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Quantitative Proteomic Analysis of *Staphylococcus aureus* Treated With Punicalagin, a Natural Antibiotic From Pomegranate That Disrupts Iron Homeostasis and Induces SOS

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***Staphylococcus aureus*, a bacterial, food-borne pathogen of humans, can contaminate raw fruits and vegetables. While physical and chemical methods are available to control *S. aureus*, scientists are searching for inhibitory phytochemicals from plants. One promising compound from pomegranate is punicalagin, a natural antibiotic. To get a broader understanding of the inhibitory effect of punicalagin on *S. aureus* growth, high-throughput mass spectrometry and quantitative isobaric labeling was used to investigate the proteome of *S. aureus* after exposure to a sublethal dose of punicalagin. Nearly half of the proteins encoded by the small genome were interrogated, and nearly half of those exhibited significant changes in accumulation. Punicalagin treatment altered the accumulation of proteins and enzymes needed for iron acquisition, and it altered amounts of enzymes for glycolysis, citric acid cycling, protein biosynthesis, and purine and pyrimidine biosynthesis. Punicalagin treatment also induced an SOS cellular response to damaged DNA. Transcriptional comparison of marker genes shows that the punicalagin-induced iron starvation and SOS responses resembles those produced by EDTA and ciprofloxacin. These results show that punicalagin adversely alters bacterial growth by disrupting iron homeostasis and that it induces SOS, possibly through DNA biosynthesis inhibition.**

1. Introduction

Staphylococcus aureus is a Gram-positive bacterium that contaminates fresh produce and causes poisoning in humans by secreting enterotoxins after ingestion.[1] Although there are physical and chemical methods that can control food-borne pathogens like *S. aureus*, natural phytochemicals from edible plants show promise as antibiotics. Among these is punicalagin, a polyphenol compound of known structure from pomegranate that has anticancer, anticarcinogenic, and anti-inflammatory attributes.[2] Punicalagin also has antibiotic activity against *Candida* spp. and *Salmonella* spp.,[3,4] and it reduces gene expression for swimming and swarming motility and virulence in *Salmonella* spp.[4] Punicalagin has a similar antibiotic effect on *S. aureus*, resulting in reduced growth kinetics, cell deformation, a release of potassium, and a reduction in biofilm formation.[5]

Punicalagin can chelate iron and can interfere with antibody binding to the *S. aureus* enterotoxin,[6,7] but its biochemical mechanistic action on microbial

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Significance Statement

Punicalagin is a natural compound from pomegranate with antibacterial activity to *Staphylococcus aureus*, a bacterium that causes food poisoning. How punicalagin affects *S. aureus* is unknown, so we used mass spectrometry and chemical labeling to evaluate proteins in *S. aureus* after exposure. Proteomic changes showed that the cells were deprived of iron and that the SOS response, a cell survival mechanism to preserve reproduction after DNA damage, was induced. Iron supplements restored cell growth in the presence of punicalagin, and gene transcriptional responses to punicalagin were similar to the iron-chelator EDTA and to the SOS-inducer ciprofloxacin. These results suggest an overlapping response mechanism between iron starvation and DNA damage. Overall, the data support the theory that punicalagin acts as an antibiotic by disrupting iron homeostasis. These results will be useful to scientists who research antibiotics and *Staphylococcus* with the goal of protecting people from disease and improving the safety of the food supply.

proteins is unknown. Aspiring to understand the mechanistic action of punicalagin on *S. aureus*, we performed a quantitative, comparative analysis of the proteome of *S. aureus* exposed to punicalagin. The identification of proteins and enzymes affected by punicalagin is an important step toward unraveling its mechanism of efficacy.

2. Experimental Section

2.1. Bacterial Strains and Growth Conditions

S. aureus ATCC 25923 is a type of strain from American Type Culture Collection (ATCC, Manassas, VA). An aliquot of diluted bacterial suspension (about 10^7 CFU mL^{-1}) was spread on tryptic soy agar (TSA) containing glucose (3 mg L^{-1}), and phosphate (2.5 g L^{-1}). A sublethal concentration (0.05 mg mL^{-1}) of punicalagin (≥98%, Must Bio-Technology, Chengdu, China) was also added to the TSA. This concentration is 1/5 of the previously determined minimal inhibitory concentration for *S. aureus*.[5] The cultures were incubated at 37 °C for 24 h.

