interestingly, none of the marker proteins are specifically involved in ROS detoxification, although Dps protects DNA upon oxidative stress among other adverse conditions through co-crystallization and by removing Fenton reaction products in its close proximity.\(^{46}\) Like H-NS, Dps restricts DNA accessibility for the transcription machinery during the stationary phase\(^{47}\) by forming tightly packed toroidal DNA-Dps co-crystals.\(^{48,49}\) The d-alanylation of lipoteichoic and teichoic acids in acid synthesis and modification system, which is involved in teichoic acids.\(^ {63}\) Free aminogroups that partially mask the negative charge of the irreversibly damaged proteins from the cellular pool,\(^ {55}\) the teases (ClpCP) counteract protein folding stress and remove chaperones (GroESL and HslO (Hsp33 analogue)) and proteases. In response to ROS, RSS, and electrophiles is complemented by organic hydroperoxide (OhrA),\(^ {51}\) superoxide anions (SodA),\(^ {52}\) and electrophilic quinone-S-adducts (AzorR, ArsC).\(^ {33,34}\) The response to ROS, RSS, and electrophiles is complemented by an upregulation of protein quality and repair systems. While chaperones (GroESL and HslO (Hsp33 analogue)) and proteases (ClpCP) counteract protein folding stress and remove irreversibly damaged proteins from the cellular pool,\(^ {35}\) the thioredoxin (TrxA and TrxB) and bacilli-thioredoxin (BrxA and BrxB) systems are involved in repair and prevention of oxidative damage to crucial thiol groups.\(^ {56}\) The bacilli-thioredoxin system is also involved in the regulation of several pathways such as cysteine and methionine synthesis-associated pathways, translation, and quality control by debacillithiolation of S-bacilli-thiolated proteins.\(^ {57}\) This mechanism is analogous to S-glutathionylation in *E. coli*, which serves to protect critical cysteine residues against irreversible states of over-oxidation.\(^ {36}\) In addition, SufA, an A-type scaffold that is involved in maturation of oxygen-sensitive [Fe4-4S] cluster proteins, was upregulated.

Allicin was the first antibacterial for which we observed an upregulation of NagBB and DltE, enzymes involved in peptidoglycan degradation and teichoic acid modification in *B. subtilis*. NagBB is part of amino sugar metabolism and is required to utilize GlcNAC as an alternative carbon source. It is hypothesized that *B. subtilis* and other bacilli may use this system in the rhizosphere for biofilm formation.\(^ {39}\) DltE is part of the teichoic acid synthesis and modification system, which is involved in γ-alanylation of lipoteichoic and teichoic acids in *B. subtilis*.\(^ {60}\) It has been shown that this system contributes to lantibiotic\(^ {61}\) and lysozyme resistance\(^ {62}\) by introducing positively charged free aminogroups that partially mask the negative charge of the phosphodiesters and thus decrease the net negative charge of teichoic acids.\(^ {63}\)

In response to allicin, various reduction factor-dependent or generating enzymes (HypO, NfrA1, NfrA2, YqiG, Ydal) involved in different central metabolic pathways were upregulated. Some of them were shown to participate in the bacterial stress response. NfrA1, a potent NADH oxidase, regulates hydrogen peroxide levels in *B. subtilis* during oxidative stress by scavenging H$_2$O$_2$ through conversion of the corresponding residues into sulfonides.\(^ {64}\) In addition, the HypR-controlled nitroreductase HypO was shown to protect against NaOCl and diamide stress.\(^ {65}\) The NADH-dependent flavin oxido-reductase YqiG was upregulated in response to the antibiotic merasacidin, which targets lipid II and impairs cell wall biosynthesis.\(^ {13}\)

Despite *B. subtilis* being among the best characterized model organisms, proteins of unknown function form the largest group of upregulated proteins revealing substantial gaps in our current understanding of the bacterial stress response to thiol-targeting compounds.

