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Unraveling the Hydroxypropionaldehyde (HPA) System: An Active Antimicrobial Agent against Human Pathogens

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The hydroxypropionaldehyde (HPA) system is a natural defense system synthesized by the probiotic bacterium *Lactobacillus reuteri*. To elucidate which of the molecules composing the HPA system (3-hydroxypropionaldehyde (3-HPA), reuterin (HPA dimer), and HPA hydrate) is responsible for the potent antimicrobial activity in biological systems, a combination of biochemical, genetic, and proteomic assays was used. The HPA system reacts with sulfhydryl-containing compounds such as cysteine and reduced glutathione (GSH) in solution. In situ, GSH knock-out *Escherichia coli* is significantly more susceptible to HPA-mediated cell death than *E. coli* wild type; GSH supplementation protects either bacteria from HPA attack. Proteomic analysis of HPA-treated bacteria (*Haemophilus influenzae*) revealed induction of redox- and heat shock-related proteins. A new antimicrobial mechanism of HPA is proposed, whereby the activity of HPA leads to depletion of free SH− groups in GSH and proteins through the action of 3-hydroxypropionaldehyde, causing an imbalance of the cellular redox status, ultimately resulting in cell death.

KEYWORDS: 3-Hydroxypropionaldehyde (3-HPA); reuterin; mode of action; bioactive

INTRODUCTION

The antimicrobial activity of the hydroxypropionaldehyde (HPA) system is directed against a wide range of organisms such as Gram-positive and Gram-negative bacteria, against the yeast *Saccharomyces cerevisiae*, and against the protozoan *Trypanosoma cruzi* (*1*). Pathogens such as *Listeria monocytogenes* and *Escherichia coli* O157:H7 in food are also inhibited (*2*). HPA showed synergistic or additive effects on *L. monocytogenes* and *Staphylococcus aureus* growth when used in combination with other antimicrobial substances (*3*). HPA was also shown to be effective in sanitizing biological tissues and for hemoglobin polymerization in blood substitute preparations (*4*, *5*). Accordingly, several authors have proposed the use of HPA as an antimicrobial agent in food and pharmaceutical preparations [reviewed by Vollenweider and Lacroix (*6*)]. 3-Hydroxypropionaldehyde (3-HPA) is produced by a bacterial species, *Lactobacillus reuteri*, that has been used as a probiotic for over two decades in a variety of functional foods and healthcare products (*7*). The probiotic activity of this strain, both in vitro and in vivo, is partly attributed to its ability to produce HPA (*8*, *9*). 3-HPA is produced from glycerol with a coenzyme B_{12}-dependent glycerol dehydratase and further reduced to 1,3-propanediol (**Figure 1**) (*6*). Once in the medium, 3-HPA undergoes a reversible dimerization and hydration, resulting in an active equilibrium (here referred to as HPA, HPA system) between 3-HPA, HPA dimer (reuterin), and HPA hydrate (**Figure 1**). The composition of HPA is pH- and temperature-dependent, forming mainly aldol dimers and polymers, or hemicacetal dimers, acetal trimers, and polymers in biological tissues at elevated temperatures (*10*, *11*). The presence of the different HPA forms and derivatives makes determination of the mechanism of action of an individual

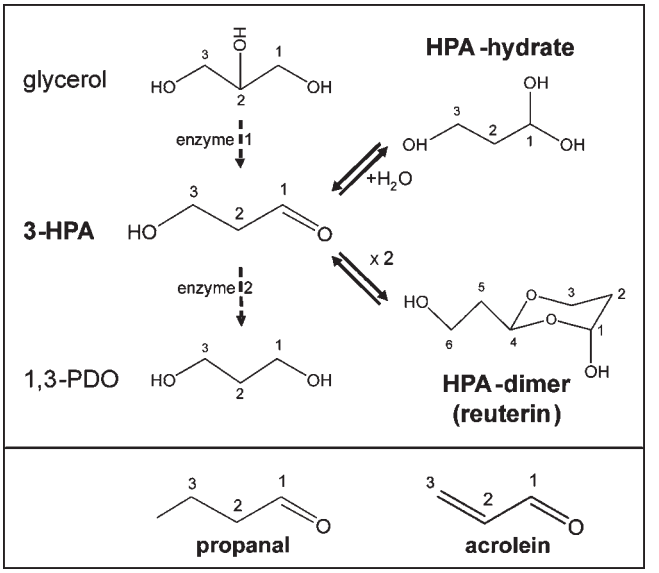

Figure 1. Formation of 3-Hydroxypropionaldehyde (3-HPA), HPA hydrate, HPA dimer, and 1,3-propanediol from glycerol and the compounds propanal and acrolein. Enzyme 1: coenzyme B_{12}-dependent glycerol dehydratase; enzyme 2, NADH-dependent 1,3-propanediol oxidoreductase.

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molecule very complicated, and the mode of action is not fully understood.

The HPA dimer, also called "antibiotic reuterin", was considered to be the antimicrobial compound, competing with its D-ribose-like structure for ribonucleotides in the binding site of ribonucleotide reductase, thus inhibiting DNA synthesis and leading to cell death (*1*). The above hypothesis does not explain the observed inhibition of thioredoxin, a protein participating in cellular redox regulation (*1*, *12*), and various authors have referred to reuterin as the mixture of all three components. At a concentration of 30 mM, where HPA acts as antimicrobial, the HPA system is composed of HPA hydrate (69%), 3-HPA (27%), and a minor percentage of HPA dimer (reuterin) as shown by NMR analysis (*13*). The hydrated and aldehyde forms are therefore most likely the active forms of HPA. Because HPA has attracted attention from the food and pharma industries for its potential use in the preservation of meat and milk products and in the sterilization of biological material, the safe use of this compound requires an understanding of its mode of action. In this study we elucidated the mode of action of HPA, using indirect methods.

EXPERIMENTAL METHODS

Reagents and Materials. Unless otherwise stated, chemicals were purchased from Sigma-Aldrich. Acrolein was freshly distilled; NAD^+ was prepared as a filter-sterilized stock solution (10 mg/mL) and stored at −20 °C.

Production and Purification of 3-HPA. Biotechnological production and purification of 3-HPA was done on site as previously described (*13*).

Reaction of Aldehydes with Amino Acids. Individual stock solutions (10 mM) of amino acids, GSH, and aldehydes were prepared in distilled water. For reactivity tests, stock solutions were diluted to 2 mM with potassium phosphate solution (0.12 M final concentration) at pH 4.4, 7.0, or 8.9.

Unbound 3-HPA and acrolein were quantified using the colorimetric method (Trp−HCl test) (*13*). Sulfhydryl groups (SH− groups) in cysteine and GSH were determined using Ellman's reagent (*14*). Standards (0−80 μM) of cysteine, cysteine−HCl, or reduced glutathione (GSH) were prepared in potassium phosphate buffer (0−80 μM, 0.1 M, pH 7.0). Samples were diluted to a concentration range of 0−80 μM in a total volume of 990 μL with potassium phosphate buffer (0.1 M, pH 7.0). The reaction was started with 10 μL of Ellman's reagent [10 mg of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 250 μL of dimethyl sulfoxide (DMSO)]. Absorption at 412 nm was read after 10 min of incubation at room temperature (Uvikon 810 P, Kontron Instruments or Varian UV−vis spectrophotometer, Cary Bio 1, Cary Inc.).