2.2. Protein Extraction

Bacteria were collected and suspended in PBS, followed by centrifugation at 4500 × *g* for 15 min. Cells were washed twice with PBS and resuspended in 1 mL of lysis buffer containing 6 M urea, 100 mM Tris-HCl (pH 7.8), 1% NP-40, and protease inhibitor cocktail (Roche, Basel, Switzerland). The cells were lysed using a bead-beating homogenizer (Omni International, Kennesaw, GA) at 6.8 m s^{-1} for a total of 5 min. The tubes were cooled on ice for 1 min at every 1 min interval. Cell debris was removed by centrifugation at 10 000 × *g* for 15 min. Supernatant protein was precipitated in 10% trichloroacetic acid and acetone, washed in cold acetone, and resuspended in 6 M urea and 100 mM Tris-HCl. The protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL).

2.3. Polyacrylamide Gel Electrophoresis

Proteins (16 μg per well) were separated by electrophoresis at 100 V for 45 min in 15% w/v polyacrylamide gels using a Tris/glycine/SDS buffer system according to the manufacturer's instructions for the Mi-Protean Tetra system (Bio-Rad, Hercules, CA). The gel was stained using Bio-Safe Coomassie G-250 (Bio-Rad).

2.4. Peptide Preparation

Proteins (≈300 μg) were reduced in Tris (2-carboxyethyl) phosphine, carboxyamidomethylated with iodoacetamide, and digested overnight at 37 °C with Poroszyme immobilized trypsin (Thermo Fisher Scientific). The digested peptides were purified using SPEC-PLUS PT C18 columns (Varian, Lake Forest, CA). Sixty-eight micrograms of peptides from each sample were labeled with tandem mass tag (TMT) 6-plex reagents according to instructions (Thermo Fisher Scientific). The samples were dried and resuspended in 0.1% TFA, and peptides were C18 purified, and quantified with the Pierce Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific). Equivalent quantities of the six tagged samples were combined to yield a 2 μg sample which was analyzed by mass spectrometry to determine TMT label incorporation percentage (>99%) and to estimate quantitative ratios between samples. On the basis of the quantitative ratios, labeled samples were mixed together and fractionated with the Pierce High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific).

2.5. Mass Spectrometry

Peptides (≈500 ng per fraction) were separated on a 75 μm (inner diameter) fused silica capillary pulled to a 5 micron tip with a P-2000 Sutter laser puller (Sutter Instrument, Novato, CA), packed with 22 cm of 2.5 micron Synergi Hydro-RP C18 (Phenomenex, Torrance, CA), and coupled directly to a Dionex UltiMate 3000 RSLC nano System (Thermo Fisher Scientific) controlling a 180 min linear gradient from 3.2% to 40% ACN and 0.1% formic acid at a flow rate of 300 nL min^{-1}. Peptides were electrosprayed at 2.4 kV into an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) operating in data-dependent mode with positive polarity and using 445.12003 m/z as an internal mass calibrant. Quadrupole isolation was enabled and survey scans were recorded in the Orbitrap at 120 000 resolution over a mass range of 400–1600 m/z. The instrument was operated in Top Speed mode using the multinotch MS^3 method with a cycle time of 3 s.[8] The automatic

gain control (AGC) target was set to 200 000 and the maximum injection time was set to 50 ms. The most abundant precursor ions (intensity threshold 5000) were fragmented by collision-induced dissociation (35% energy) and fragment ions were detected in the linear ion trap (AGC 10 000, 50 ms maximum injection). Analyzed precursors were dynamically excluded for 45 s. Multiple MS^2 fragment ions were captured using isolation waveforms with multiple frequency notches and fragmented by high energy collision-induced dissociation (65% normalized collision energy). MS^3 spectra were acquired in the Orbitrap (AGC 100 000; maximum injection time 120 ms, 60 000 resolution scanning from 100–500 m/z). These mass spectrometry data files can be retrieved from massive.ucsd.edu (MSV000081714).