### 3.4. Allicin Treatment Caused Cell Wall Damage

In all three species, the allicin responses encompassed upregulation of proteins that are important for the cell envelope, its structure, and its function (Figure 2B), be it the major outer membrane proteins OmpA and Lpp in *E. coli*, the tRNA-ligase AlaS indirectly involved in membrane restructuring in *P. aeruginosa*, or peptidoglycan degrading enzyme NagBB and teichoic acid-modifying enzyme DltE in *B. subtilis*. Allicin could either have a direct impact on the integrity or functionality of membrane or cell wall constituents, or their regulation could be an attempt to decrease allicin influx into the cytosol. In *B. subtilis*, a set of microscopy-based assays has been established to test for membrane depolarization, pore formation, and cell wall integrity. We used these assays to probe for effects of allicin on envelope integrity and function (Figure 3). We first tested if allicin causes pore formation in the cytoplasmic membrane as described for tonoplasts and artificial bilayers\(^ {31}\) using nisin as a positive control. Nisin integrates into the membrane and forms large pores that lead to dissipation of the membrane potential and nutrient and ion leakage.\(^ {66}\)

We probed for pore formation using a modified LIVE/DEAD assay. The green fluorescing SYTO9 enters all cells, while propidium iodide only enters cells with impaired membrane integrity. As shown for the nisin control, pore formation results in propidium iodide influx and an orange staining of the cells caused by overlapping red and green fluorescence signals. In addition, we tested for membrane depolarization using a GFP-MinD assay.\(^ {14}\) MinD is usually localized at the cell poles and at mid cell, but delocalizes when the membrane potential is altered by membrane-depolarizing agents such as gramicidin S or valinomycin,\(^ {15}\) or by pore-forming compounds such as nisin.\(^ {13}\) Despite previous observations that allicin caused pores in tonoplasts and artificial membranes,\(^ {11}\) allicin, at the concentration tested here, neither caused pore formation nor membrane depolarization in *B. subtilis*. In congruence with allicin-response-associated-proteins indicating an impairment of the cell envelope, the “bubble assay” indicated reduced cell wall integrity. After 15 min of allicin treatment, cells were subjected to acetate/methanol fixation. We observed membrane extrusions through holes in the cell wall indicating corrosion of cell wall integrity (Figure 3). Similar effects have been observed after inhibition of membrane-bound steps of peptidoglycan biosynthesis by nisin and vancomycin treatment, but not by treatment with β-lactam antibiotics.\(^ {12}\) The inhibition
Figure 3. Influence of 15 min allicin treatment of B. subtilis on pore formation, cell membrane potential, and cell wall integrity. The BacLight LIVE/DEAD assay was modified to monitor pore formation at the time-point and concentrations equivalent to those in the proteomic experiment. All cells were stained green with SYTO9, while the red-fluorescing propidium iodide selectively stained cells with an impaired cell membrane barrier due to pore formation. These cells appear orange due to the overlay of both channels. GFP-MinD delocalization indicates membrane depolarization. In contrast to nisin, GFP-MinD delocalization was not observed after allicin treatment. The “bubble assay” was used to assess cell wall integrity. Cell membrane extrusions occur after acetic acid/methanol fixation in allicin and nisin treated cells (black arrows) but not in untreated controls.

3.5. Comparing the Proteomic Response of B. subtilis to Allicin with an Antibiotic Response Library

To identify commonalities of the physiological impact of allicin to that of other antibacterial agents, we compared the proteomic response to allicin to a response library containing 44 proteomic responses of B. subtilis to known antimicrobial agents.[16,17,21,22,67–76] Regarding the number of induced proteins, with 56 marker proteins, allicin evoked the most complex response in B. subtilis among the tested compounds. Thirty of the 56 marker proteins were only upregulated in response to allicin but not in response to other tested antibacterial agents. Stress responses to DNA-damaging, membrane-disrupting, and oxidative stress-causing antibiotics were the closest matches to the allicin response (Figure 4). The largest overlap in marker proteins was identified for the functional groups of maintenance of proteostasis (protein folding, protein degradation, translation) and detoxification of ROS, RSS, and quinone-S-adducts. Interestingly, these were also the major functions for which responder proteins were identified in E. coli by Müller et al.[7]