Toxicity of Aldehydes to *E. coli*. *Bacterial Cultures*. The *E. coli* K-12 AB1157 wild type strain and its GSH deficient (gshA20::Tn10kan) derivative JTG10 were obtained from the *E. coli* Genetic Stock Center (Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT) (*15*, *16*).

Stock cultures were prepared by incubating the bacteria overnight in LB broth (Difco) at 37 °C. Kanamycin was added (50 μg/mL) to the medium for the propagation of JTG10. Incubation was performed in Erlenmeyer flasks with baffles in a temperature-controlled (37 °C) shaker (150 rpm/min) to ensure adequate oxygenation. Stock cultures were prepared by freezing cultures of *E. coli* AB1157 and JTG10 in a solution containing 20% (v/v) glycerol at −80 °C. Experimental cultures were prepared by inoculating *E. coli* stock culture to 1% into LB broth. Cultures were propagated for 15 h (overnight) under conditions similar to those described above for the stock culture.

Incubation with Aldehydes. A 5 mL volume of an *E. coli* overnight culture was added to 100 mL of fresh medium, incubated at 37 °C as described for the stock culture, and grown to an OD_{600} of 0.6. Cells were centrifuged for 10 min at 3000*g* and 10 °C and resuspended in 105 mL of M9 salt solution (Difco). The OD_{600} was adjusted to 0.5 with M9 salt solution. Five milliliters of the cell suspension was added to 50 μL of freshly prepared aldehyde at the desired concentration or to the M9 salt solution. Cell viability and SH− content were determined at $t = 0$ and after 60 min of incubation at room temperature. At least three independent repetitions of this experiment were done with triplicate sampling.

Cell Viability. For viable cell enumeration of the JTG10 strain, samples were diluted in M9 salt solution, and 10 μL was plated in duplicate on LB agar containing kanamycin (50 μg/mL) and incubated for 24 h at 37 °C. Viable cell numbers were determined as colony-forming units (CFU/mL).

Determination of free SH− groups. SH-groups in cysteine and GSH were determined using Ellman's test (*14*). Standards in a concentration range from 0 to 80 μM were prepared with cysteine, cysteine−HCl, or reduced glutathione (GSH) in potassium phosphate buffer (0.1 M, pH 7.0). Samples were diluted with 990 μL of potassium phosphate buffer (0.1 M, pH 7.0). The reaction was started with 10 μL of Ellman's reagent (DTNB) in 250 μL (DMSO). Absorption was read after 10 min of incubation at room temperature at 412 nm. Cell suspensions (5 mL each, prepared as described under Incubation with Aldehydes) were centrifuged for 10 min at 6000*g* and 10 °C. The pellets were resuspended with a pipet, avoiding oxygenation, in 300 μL of B-PER lysis buffer (Pierce). A volume of 130 μL of this suspension was mixed with the same amount of 2.5% (w/v) sulfosalicylic acid (SSA) for GSH determination or with M9 salt solution for determination of total SH− content (*17*). The suspensions were incubated for 15 min at 4 °C and centrifuged for 15 min at 10000*g* and 4 °C. The supernatant (150 μL) was transferred into 840 μL of potassium phosphate buffer (0.15 M, pH 7.0), and 10 μL of Ellman's reagent was added. The samples were mixed by inversion and incubated for 10 min at room temperature. Absorption was read at 412 nm against the corresponding blank, similar to the sample but lacking bacterial cells.

Aldehyde Toxicity on *H. influenzae* RDKW20. The nonpathogenic strain of *H. influenzae* (ATCC 51907) was obtained from the American Type Culture Collection (Rockville, MD). Strain and culture preparation was done as described (*18*). Cells were inoculated into MHY (Mueller−Hinton broth, Merck, 38.0 g/L) supplemented for stock preparation, with the addition of yeast extract (5.0 g/L), NAD^+ (15 mg/L), and hemin (15 mg/L), grown to an OD_{595} of 0.3−0.4 and stored at −80 °C in 20% glycerol. A volume of 25 mL of minimal medium (*19*) was inoculated with 2 mL of stock culture and incubated overnight at 37 °C under shaking (200−220 rpm). Overnight cultures were diluted with minimal medium to an OD_{595} of 0.05 and grown to an OD_{595} at 0.4. Five milliliters of these cultures was transferred into 10 mL sterile round, screw-capped glass cuvettes (WTW), and 50 μL of aldehyde stock solution was immediately added to a final concentration of 0−10 mM. The cuvettes were incubated in a shaking water bath (150 rpm) at 37 °C. Bacterial growth was monitored by reading the optical density with a WTW photometer at 595 nm.

Proteomic Analysis. Proteomic analysis of *H. influenzae* was done as previously described (*20*). Immobilized pH gradient (IPG) strips were purchased from Amersham Pharmacia Biotechnology, acrylamide was purchased from Serva, and the other reagents for the polyacrylamide gel preparation were obtained from Bio-Rad (Hercules). Carrier ampholytes (pH 3.5−10 "Resolyte") were purchased from BDH Laboratory Supplies. CHAPS, thiourea, and benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) were from Sigma. Urea, dithioerythritol, and EDTA were obtained from Merck.

Preparation of H. influenzae Cells for Metabolic Labeling. *H. influenzae* was grown in minimal medium with reduced methionine content (0.6 μM) to an OD_{595} of 0.4 (*19*). The cultures were then transferred into 5 mL tubes, inhibitors were added, and incubation was continued in a shaker at 150 rpm and 37 °C. Cells incubated in the absence of inhibitors were used as a control. Aliquots (1 mL) were removed from the cultures after 10 and 30 min of incubation, 0.7 MBq of L-[^{35}S]-methionine (Amersham Radiochemicals, GE Healthcare Europe GmbH) was added, and incubation was continued for 2 min. Cells were then rapidly chilled on ice, centrifuged, washed in PBS buffer, and resuspended directly in sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris base, 65 mM 1,4-dithioerythritol, and 2% carrier ampholytes. The extracts were centrifuged at 100000*g*, and the supernatant was recovered. The amount of radioactivity incorporated in the supernatant was determined in a model 2500 TR liquid scintillation counter (Packard Instrument Co.).

Two-Dimensional (2-D) Electrophoresis. The 2-D gel electrophoresis was performed as reported (*21*). Aliquots containing 4×10^6 cpm of radioactivity were applied on IPG strips at the basic end of the strips. Isoelectric focusing was initiated at 200 V, and the voltage was gradually

increased to 5000 V over 8 h and kept at 5000 V for a further 12 h. Separation into the second dimension was performed on 1 mm thick 12% polyacrylamide gels. After electrophoresis, gels were dried on 3MM Whatman filter paper and exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA). Images were analyzed with PDQuest software (Bio-Rad).

Two-dimensional gels were matched and data stored in an Oracle database. Parallel gels (one sample per time point and concentration with the corresponding control) were run with the same batch of strips, the same isoelectric focusing run, the same batch of SDS-PAGE gels, and the same SDS-PAGE run. Three pairs of gels obtained under identical conditions were generated for each inhibitor, concentration, and incubation time. The spot intensities were normalized such that the sum of all spot intensities was equal for all gels. Spot intensity data were exported to Microsoft Excel. Means of spot intensities and the p values (paired t test) from all three gels were calculated. Gels were matched with a reference gel to determine spot identities (*18*, *20*). Representative 2-D images of the soluble protein fraction analyzed on pH 3−10 IPG strips are shown in the Supporting Information (Figure S1). Approximately 1100 spots were counted with PDQuest software. The reproducibility of separation was high, although some variations were observed, mainly in the acidic, high molecular mass region.