2.6. Peptide Matching and Statistics

Mass spectrometry data were processed with Proteome Discoverer 2.1 (Thermo Fisher Scientific) which extracted MS^2 spectra for peptide identification and MS^3 spectra for peptide quantitation. MS^2 spectra were searched with MASCOT 2.5.1 against the *S. aureus* ATCC 25923 protein database (2672 chromosome and plasmid records acquired from GenBank (taxid:46170)[9] appended with a list of 172 sequences to detect common contaminants and 23 690 randomized decoy sequences to raise the MASCOT Identity score to ensure that peptides were identified by higher-quality peptide-spectrum matches [PSM]).[10,11] Search parameters were for tryptic digests with two possible missed cleavages, fixed amino acid modification for chemically modified cysteine and labeled N-terminal and internal lysine (+57.021 Da, C; +229.163 Da, K), variable oxidized methionine (+15.995 Da, M), monoisotopic mass values, ±10 ppm parent ion mass tolerance, and ±0.6 Da fragment ion mass tolerance. PSM were processed by Percolator[12] using delta Cn (0.05), strict FDR (0.01), relaxed FDR (0.05), and PEP (0.05) settings. Additional filters limited MASCOT Ions scores (greater than or equal to 13) and PSM and peptide PEPs (strict 0.01; relaxed 0.05). The estimated PSM strict and relaxed FDR was 0.002. Peptides were assigned to logical protein groups using parsimony. Proteins were quantified on summed signal-to-noise (S/N) ratios for each TMT channel for 28 702 qualified PSMs for unique peptides (isolation interference <25%; S/N ratio average of all channels >8; at least medium confidence). The most confident centroid within 0.003 Da of the expected mass of the reporter ions was used. TMT signals were also corrected for isotope impurities (lot specific data provided by the manufacturer). Missing TMT signal values were replaced with a minimum value of 1 for 204 qualified PSMs. Matches to contaminants and decoys were removed from the dataset as were proteins with quantitative signal sums across all six channels <50. Protein quantitative values for each channel were normalized and then scaled to 100 across the channels. A *t*-test was used to measure significant differences, and the Benjamini and Hochberg correction was applied to limit false discovery to ≤0.05. The amino acid sequences for quantified proteins were assigned KO numbers and mapped to biochemical and cellular pathways annotated in KEGG (http://www.kegg.jp).

2.7. Free Iron Estimation

S. aureus grown on TSA and TSA with 0.05 mg mL^{-1} punicalagin was harvested and suspended in water. CFU was determined and cells were lysed and centrifuged. The supernatant was used to measure free Fe^{2+} and Fe^{3+} using the QuantiChrom Iron Assay Kit (BioAssay Systems, Hayward, CA).

2.8. RNA Extraction and Reverse-Transcription Quantitative PCR

The transcripts of 11 *S. aureus* genes, *SbnG*, *IsdB*, *IsdI*, *SbnF*, *IsdG*, *Fur*, *DtxR*, *FtnA*, *RecA*, *RuvA*, and *HolB*, were analyzed by RT-qPCR using primers in Supporting Information. Treatments included *S. aureus* grown in TSA, TSA with 20 mM EDTA, TSA with 0.05 mg mL^{-1} punicalagin, and TSA with 0.78 μg mL^{-1} ciprofloxacin. Using titrations of RNA and melting curve analysis, primers were confirmed to amplify at >99% efficiency and to not produce off-target products. The Ct values for the tested genes were normalized to the expression of *gmK*. Reactions were performed in triplicate and three independent experiments were evaluated. Statistically significant differences were determined for relative quantitation values by analysis of variance ($p < 0.05$) followed by the Bonferroni and Dunnett post hoc multiple comparisons (SAS Institute, Cary, NC).