4. Discussion

Allicin is known to severely disturb bacterial cell homeostasis. As a reactive sulfur species (RSS), allicin can undergo a thiol-disulfide-type exchange reaction with cellular thiol groups.[77] This reactivity is thought to be responsible for allicin’s antimicrobial properties.[7] Free thiols are depleted and S-allylmercapto modification of cysteine residues in proteins by allicin or S-allylmercaptoglutathione (GSSA) coincided with enzyme inhibition in vitro.[78] Depletion of the glutathione pool is further thought to result in
oxidative stress and thus may disrupt proteostasis.[7] However, previous studies emphasized that allicin susceptibility highly varies between different bacterial species suggesting species-specific strategies to overcome allicin stress.[7] Our analysis revealed major processes upregulated in the acute response to allicin: i) cell envelope related processes, ii) processes related to translation and maintenance of protein quality, and iii) redox homeostasis-related processes (Figure 2).

### 4.1. Cell Envelope

The physiologically effective concentration that impairs growth of exponentially growing cells was much higher for the Gram-negative species than for *B. subtilis*. The Gram-negative cell envelope with the two membranes that have complementary diffusion barrier functions[89] is known to be more effective in minimizing entry of toxic substances into the cytosol than the Gram-positive cell envelope. The stress-induced modification of the outer membrane provides an additional means of protection for Gram-negative bacteria. Indeed, in all three species, the proteomic response indicated that improving the envelope’s barrier function is one of the response strategies (Figure 2).

We observed an upregulation of proteins that participate in the modification of the bacterial cell envelope known to prevent an interaction with toxic agents or their reaction in the cytosol. In *P. aeruginosa*, Ala-tRNAAla is required for altering the charge of phosphatidylglycerol (PG) in cell membranes.[80] The conversion of the negatively charged headgroup of PG into the zwitterionic aminoacyl ester alanyl-PG (aa-PG) results in a reduced negative net charge of the membrane.[81] This modification finetunes biochemical properties of the membrane and prevents electrostatic interactions with cell membrane targeting compounds, such as cationic antimicrobial peptides.[82] In *B. subtilis*, DltE is part of the teichoic acid synthesis and modification system, which is involved in d-alanylation of lipoteichoic and teichoic acids.[60] It has been shown that the modification of lipoteichoic acids contributes to lantibiotic[61] and lysozyme resistance[62] by introducing positively charged free amino groups that partially mask the negative charge of the phosphodiester linkages and thus the teichoic acids.[83] In *E. coli*, important structural constituents of the cell envelope were upregulated by allicin treatment. Lpp, the most abundant protein in *E. coli* even under non-stress conditions, is further upregulated in response to allicin. It tethers the outer membrane to the peptidoglycan layer and serves as a barrier for influx of toxic compounds including antibiotics.[84] OmpA is not only a major transporter in the outer membrane, but also a major structural component that is anchored in the peptidoglycan layer mediating resistance to membrane stress.

In *B. subtilis*, DltE and NagBB are marker proteins of cell wall restructuring. DltE is involved in teichoic acid and NagBB is involved in N-acetylglucosamine (GlcNac) synthesis. NagBB is part of the catabolic processes for utilization of GlcNac from chitin or plant-derived amino-sugars as an alternative carbon source.[84,85] In our experimental setup, cellular peptidoglycan and its precursors were the only source for GlcNac. NagBB was also upregulated in response to tunicamycin (Senges, unpublished results), which inhibits cell wall biosynthesis by blocking N-acetylglucosamine transferases and preventing formation of N-acetylglucosamine lipid intermediates.[86] Microscopy-based follow-up experiments further confirmed that cell wall synthesis steps are likely impaired by allicin. Allicin seems to impair bacterial cell wall integrity at a concentration (here 14 μg mL−1) that did not appear to influence the membrane potential or cause pore formation. Like the lipopeptide antibiotic daptomycin,[74] the lantibiotic nisin,[87] or the cationic antimicrobial peptide MP-196,[16] it seems to interfere with membrane-associated steps of cell wall biosynthesis.