Statistics. All average results are presented as mean ± SE. ANOVA was performed using the general linear model of SPSS 16.0. Significant differences between treatments were determined using the LSD multiple-comparison test, with a significance level of ≤0.05. For the proteomic analysis the paired t test (Excel, 2003) was used.

RESULTS AND DISCUSSION

In this study, the chemical reactivity of HPA was studied in vitro using simple amino acid based molecules. These results were validated in a biological system, using bacterial cells (*E. coli*) containing defined metabolic mutations. Comparison of protein expression patterns of HPA−treated and control *H. influenzae* provided important new insights into the metabolic response of bacteria to HPA treatment. Together, the results led to the hypothesis that 3-HPA is the active compound.

The chemical reactivities of the HPA hydrate and HPA dimer are expected to be low because of their trihydroxy respectively ribose-like structure. In contrast, the chemical reactivity of 3-HPA, containing both a hydroxyl and an aldehyde functional group, is expected to be higher (*22*−*25*). Aldehydes can react with DNA, amino, sulfhydryl (SH− groups), or hydroxyl groups (OH groups).

Chemical Reactivity of HPA with Amino Acid Based Molecules. The HPA binding to individual amino acids was tested to better understand the mechanism of protein binding and enzyme inhibition. Unbound HPA was quantified after 1.25 h of incubation with equimolar amounts (2 mM) of HPA with neutral (G, A, S, V, L, I), basic (R, K, H), aromatic (F), heterocyclic (W), or sulfur-containing (M, C, C−HCl) amino acids at defined pH using the Trp−HCl test. HPA concentrations were not significantly different in controls (no amino acid) and samples with neutral, basic, or aromatic amino acids (results not shown). In contrast, a strong and pH-dependent decrease of unbound HPA was observed after incubation with cysteine−HCl and cysteine, both of which contain a free sulfhydryl group. The results showed a higher reactivity of free cysteine than of cysteine−HCl, reflecting the higher stability of cysteine−HCl. HPA did not react with methionine (M) containing a protected SH− group. A small but significant binding of HPA to tryptophan (W) and histidine (H) was observed at all pH values (4.4, 7.0, 8.9) (results not shown).

To validate the above results obtained by measuring free HPA, additionally SH− groups were measured (see **Figures 2** and **3**). Reduced glutathione, a tripeptide containing one SH− group (γ-glutamyl-cysteinyl-glycine, GSH), was also tested. Concentrations of SH− groups (Ellman) and HPA (Trp−HCl) showed

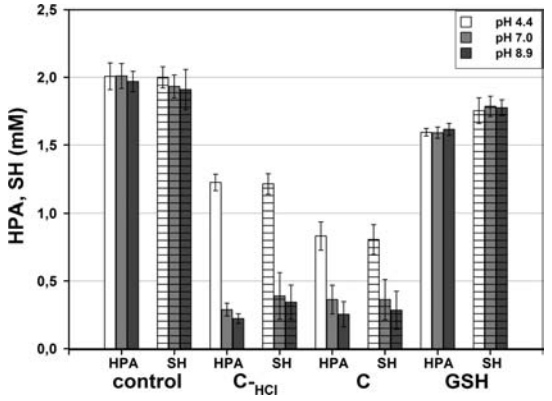

Figure 2. Reactivity of HPA with cysteine−HCl ($C-_{HCl}$), cysteine (C), and reduced glutathione (GSH). Equimolar amounts (2 mM) of HPA and cysteine or GSH were incubated in water or buffer. After 1.25 h, the amount of free 3-HPA or SH− groups was determined (mean of $n = 3-14$, ± SD).

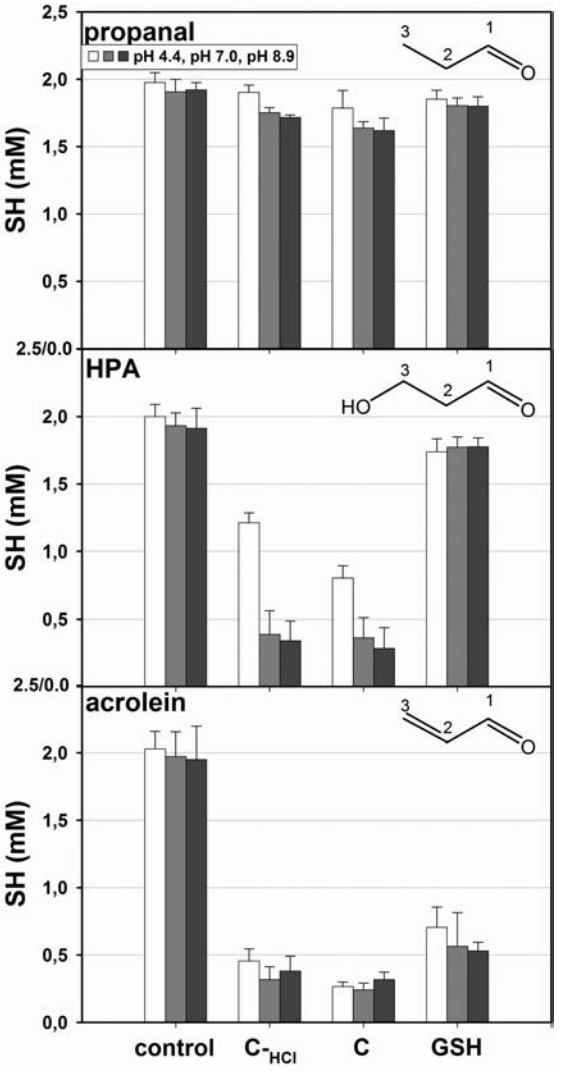

Figure 3. Reactivity of propanal, HPA, and acrolein with cysteine and reduced glutathione (GSH). Equimolar amounts (2 mM) of aldehydes and cysteine−HCl ($C-_{HCl}$), cysteine (C), or GSH were incubated in water or buffer. After 1.25 h, the amount of free sulfhydryl groups was determined. Univariate analysis of variance was performed using the Tukey HSD as post hoc test.

no significant differences in the control. The amount of HPA and SH− groups of cysteine measured by either test gave identical

results ($p > 0.05$). HPA was bound to GSH, albeit to a lesser extent than to cysteine, even though cysteine and GSH have similar reduction potentials (−0.25 and −0.264 mV, respectively) and pK_a values for protonation of the thiol group (8.3 and 8.6, respectively). However, the reaction of the α-amino group of cysteine with glutamate and the addition of glycine to the C-terminus reduce the metal-catalyzed thiol oxidation of cysteine in GSH. Therefore, the reactivity of cysteine is higher than GSH reactivity.

In Vitro Reactivity of Propanal, HPA, and Acrolein with Cysteine and GSH. Reactivity of HPA was compared to that of other aldehydes. Two closely related 3-carbon aldehydes were chosen: propanal, a simple *n*-alkanal, and acrolein, an α,β-unsaturated aldehyde.

Figure 3 shows the amount of free SH− groups after the reaction of the aldehydes with C, C−HCl, and GSH at different pH values. The concentration of free SH− groups without aldehyde addition (control) was not significantly affected by pH. Aldehyde addition decreased the concentration of free SH− groups in a pH- and aldehyde-dependent manner. Propanal resulted in a small but significant decrease of free SH− groups after incubation with C and C−HCl for all tested pH values, except at pH 4.4 with C, and showed no significant reactivity with GSH. HPA showed a strong and pH-dependent reactivity with C and C−HCl, but no significant reactivity with GSH. Acrolein reacted strongly with all SH-containing molecules, leaving ≤25% free SH− groups (except GSH, pH 4.4, 35%).