3. Results

3.1. Changes in Protein Profiles After Punicalagin Treatment

The growth inhibition of *S. aureus* by punicalagin is established,[2,5] but the mechanism of action is not resolved. Because full growth inhibition would be impractical for proteomic study, we applied a sublethal concentration of punicalagin and monitored changes. In the presence of punicalagin at 0.05 mg mL^{-1}, *S. aureus* colonies exhibited a rough surface similar to that which was reported previously,[5] and overall growth was reduced, resulting in a lawn that was uniform but thin. In comparison, *S. aureus* in the absence of punicalagin formed a thick, uniform lawn after 24 h incubation. These cells were harvested and proteins were purified from approximately equal masses of treated and untreated *S. aureus* cells. Three replicate experiments were performed. SDS-PAGE analysis and Coomassie staining revealed obvious changes in banding patterns, including decreased band intensity for clusters of proteins with molecular weights of 20 kDa and 60 kDa, and increased band intensity for proteins with molecular weights of 35 kDa and 45 kDa (**Figure1**A). These results demonstrated that punicalagin consistently altered the proteome of *S. aureus*.

The proteins isolated from equivalent masses of cells were digested with trypsin, differentially labeled with TMT reagents, and analyzed by mass spectrometry. We detected 11 574 unique peptides which constituted 1202 proteins with unique TMT reporter ion signals (Supporting Information). These signals were used to establish relative amounts of accumulation for each protein per replicate treatment. The quantitative amounts for each replicate treatment clustered into distinct groups, with the