Allicin was shown previously to enhance the activity of cell membrane-disrupting polymyxins in *E. coli*,[11] which is in agreement with allicin also affecting the cell envelope of Gram-negative species. When allicin reaches the periplasm, it may also cause damage to the Gram-negative cell envelope by impairing cell envelope-associated processes that are required for structural integrity. For instance, YbiS, a periplasmic t-d-transpeptidase that covalently attaches Lpp to the peptidoglycan layer,[88] contains a catalytic cysteine residue that is prone to S-allylmercapto modifications and which cannot be reduced by disulfide bond isomerases.[89] In *E. coli*, a total of ≈500 proteins fulfilling different functions are targeted to the periplasm. More than 40% of the envelope proteome is known or predicted to interact with the DsbAB disulfide bond formation systems.[90] Such DsbAB targets are putatively sensitive to thiol modifications during allicin stress, as well as the DsbAB system itself, which relies on CXXC motifs.[91,92] Hence, not only in Gram-positive species, allicin might damage the cell wall by impairing enzymes that maintain cell wall integrity. Indeed, this conclusion is congruent with the previously reported bacteriolytic activity of garlic extract against both *S. aureus* and *P. aeruginosa*.[93] Based on our data, the reported rupture of the cell envelope might be attributed to the impairment of critical cell envelope maintenance and synthesis systems by allicin. Our data further supports the notion of Boboye et al.[93] that the described species-specific efficacy of garlic extract mirrors species-specific cell protective strategies to cope with allicin stress rather than species-specific modes of action. We conclude that both, Gram-positive and Gram-negative species, suffer from impairment of cell envelope integrity by allicin treatment and that limiting the influx of allicin to the cytosol by modifying the envelope is one of the key defense strategies to cope.

### 4.2. Translation and Maintenance of Protein Quality

At allicin concentrations that inhibit growth in exponential phase, global changes to the proteome provide insights into fundamentally different strategies of Gram-negative species and *B. subtilis* (Figure 2B). In *E. coli* and *P. aeruginosa*, many of the proteins synthesized in untreated cells are no longer present following allicin treatment (Figure 1C). The major DNA-binding proteins H-NS (histone-like nucleoid structuring protein) of *E. coli* and Dps (DNA protection during starvation protein) of *P. aeruginosa*, were significantly upregulated. H-NS and Dps are highly abundant constituents of the nucleoid[94] that condense DNA during stationary phase and under oxidative stress conditions.[95,96] In addition, Dps of *P. aeruginosa* is known to protect DNA during hydrogen peroxide exposure by removing Fenton reaction products[97] thus potentially adding an ROS...
detoxification function in the presence of allicin. Both proteins restrict DNA accessibility for the transcription machinery by altering DNA curvature on a global scale. Using L-[35S]-methionine incorporation measurements, Schröder et al. showed that translation capacity is reduced by H-NS-mediated repression of the rRNA core promoter rrnB P1 to prevent oxidative damage of RNA and tRNA. These results are fully congruent with the reduction of protein synthesis rates observed in this study. The global decrease in gene expression likely is a key contributing factor in decreasing the vulnerability of both Gram-negative species to the disruption of cellular processes by allicin.

Particularly proteins assisting in protein folding and refolding were found to be upregulated in response to allicin in all three species. Especially in *P. aeruginosa*, translation capacity was almost exclusively dedicated to chaperone synthesis. Even under control conditions, basal levels of heat shock proteins are significantly higher in *P. aeruginosa* than in *E. coli* or *B. subtilis*. Chaperones are essential responders to heat shock, oxidative stress and disulfide stress. They were previously reported to be upregulated in *E. coli* encountering allicin stress, which may initially prevent protein unfolding and aggregation and thus their high levels may contribute to *P. aeruginosa* tolerating comparably high allicin concentrations.