HPA Inhibition of Microbial Growth and Influence on Intracellular GSH Concentration. In vivo, GSH can be maintained in millimolar concentrations and has a function as a redox buffer. *E. coli* K12 (AB1157) wild type (wt) was chosen for this study, especially because the intracellular GSH concentration is well-known and has been monitored after treatment with various toxic molecules including H_2O_2 (*15*), acrolein (*26*), and chlorine (*27*). Studies in our laboratory showed that the minimal inhibition concentration (MIC) for *E. coli* is between 7.5 and 15 mM HPA. The MIC for lactobacilli was in the range of 15−40 mM and that for *L. reuteri* in the range of 30−50 mM (*28*). We chose short-term incubations with concentrations of propanal and HPA as high as 15 mM in this study to prevent an adaptive metabolic response to aldehydes and because this is the MIC for these bacteria (*28*). The toxic effect of acrolein was too strong at 15 mM concentration and was decreased to 0.5 mM, resulting in an equal toxicity as HPA (15 mM). The viability of *E. coli* was not decreased by different pH values in the solvent alone (M9 salt solution, control, **Figure 4**). Addition of HPA and acrolein decreased cell viability with increasing pH, with no toxicity at pH 5 and maximal viability losses of log 3.5 and log 4.5, respectively, after 60 min of incubation at pH 8.9. These data are consistent with the in vitro tests.

A pH of 7.4, nearest the cytoplasmic pH of *E. coli* (*29*), was chosen for further experiments. The potential role of GSH in the detoxification of HPA, propanal, and acrolein was tested by adding or not adding external GSH to bacterial cultures of wild type *E. coli* (AB1157) or a mutated *E. coli* with a deletion in the gene encoding the GSH formation (JTG10) (**Figure 5**). No loss of bacterial viability was observed after incubation in M9 salt solution (control) and propanal with or without exogenously added GSH (15 mM) ($p > 0.05$). HPA and acrolein showed a higher toxicity for JTG10 mutant than for the parent wild type strain AB1157. GSH addition (15 mM) completely restored cell viability. Quantification of intracellular SH− groups was done in the following experiments to investigate whether HPA and acrolein altered the content of bacterial sulfhydryl groups (SH− groups) in vivo. SH− group containing molecules that are not

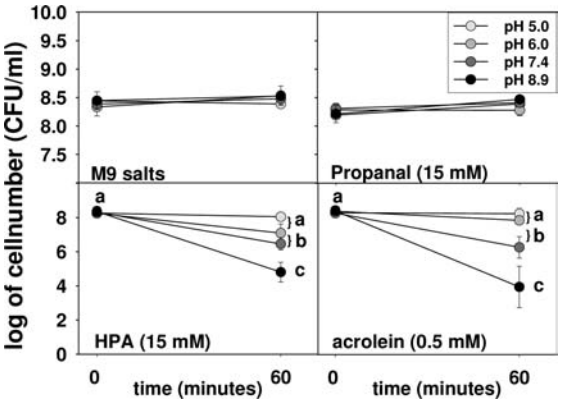

Figure 4. Toxicity of 3-carbon aldehydes to *E. coli* wild type (AB1157) at different pH values. *E. coli* in the exponential growth phase was resuspended in M9 salts alone or in M9 salts containing propanal (15 mM), HPA (15 mM), or acrolein (0.5 mM). After 0 and 60 min of incubation at room temperature, cell viability was determined (mean of $n = 3-4$). Univariate analysis of variance was performed using the Tukey HSD as post hoc test.

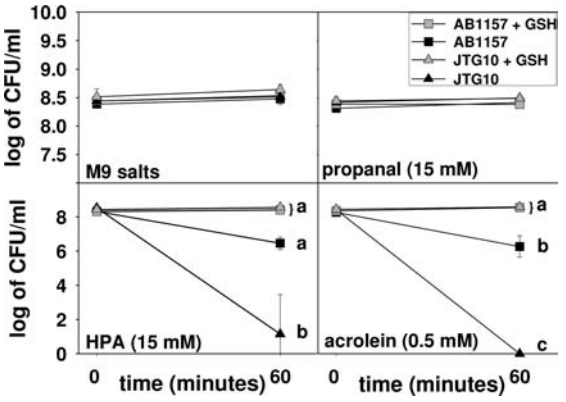

Figure 5. Toxicity of 3-carbon aldehydes to *E. coli* (AB1157) and *E. coli* lacking GSH (JTG10) at pH 7.4. Treatment was as described in **Figure 4**.

precipitated with trichloroacetic acid (TCA) can be attributed to GSH (the most abundant soluble, low molecular weight SH compound), whereas TCA-precipitated SH− groups can be attributed to protein-linked SH− groups (*17*).

Intracellular GSH concentration in wild type *E. coli* incubated with M9 salt solution was about 5-fold higher (**Figure 6**) than in the mutant *E. coli* (10.43 ± 2.68 vs 2.06 ± 0.53 μmol of GSH/10^{12} cells) and is in agreement with reported data (*27*). The amount of GSH in the wild type strain AB1157 did not significantly change after treatment with propanal. In contrast, HPA and acrolein significantly reduced the intracellular GSH level in AB1157 immediately after aldehyde addition, depleting almost all intracellular GSH after 60 min. When GSH (15 mM) was added to the medium, the intracellular GSH level in AB1157 cells increased immediately after aldehyde treatment on average about 6-fold (62.5 ± 22.2 μmol of GSH/10^{12} cells), and after 60 min approximately 9-fold (89.6 ± 25.6 μmol of GSH/10^{12} cells). No significant differences were observed for the three aldehydes.

In the JTG10 strain, HPA and acrolein treatment significantly decreased the GSH levels after 60 min compared to the control. When GSH (15 mM) was added to the medium, the intracellular GSH level in JTG10 cells increased to values nearly as high as for the wild type.

Depletion of GSH has a negative influence on the redox status of proteins and the amount of unprotected protein-bound SH− groups (PSH− groups) after aldehyde treatment. PSH− groups

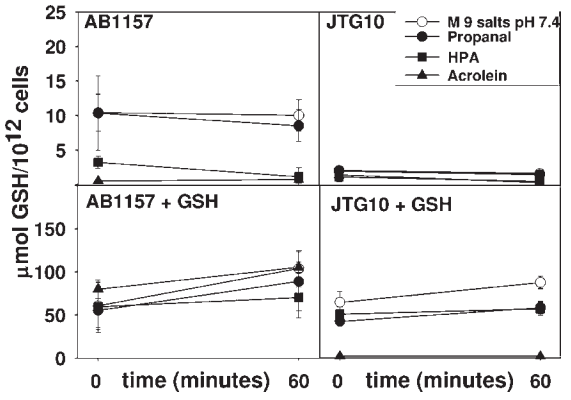

Figure 6. Intracellular GSH content of *E. coli* after treatment of 3-carbon aldehydes with or without the presence of external GSH. *E. coli* (AB1157) and mutant, lacking GSH (JTG10), in the exponential growth phase were resuspended in M9, pH 7.4, salts alone or in M9 salts containing propanal (15 mM), HPA (15 mM), or acrolein (0.5 mM) with or without supplementation of GSH (15 mM). After 0 and 60 min of incubation at room temperature, intracellular GSH content was determined (mean of $n = 3$).