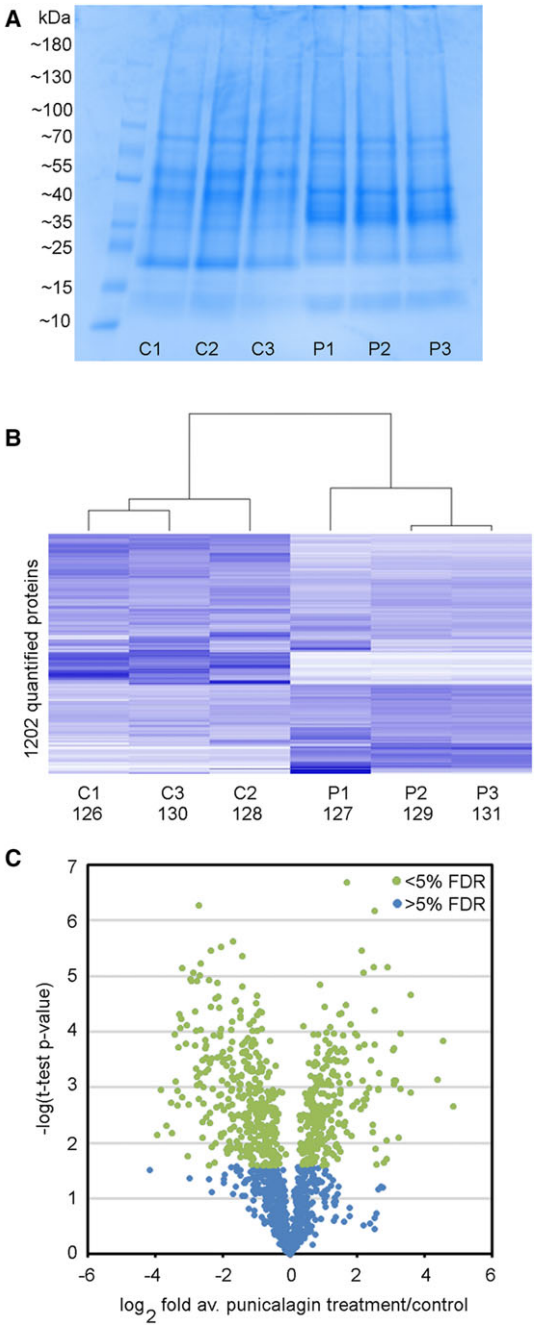

Figure 1. Protein profiles of *S. aureus* without and with punicalagin treatment. A) SDS-PAGE profiles of proteins from *S. aureus* cells from three independent experiments. Lanes from left to right: Molecular weight marker with estimated sizes in kDa; C1, C2, C3 (*S. aureus* controls); P1, P2, P3 (punicalagin-treated *S. aureus*). B) Hierarchical clustering of the normalized and scaled TMT reporter ion (126, 127, 128, 129, 130, 131) summed S/N values for proteins from three biological replicates. Blue is the highest amount of relative expression and white is the lowest. Clustering was performed with Treeview 3.0. C1, C2, C3 (*S. aureus* controls); P1, P2, P3 (punicalagin-treated *S. aureus*). C) Volcano distribution of $\log_2$ average normalized and scaled TMT reporter ion S/N values for proteins from *S. aureus* with punicalagin treatment divided by the same for *S. aureus* controls versus the $-\log(t\text{-test } p\text{-value})$ for each set of reporter ion S/N values for each protein. Proteins with *p*-values with ≤5% false discovery rate (FDR) are in green and those with >5% FDR are in blue.

untreated and treated samples clustering as separate groups (Figure 1B), demonstrating that the response to punicalagin was reproducible. Among the 1202 proteins quantified, 558 that exhibited significantly different ($p < 0.05$) accumulation levels between treatments (Figure 1C; Supporting Information). There were 312 that exhibited decreased accumulation, and 246 that exhibited increased accumulation due to punicalagin treatment (Supporting Information).

3.2. Iron Homeostasis Enzymes Affected by Punicalagin

Among proteins with the greatest increases in accumulation after cells were exposed to punicalagin were siderophore biosynthesis proteins, heme and iron transporters, and a heme-degrading monooxygenase (Table 1, Supporting Information). Overall, there were at least 12 proteins associated with bacterial iron acquisition that had increased accumulation (**Table 1**). These results imply that the bacterium was scavenging iron after punicalagin treatment. The genes for these proteins are known to be negatively regulated by the ferric uptake regulator (Fur), a transcription factor that binds iron and blocks transcription of iron acquisition genes.[13–15] In the absence of iron, Fur ceases to block transcription and iron uptake ensues.[13,14] Interestingly, among the proteins we identified with decreased accumulation after punicalagin treatment was a Fur-family homolog with 32% identity to the canonical *Escherichia coli* Fur (Table 1). Thus, it is possible that the decreased amounts of this Fur homolog protein led to the derepression of the iron acquisition genes and the subsequent accumulation of their cognate proteins. One of those, SbnI, also is an iron-binding transcription factor that controls *SbnD-I* expression.[16] In the presence of iron, SbnI does not bind DNA.[16] Thus, the decrease in the Fur homolog likely led to the accumulation of SbnI which likely led to the accumulation of SbnF and SbnG (Table 1). Similarly to the Fur homolog, there was decreased accumulation of a DtxR-family transcription factor (Table 1).[17] Canonical DxtR from *Corynebacterium diphtheria* acts as a transcriptional repressor in the presence of iron.[18] It is possible that the DtxR homolog decreased in an iron-regulated manner, leading to its derepression and activation of other genes. Also among proteins with decreased accumulation was ferritin (Table 1), a protein common to eukaryotes and prokaryotes that stores iron and balances iron homeostasis.[15] It is possible that *S. aureus* decreased ferritin to liberate internal iron stores needed to sustain its metabolic processes. Altogether, these results support the hypothesis that punicalagin in the growth medium chelated iron and caused *S. aureus* to be iron deficient, and that the cells responded by altering their proteomes to acquire iron.

Under iron deficiency, bacteria decrease their metabolic demands for iron by reducing the accumulations of enzymes that use iron as a cofactor, often switching to a homolog that uses another metal instead.[19] One such protein is superoxide dismutase,[20] and we observed a decrease in one isoform of superoxide dismutase, although there also was an increase in another isoform after punicalagin treatment (Table 1). Furthermore, enzymes of the citric acid cycle also use iron cofactors,[19] and *S. aureus* treated with punicalagin exhibited decreased amounts of fumarase and aconitase (Figure 2, Supporting Information).

Table 1. Proteins related to iron acquisition and sequestration and iron-modulated transcriptional regulation with significantly altered accumulation after punicalagin treatment.