### 4.3. Redox Homeostasis

Although *P. aeruginosa* is naturally confronted with a variety of oxidants, e.g., during the respiratory burst of human phagocytes (HOCl, HOBr, HOSSCN), except for Dps, no known components of the oxidative stress response (e.g., catalase, alkyl hydroperoxide reductase, glutathione peroxidase, glutathione reductase) were upregulated while in *E. coli* and *B. subtilis* chaperones are complemented by proteins involved in thiol group regeneration (Trx and Brc systems), repair of damaged iron sulfur proteins (NfuA (*E. coli*), SufA (*B. subtilis*)), and ROS detoxification.

In contrast to *P. aeruginosa* and *E. coli*, in which most proteins are downregulated, many cytosolic proteins of *B. subtilis* are upregulated. This may be a reflection of more allicin entering the cytosol due to a less effective barrier function of the cell envelope. For instance, an increased synthesis of (oxidoreductases and monooxygenases that reduce free flavins (riboflavin, FMN, FAD) to FMNH2 and FADH2 was observed for *B. subtilis*, which has not been observed for other antibiotics tested. These cofactors are involved in a variety of cellular processes, predominantly in iron sulfur protein assembly, methemoglobin reduction, oxygen reduction, short-chain fatty acid degradation degradation, or desulfuration processes. Most essentially, FADH2 is required for providing reduction equivalents in the electron transport chain that builds up the proton motive force for the synthesis of ATP. Usually, in growing cells, reduction equivalents are generated by glycolysis and the TCA cycle in sufficient amounts. It is striking that in response to allicin, we found three FMN/FAD reducing (oxidoreductases (NfrA 1, NfrA 2, and YqG), one FADH2-dependent monooxygenase (YcnE), and two NAD(P)H-dependent nitroreductases (HyPO and Ydgl) upregulated. Nitroreductases are often involved in anaerobic processes that utilize quinones to provide reducing power. Upregulation of FMN:oxidoreductases has been observed in response to superoxide, peroxide and disulfide stress. This enzyme class is proposed to be involved in maintaining redox homeostasis during the stationary phase when activities of glycolysis and the TCA cycle are reduced. In vitro, allicin was shown to reduce enzyme activity by modifying cysteines. Isocitrate lyase AceA and GapA activities were reduced in allicin treated *E. coli* cell lysates. However, the catalytical cysteines of both enzymes remained unmodified indicating that allosteric modifications were responsible for the loss in enzyme activity.

In this context, FMN:oxidoreductases might present an alternative or supporting system for ensuring redox homeostasis in *B. subtilis* when activities of oxidation-sensitive components of glycolysis and the TCA cycle components are diminished during allicin exposure.

### 5. Conclusion

The cellular stress responses of *P. aeruginosa, E. coli*, and *B. subtilis* to allicin revealed a common set of strategies that are given different emphasis. Cell envelope integrity is addressed in all three species. In *P. aeruginosa* and *B. subtilis*, alanylation of phosphatidylglycerol headgroups and teichoic acid, respectively, counteract allicin influx. *B. subtilis* growth was affected at a 20× lower concentration than those shown to inhibit growth of the Gram-negative species. Even at low concentrations, cell envelope integrity in *B. subtilis* was compromised. *B. subtilis* upregulates proteins indicating the loss of function of cytoplasmic enzymes. For example, the upregulation of flavin-reducing enzymes could play an important role in mediating redox-homeostasis upon allicin-related protein modification causing inactivation of key metabolic enzymes. Due to the influx of allicin, ROS and RSS detoxification is of particular importance for *B. subtilis*. For both Gram-negative species, a drastic reduction in global gene expression mediated by Dps or H-NS-based DNA condensation together with chaperone-based protection of cytoplasmic proteins, coupled with limited ROS protection seem to be the strategy to outlast the allicin stress.

### Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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### Conflict of Interest

The authors declare no conflict of interest.
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