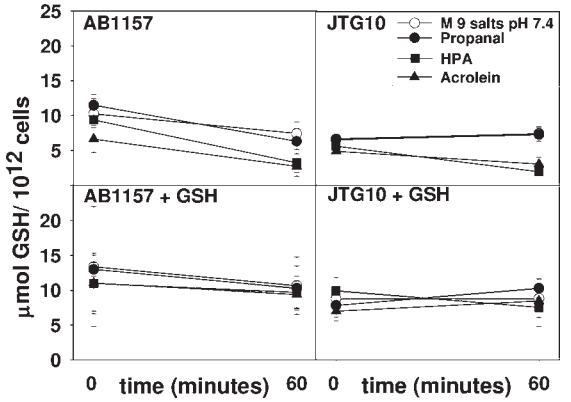

Figure 7. Protein-bound SH− content of *E. coli* after treatment of 3-carbon aldehydes with or without external GSH addition. Treatment was as described in **Figure 6**. After 0 and 60 min of incubation at room temperature, intracellular protein-bound SH− content was determined (mean of $n = 3$).

in *E. coli* in M9 salt solution were significantly lower in JTG10 (GSH-deficient) than in the AB1157 (wild type) strain (6.73 ± 0.55 vs 10.23 ± 2.01 μmol of PSH/10^{12}) (**Figure 7**).

The amount of PSH− groups in AB1157 cells decreased significantly after 60 min for the control (M9 salts, 30%) and aldehyde treatments (60%). In JTG10, the initial level of PSH− groups tested was lower than in the AB1157. After 60 min, the PSH− content of cells incubated with M9 salts or propanal showed no further decrease, in contrast to cells incubated with either HPA or acrolein (which were not significantly different from each other). When GSH was added, neither AB1157 nor JTG10 showed significant decreases in intracellular PSH− groups (**Table 1**).

The treatment of *E. coli* AB1157 and JTG10 with HPA or acrolein significantly decreased PSH− levels after 60 min in both strains. Exogenous GSH addition prevented this decrease in the AB1157 strain and even significantly increased PSH− over the control level in JTG10 (6.73 ± 0.55 μmol of PSH/10^{12} cells).

In Vivo Inhibition of *H. influenzae*. The mechanism of HPA toxicity was further elucidated by analyzing HPA-induced metabolic changes in a living model system. We chose *H. influenzae* as our model system, because the protein map of this organism has been fully described (*20*). Dose-dependent toxicity of propanal and HPA on growth was determined to identify the optimal concentration for metabolic analysis (**Figure 8**).

Table 1. Amount of Exposed Protein-Bound SH− Groups (PSH− Groups, Micromoles of PSH/10^{12} Cells)[a]

min	AB1157	AB1157 GSH	JTG10	JTG10 GSH
0	7.56 ± 2.57 a	10.96 ± 3.72 c	5.33 ± 0.61 d	8.23 ± 2.15 f
60	2.86 ± 1.20 b	9.51 ± 1.91 c	2.39 ± 0.86 e	7.99 ± 2.44 f

[a] Values are the average of HPA and acrolein treatments. Data are presented as means of 6−10 replicates. The same letters indicate no significant difference.

Propanal showed no growth inhibition at 0.1, 0.5, or 1.0 mM and minor but significant growth inhibition after 120 min for 5 and 10 mM; HPA, in contrast, significantly inhibited growth in a dose-dependent manner at concentrations of 0.5−10 mM. For 5 and 10 mM HPA, all growth was already stopped after 20 min of incubation.

Protein Expression Profile in Response to HPA and Propanal. For proteomic studies *H. influenzae* cells were incubated in minimal medium without (control) or with the presence of propanal or HPA (1 mM). After 10 and 30 min, proteins were labeled with L-[^{35}S]-methionine. Extracted, soluble proteins were then separated by 2-D gel electrophoresis (Figure S1, Supporting Information). The expression of 570 proteins was analyzed. Proteins showing a significant ($p \leq 0.05$) difference in expression to the control (paired t test) were selected for further analysis. About 65% of these proteins could be identified by mass spectrum analysis and subsequent comparison with the databank, resulting for the propanal treatment in 29 proteins (10 min) and 37 proteins (30 min) and for HPA treatment in 37 proteins (10 min) and 47 proteins (30 min). After 10 and 30 min of propanal treatment, 31 and 22% proteins changed expression, respectively, whereas HPA treatment changed the expression of 69 and 85% proteins after 10 and 30 min, respectively.

Protein induction was at a level of 24% after 10 min for propanal and HPA. After 30 min, the number of induced proteins dropped to 5 and 19%, respectively (**Figure 9A**). In contrast, Protein repression after 10 min was at a level of 7 or 45% for propanal or HPA, respectively. After 30 min, even more proteins were repressed, with 16 and 66%, respectively (**Figure 9A**).

Propanal had little effect on protein expression, as shown by the small mean induction and repression (**Figure 9B**). HPA, in contrast, showed a higher mean induction, with maximal values of 234.4 and 160.0, respectively, after 10 and 30 min. The mean repression was smaller and comparable for both time points with maximal values of 19.0 and 25.8. The proteins repressed by HPA treatment were mainly housekeeping proteins from different pathways (amino acids, fatty acids, purines, energy, translation, transport). Proteins increased after 10 min of HPA treatment either are involved in the redox status of the cell (GSH reductase, gor; peroxidoxin, tagD), are stress-related proteins (groES, surA), or reinforce the cell envelope (ADP-L-glycero-D-mannoheptose-6-epimerase, rfaD). After 30 min, in addition to the housekeeping proteins, proteins from energy metabolism pathways were repressed, whereas four heat shock-related proteins (groES, grpE, hsIV, htrA) were induced. A detailed list of the expression profiles is presented in the Supporting Information.

Reactivity of 3-HPA, HPA Hydrate, HPA Dimer, Propanal, and Acrolein. 3-HPA was shown to react with sulfhydryl (SH−) groups present in the amino acid cysteine, in reduced glutathione (GSH), and in proteins. 3-HPA, as a bifunctional compound containing a hydroxyl and a carbonyl group, showed intermediate reactivity compared to propanal and acrolein. Propanal, a simple alkanal, showed low reactivity with SH− groups, and the highly electrophilic, α,β-unsaturated acrolein showed a high

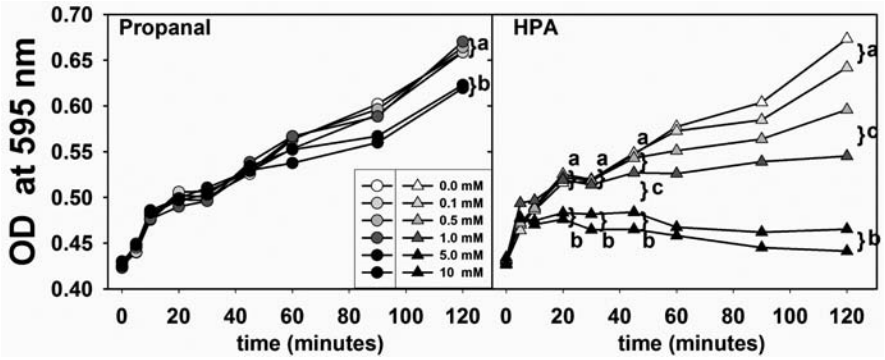

Figure 8. Growth inhibition of *H. influenzae* after treatment with propanal or HPA. *H. influenzae* was grown to an OD_{595} of 0.430 in a minimal medium, and then propanal (0−10 mM) or HPA (0−10 mM) was added and the OD_{595} was followed. Different letters (a−c) indicate significant differences (mean of six independent experiments with $n = 3$). Univariate analysis of variance was performed using the Tukey HSD as post hoc test.

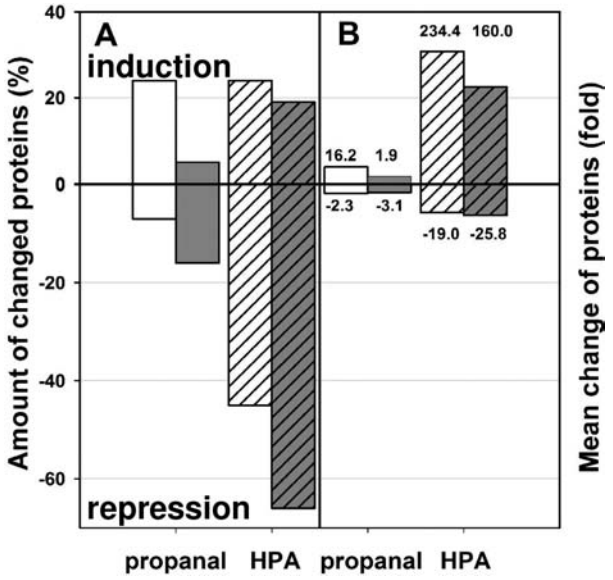

Figure 9. Protein expression of *Haemophilus influenzae*: (**A**) number of proteins (%) induced or repressed after treatment; (**B**) mean induction or repression after treatment (with maximal values). *H. influenzae* was grown to an OD_{595} of 0.430 in a minimal medium, with propanal (solid) or HPA (shaded) addition (1 mM final concentration). Proteins were extracted after 10 (white) and 30 (grey) min, and their expression was compared to the control (minimal medium).

reactivity as already found by other researchers (*30*−*34*). Physically, the differences in reactivity can be explained by comparison of the ^{13}C and/or $\{^{1}H\}$ NMR data for propanal, acrolein (*35*), and HPA (*13*). The NMR studies of these three aldehydes revealed important differences in the shifts (in ppm) of their carbon atoms [C1 (aldehyde), C2, C3]. The aldehydes have a higher electronegativity (≈200 ppm) in C1 than the HPA hydrate (88.6, 39.3, 57.7 ppm) and the HPA dimer (97.5, 32.1, 65.0, 94.5, 36.4, 56.8 ppm), although the latter is not directly comparable as it possesses six carbon atoms (**Figure 1**), making the HPA aldehyde probable to be the active ingredient of the HPA system (*13*). The differences in the reactivity of these aldehydes can be seen in C2 and C3: propanal (203.21, 37.28, 6.04 ppm), acrolein (194.44, 138.53, 137.96 ppm), 3-HPA (203.3, 43.1, 60.4 ppm). The reactivity of C2 in acrolein is due to the α,β-unsaturated double bond. Comparison of the C3 carbon atoms reveals also important differences, explaining the differences in reactivity between the molecules. Higher shifts (ppm) are correlated with lower electron density, indicating that the molecule became more electrophilic, resulting in a higher reactivity with nucleophilic molecules such as SH− groups. The differences are also reflected by the relative toxicity of these molecules. Acrolein is highly toxic [oral lethal dose (LD_{50}) for rats < 0.046 g/kg] due to the presence of an aldehyde group combined with a double bound (C2−C3, α,β-unsaturated aldehyde), reacting mainly with lysine and sulfhydryl groups (*36*). Propanal does not contain a double bound and is far less reactive and therefore less toxic (oral LD_{50} for rats = 1.4 g/kg) (*37*). Ribose, the HPA dimer related compound, is not toxic. Following the rules of the structure−activity relationship of molecules (*36*), the above facts lead us to the conclusion that 3-HPA is the active ingredient in the HPA system.

Further proof for this was found in the chemical reactivity of HPA with cysteine and GSH, which is increased when the SH− group becomes more negative with increasing pH (pH 7.0 or 8.9) (*37*). This indicates that the aldehyde (3-HPA) is the reactive molecule in the HPA system, forming adducts with accessible SH− groups in proteins, leading to their inhibition. This hypothesis explains also the observed inhibition of thioredoxin and ribonucleotide reductase, both enzymes with SH− groups participating in their catalytic activities (*38*−*40*). Our in vivo data from experiments with *E. coli* support this hypothesis. HPA induces a depletion of GSH in *E. coli* followed by an attack on protein-linked SH− groups, ultimately leading to cell death. Exogenous GSH addition increased intracellular GSH concentrations up to 9-fold (except for the acrolein-treated mutant, with which no GSH increase could be observed) and prevented alteration of protein thiols in all treatments, thus rescuing the bacteria.

The proteomic approach showed the first insights into the intracellular response of the model bacterium *H. influenzae* to subtoxic concentrations of propanal and HPA. After incubation of cells with the aldehydes, proteins were extracted and identified by sequence matching with the database (*20*). Analysis of protein expression profiles after propanal treatment (1 mM) revealed only minor changes of a few housekeeping proteins (see Supporting Information).

HPA treatment in contrast significantly altered the expression of several proteins, with more proteins repressed than induced, although the expression levels of induced proteins underwent more change than repressed proteins. Repressed proteins could be assigned to enzymes involved in the synthesis and processing of DNA, RNA, and proteins, the major metabolic pathways (housekeeping proteins), or to energy metabolism. Specifically, proteins showing increased expression after 10 min of HPA treatment were involved in the redox status of the cell. These include glutathione reductase (gor, 3.4-fold induced), which was also observed in *E. coli* treated with acrolein (*26*), and in vitro (bovine GSH reductase) by acrolein, crotonaldehyde, and cinnamaldehyde (*38*).

Thioredoxin-linked peroxidase activity (tagD, also tpx, 4.8-fold induced) or is an enzyme with a conserved cysteine, using thioredoxin to reduce peroxides and protect enzymes (*39*). Similarly, HI0719 (19.7-fold induced) is involved in the binding of α-keto acids or α,β-unsaturated acids and was suggested to be involved in the isoleucine biosynthetic pathway (*40*). Other proteins involved in cell survival that were also found to be induced include surA (1.1-fold induced) and groES (9.7-fold induced), both of which ensure correct protein folding (*39*, *41*), as well as rfaD (also known as htrM; 234.41-fold induced), which has a role in cell envelope synthesis and the protection of outer membrane proteins during folding (*42*). Interestingly, the latter protein is induced by heat shock proteins. Prolonged treatment of the cells with HPA (30 min total) resulted in the induction of other heat shock induced proteins, including grpE, preventing the aggregation of stress-denatured proteins in association with DnaK (*39*) or participating in stabilizing mRNAs (*43*), as well as hsIV and htrA, two proteases involved in degradation of abnormal proteins (*44*, *45*).

Recently, authors reported the expression of genes within the OxyR regulon of *E. coli* after HPA treatment (*46*). *E. coli* mutated in oxyR was more susceptible for HPA treatment, and cysteine-supplemented *E. coli* or *Clostridium difficile* was more resistant to HPA treatment. The results found by these authors that HPA induces oxidative stress and modifies cellular thiol groups correspond to our findings.

The involvement of SH− groups explaining the inhibitory mechanism of HPA on both ribonucleotide reductase and thioredoxin was already considered by Sjöberg (*1*, *47*). Both enzymes are indeed tightly involved in the SH− redox reactions of the cell, having unprotected SH− groups in their active center, and their reactivity is directly dependent on the availability of redox molecules (*48*−*50*). Ribonucleotide reductases are especially vulnerable because they require a protein radical for activity (*51*, *52*). Another target might be coenzyme B_{12}-dependent glycerol dehydratase. This enzyme is inhibited during the bioconversion of glycerol to 3-HPA by substrate analogues (*53*), but it uses a radical mechanism for activity and might therefore be inhibited by the aldehyde 3-HPA.

Our data provide important new insights into the mechanism of the HPA activity. We could pinpoint the antimicrobial active form through a novel approach. We demonstrated that the aldehyde, 3-HPA, is responsible for the antimicrobial activity. After treatment with a HPA mixture, the cell orchestrates an antioxidant defensive response against the action of HPA, by increasing the expression of enzymes regenerating the redox system. Depending on 3-HPA concentration, GSH depletion leads to loss of protein-bound SH− groups. Then the cell responds by inducing repair proteins, preventing aggregation of proteins and helping to refold denatured proteins, especially to reinforce the outer membrane. In addition, misfolded proteins are degraded. Ultimately, however, this response will be insufficient for cells to survive continued stress. Our data are supported by the recently published work of Schaefer et al. (*46*).

The use of HPA as an antimicrobial in the food industry has therefore to be considered carefully in each case, as the increased reactivity of the aldehyde and formation of acrolein at elevated temperatures could affect the taste and safety of the products. The ability to control HPA activity by means of pH and temperature opens new prospects for the use of HPA in pharmaceutical and medical applications, in which a "low reactive" precursor could be highly desirable. 3-HPA could be used alone or in combination with other treatments to inhibit pathogenic bacteria or fungi in humans, animals, or plants or proliferating cells. Tests on the inhibition of individual enzyme activities are under way to further elucidate the reactivity of HPA with proteins.

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Supporting Information Available: Supplementary figure and table. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Dobrogosz, W. J.; Lindgren, S. E. Antibiotic reuterin. U.S. Patent WO/1988/008452A1, **1988**.

(2) El-Ziney, M. G.; Debevere, J. The effect of reuterin on *Listeria monocytogenes* and *Escherichia coli* O157:H7 in milk and cottage cheese. *J. Food Prot.* **1998**, *61* (10), 1275–1280.

(3) Arqués, J. L.; Rodriguez, E.; Nuñez, M.; Medina, M. Antimicrobial activity of nisin, reuterin, and the lactoperoxidase system on *Listeria monocytogenes* and *Staphylococcus aureus* in cuajada, a semisolid dairy product manufactured in Spain. *J. Dairy Sci.* **2008**, *91* (1), 70–75.

(4) Liang, H. F.; Chen, C. N.; Chang, Y.; Sung, H. W. Natural antimicrobial agent (reuterin) produced by *Lactobacillus reuteri* for sanitization of biological tissues inoculated with *Pseudomonas aeruginosa*. *Biotechnol. Bioeng.* **2003**, *84* (2), 233–239.

(5) Chen, Y. C.; Chang, W. H.; Chang, Y.; Huang, C. M.; Sung, H. W. A natural compound (reuterin) produced by *Lactobacillus reuteri* for hemoglobin polymerization as a blood substitute. *Biotechnol. Bioeng.* **2004**, *87* (1), 34–42.

(6) Vollenweider, S.; Lacroix, C. 3-Hydroxypropionaldehyde: applications and perspectives of biotechnological production. *Appl. Microbiol. Biotechnol.* **2004**, *64*, 16–27.

(7) Casas, I. A.; Dobrogosz, W. J. Validation of the probiotic concept: *Lactobacillus reuteri* confers broad-spectrum protection against disease in humans and animals. *Microb. Ecol. Health Dis.* **2000**, *12*, 247–285.

(8) Cleusix, V.; Lacroix, C.; Vollenweider, S.; Le Blay, G. Glycerol induces reuterin production and decreases *Escherichia coli* population in an *in vitro* model of colonic fermentation with immobilized human feces. *FEMS Microbiol. Ecol.* **2008**, *63* (1), 56–64.

(9) Morita, H.; Toh, H.; Fukuda, S.; Horikawa, H.; Oshima, K.; Suzuki, T.; Murakami, M.; Hisamatsu, S.; Kato, Y.; Takizawa, T.; Fukuoka, H.; Yoshimura, T.; Itoh, K.; O'Sullivan, D. J.; McKay, L. L.; Ohno, H.; Kikuchi, J.; Masaoka, T.; Hattori, M. Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. *DNA Res.* **2008**, *15* (3), 151–161.

(10) Sung, H. W.; Chen, C. N.; Liang, H. F.; Hong, M. H. A natural compound (reuterin) produced by *Lactobacillus reuteri* for biological-tissue fixation. *Biomaterials* **2003**, *24* (8), 1335–1347.

(11) Chen, C. N.; Sung, H. W.; Liang, H. F.; Chang, W. H. Feasibility study using a natural compound (reuterin) produced by *Lactobacillus reuteri* in sterilizing and crosslinking biological tissues. *J. Biomed. Mater. Res.* **2002**, *61* (3), 360–369.

(12) Talarico, T. L.; Dobrogosz, W. J. Chemical characterization of an antimicrobial substance produced by *Lactobacillus reuteri*. *Antimicrob. Agents Chemother.* **1989**, *33* (5), 674–679.

(13) Vollenweider, S.; Grassi, G.; König, I.; Puhan, Z. Purification and structural characterization of 3-hydroxypropionaldehyde and its derivatives. *J. Agric. Food Chem.* **2003**, *51* (11), 3287–3293.

(14) Ellman, G. E. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.

(15) Greenberg, J. T.; Demple, B. Glutathione in *Escherichia coli* is dispensable for resistance to H_2O_2 and γ radiation. *J. Bacteriol.* **1986**, *168* (2), 1026–1029.

(16) DeWitt, S. K.; Adelberg, E. A. The occurence of a genetic transposition in a strain of *Escherichia coli*. *Genetics* **1962**, *47* (5), 577–585.

(17) Greenberg, N. A.; Shipe, W. F. Comparison of the abilities of trichloroacetic, picric, sulfosalicylic, and tungstic acids to precipitate protein hydrolysates and proteins. *J. Food Sci.* **1979**, *44*, 735–737.

(18) Evers, S.; Di Padova, K.; Meyer, M.; Langen, H.; Fountoulakis, M.; Keck, W.; Gray, C. P. Mechanism-related changes in the gene

transcription and protein synthesis patterns of *Haemophilus influenzae* after treatment with transcriptional and translational inhibitors. *Proteomics* **2001**, *1* (4), 522–544.
(19) Barcak, G. J.; Chandler, M. S.; Redfield, R. J.; Tomb, J. F. Genetic systems in *Haemophilus influenzae*. *Methods Enzymol*. **1991**, *204*, 321–342.
(20) Langen, H.; Takacs, B.; Evers, S.; Berndt, P.; Lahm, H. W.; Wipf, B.; Gray, C.; Fountoulakis, M. Two-dimensional map of the proteome of *Haemophilus influenzae*. *Electrophoresis* **2000**, *21* (2), 411–429.
(21) Langen, H.; Roder, D.; Juranville, J. F.; Fountoulakis, M. Effect of protein application mode and acrylamide concentration on the resolution of protein spots separated by two-dimensional gel electrophoresis. *Electrophoresis* **1997**, *18* (11), 2085–2090.
(22) Grimsrud, P. A.; Xie, H.; Griffin, T. J.; Bernlohr, D. A. Oxidative stress and covalent modification of protein with bioactive aldehydes. *J. Biol. Chem*. **2008**, *283* (32), 21837–21841.
(23) O'Brien, P. J.; Siraki, A. G.; Shangari, N. Aldehyde sources, metabolism, molecular toxicity mechanisms, and possible effects on human health. *Crit. Rev. Toxicol*. **2005**, *35* (7), 609–662.
(24) Kalapos, M. P. Methylglyoxal in living organisms: chemistry, biochemistry, toxicology and biological implications. *Toxicol. Lett*. **1999**, *110* (3), 145–175.
(25) Schauenstein, E.; Esterbauer, H.; Zollner, H. *Aldehydes in Biological Systems: Their Natural Occurrance and Biological Activities*; Pion: London, U.K., **1977**; Vol. 5.
(26) Nunoshiba, T.; Yamamoto, K. Role of glutathione on acrolein-induced cytotoxicity and mutagenicity in *Escherichia coli*. *Mutat. Res*. **1999**, *442* (1), 1–8.
(27) Saby, S.; Leroy, P.; Block, J.-C. *Escherichia coli* resistance to chlorine and glutathione synthesis in response to oxygenation and starvation. *Appl. Environ. Microbiol*. **1999**, *65* (12), 5600–5603.
(28) Cleusix, V.; Lacroix, C.; Vollenweider, S.; Duboux, M.; Le Blay, G. Inhibitory activity spectrum of reuterin produced by *Lactobacillus reuteri* against intestinal bacteria. *BMC Microbiol*. **2007**, *7* (1), 101.
(29) Maurer, L. M.; Yohannes, E.; Bondurant, S. S.; Radmacher, M.; Slonczewski, J. L. pH regulates genes for flagellar motility, catabolism, and oxidative stress in *Escherichia coli* K-12. *J. Bacteriol*. **2005**, *187* (1), 304–319.
(30) Meacher, D. M.; Menzel, D. B. Glutathione depletion in lung cells by low-molecular-weight aldehydes. *Cell Biol. Toxicol*. **1999**, *15* (3), 163–171.
(31) Müller, K.; Hardwick, S. J.; Marchant, C. E.; Law, N. S.; Waeg, G.; Esterbauer, H.; Carpentar, K. L.; Mitchinson, M. J. Cytotoxic and chemotactic potencies of several aldehydic components of oxidised low density lipoprotein for human monocyte-macrophages. *FEBS Lett*. **1996**, *388* (2−3), 165–168.
(32) Alméras, E.; Stolz, S.; Vollenweider, S.; Reymond, P.; Mène-Saffrané, L.; Farmer, E. E. Reactive electrophile species activate defense gene expression in *Arabidopsis*. *Plant J*. **2003**, *34* (2), 205–216.
(33) Roy, D. R.; Parthasarathi, R.; Maiti, B.; Subramanian, V.; Chattaraj, P. K. Electrophilicity as a possible descriptor for toxicity prediction. *Bioorg. Med. Chem*. **2005**, *13* (10), 3405–3412.
(34) Benigni, R.; Passerini, L.; Rodomonte, A. Structure−activity relationships for the mutagenicity and carcinogenicity of simple and α,β-unsaturated aldehydes. *Environ. Mol. Mutat*. **2003**, *42* (3), 136–143.
(35) Saito, T.; Hayamizu, K.; Yanagisawa, M.; Yamamoto, O. In *Spectral Database for Organic Compounds*; National Institute of Advanced Industrial Science and Technology (AIST): Tokyo, Japan, 2008.
(36) Ghilarducci, D. P.; Tjeerdema, R. S. Fate and effects of acrolein. *Rev. Environ. Contam. Toxicol*. **1995**, *144*, 95–146.
(37) Milch, R. A. Aqueous solution infrared spectra of collagen-reactive aldehydes. *Biochim. Biophys. Acta* **1964**, *93*, 45–53.
(38) Vander Jagt, D. L.; Hunsaker, L. A.; Vander Jagt, T. J.; Gomez, M. S.; Gonzales, D. M.; Deck, L. M.; Royer, R. E. Inactivation of glutathione reductase by 4-hydroxynonenal and other endogenous aldehydes. *Biochem. Pharmacol*. **1997**, *53* (8), 1133–1140.
(39) Han, M. J.; Lee, S. Y. The *Escherichia coli* proteome: past, present, and future prospects. *Microbiol. Mol. Biol. Rev*. **2006**, *70* (2), 362–439.
(40) Parsons, L.; Bonander, N.; Eisenstein, E.; Gilson, M.; Kairys, V.; Orban, J. Solution structure and functional ligand screening of HI0719, a highly conserved protein from bacteria to humans in the YjgF/YER057c/UK114 family. *Biochemistry* **2003**, *42* (1), 80–89.
(41) Kerner, M. J.; Naylor, D. J.; Ishihama, Y.; Maier, T.; Chang, H. C.; Stines, A. P.; Georgopoulos, C.; Frishman, D.; Hayer-Hartl, M.; Mann, M.; Hartl, F. U. Proteome-wide analysis of chaperonin-dependent protein folding in *Escherichia coli*. *Cell* **2005**, *122* (2), 209–220.
(42) Missiakas, D.; Betton, J. M.; Raina, S. New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. *Mol. Microbiol*. **1996**, *21* (4), 871–884.
(43) Yoon, H.; Hong, J.; Ryu, S. Effects of chaperones on mRNA stability and gene expression in *Escherichia coli*. *J. Microbiol. Biotechnol*. **2008**, *18* (2), 228–233.
(44) Bochtler, M.; Hartmann, C.; Song, H. K.; Bourenkov, G. P.; Bartunik, H. D.; Huber, R. The structures of HsIU and the ATP-dependent protease HsIU-HsIV. *Nature* **2000**, *403* (6771), 800–805.
(45) Zurawa-Janicka, D.; Narkiewicz, J.; Lipińska, B. Characterization of the HtrA family of proteins. *Postepy Biochem*. **2007**, *53* (1), 27–36.
(46) Schaefer, L.; Auchtung, T. A.; Hermans, K. E.; Whitehead, D.; Borhan, B.; Britton, R. A. The antimicrobial compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with thiol groups. *Microbiology* **2010**, *156*, 1589−99.
(47) Axelsson, L.; Chung, T. C.; Dobrogosz, W. J.; Lindgren, S. E. Production of a broad-spectrum antimicrobial substance produced by *Lactobacillus reuteri*. *Microb. Ecol. Health Dis*. **1989**, *2*, 131–136.
(48) Hogenkamp, H. P. Nature and properties of the bacterial ribonucleotide reductases. *Pharmacol. Ther*. **1983**, *23* (3), 393–405.
(49) Sjöberg, B.; Sahlin, M. Thiols in redox mechanism of ribonucleotide reductase. *Methods Enzymol*. **2002**, *348*, 1–21.
(50) Nordlund, P.; Reichard, P. Ribonucleotide reductases. *Annu. Rev. Biochem*. **2006**, *75*, 681–706.
(51) Fontecave, M. Ribonucleotide reductases and radical reactions. *Cell. Mol. Life Sci*. **1998**, *54* (7), 684–695.
(52) Stubbe, J.; van der Donk, W. A. Ribonucleotide reductases: radical enzymes with suicidal tendencies. *Curr. Biol*. **1995**, *2*, 793–801.
(53) Toraya, T. Radical catalysis in coenzyme B_{12}-dependent isomerization (eliminating) reactions. *Chem. Rev*. **2003**, *103* (6), 2095–2127.

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