Accession	Description	Log_2 fold change (punicalagin/control)
685631273	Siderophore biosynthesis protein SbnA	4.5
685631279	Siderophore biosynthesis protein SbnG	4.4
685632216	Heme transporter IsdB	2.9
685631278	Siderophore biosynthesis protein SbnF	2.9
685632223	Heme-degrading monooxygenase IsdI	2.9
685631802	Iron-enterobactin transporter ATP-binding protein	2.7
685631281	Siderophore biosynthesis protein SbnI	2.6
685631322	Heme-degrading monooxygenase IsdG	2.5
685631411	Cell wall biosynthesis protein ScdA (iron–sulfur cluster)	1.3
685631895	Iron ABC transporter substrate-binding protein	1.3
685631741	Heme peroxidase	0.93
685632736	Superoxide dismutase	0.63
685631978	Fe–S cluster assembly protein SufD	0.43
685631290	Superoxide dismutase	−0.61
685632004	Hypothetical protein KQ76_04260 (iron–sulfur cluster)	−0.91
685632037	Fe–S cluster assembly protein SufT	−1.1
685633038	Fur-family transcriptional regulator	−1.1
685631789	DtxR-family transcriptional regulator	−1.7
685633072	Ferritin FtnA	−2.8

Interestingly, all enzymes of the citric acid cycle except for malate dehydrogenase exhibited decreased accumulation (**Figure 2**). Although not all citric acid cycle enzymes use iron as a cofactor, the overall decrease in citric acid cycle proteins appears to be similar to an iron-deficiency response regulated by Fur where the loss of ATP resulting from a compromised citric acid cycle is compensated by an increase in glycolysis.[21] Our results corroborate this, showing increased amounts of enzymes throughout the glycolysis pathway (Figure 2). Hence, these results support the hypothesis that *S. aureus* was responding to iron deficiency caused by punicalagin.

3.3. Protein Translation Enzymes Affected by Punicalagin

Punicalagin treatment also appeared to affect protein translation machinery. There were increased amounts for 29 subunits for 50S and 30S ribosomes in punicalagin-treated cells whereas only 4 subunits had decreased accumulation (Supporting Information). An increase in ribosomes suggests a reduction in ribosome turnover in punicalagin-treated cells. This implies that the cells were attempting to stabilize protein biosynthesis. The increased amounts of a ribosome maturation protein RimP that interacts with 30S ribosomal subunits,[22] an SmpB-like single-stranded DNA-binding protein that releases ribosomes from damaged mRNA,[23] a peptide chain release factor 1, and a ribosome recycling factor and its interaction partner elongation factor G,[24] along with coinciding decreased amounts of antitermination protein NusG[25] support the hypothesis that the treated cells attempted to stabilize protein translation. One reason could be that novel protein biosynthesis was stalled. We observed decreased amounts of enzymes involved in tRNA biosynthesis and decreased amounts of enzymes needed to produce valine, leucine, isoleucine, glycine, serine, threonine, and glutamine (Supporting Information). A reduction in available pools of amino acids could have interrupted protein translation and inhibited cell growth.

3.4. Nucleic Acid Biosynthesis Enzymes Affected by Punicalagin

The slow growth rate of *S. aureus* on punicalagin indicated that cell division was negatively affected. Slower cell division could result from stalled protein biosynthesis or from reduced DNA and RNA biosynthesis. Our studies showed that cells treated with punicalagin had decreased amounts of enzymes that form purine precursor inosine monophosphate and pyrimidine precursor uridine monophosphate. Furthermore, there were decreased amounts of ribonucleotides-diphosphate reductase and nucleoside-diphosphate kinase that form nucleotide and deoxynucleotide triphosphates, as well as polymerases that polymerize DNA and RNA (Supporting Information). It is possible that these decreases were related to iron-deficiency stress because ribonucleotide-diphosphate reductase uses iron as a cofactor and is repressed by NrdR, a Fur-regulated transcription factor.[26] We observed increased accumulation of NrdR, which is consistent with iron starvation.

3.5. SOS Response Enzymes Affected by Punicalagin

We observed increased amounts of proteins that coincide with SOS activation (Table 2, Supporting Information). The SOS response is a cellular survival response acutely induced by the

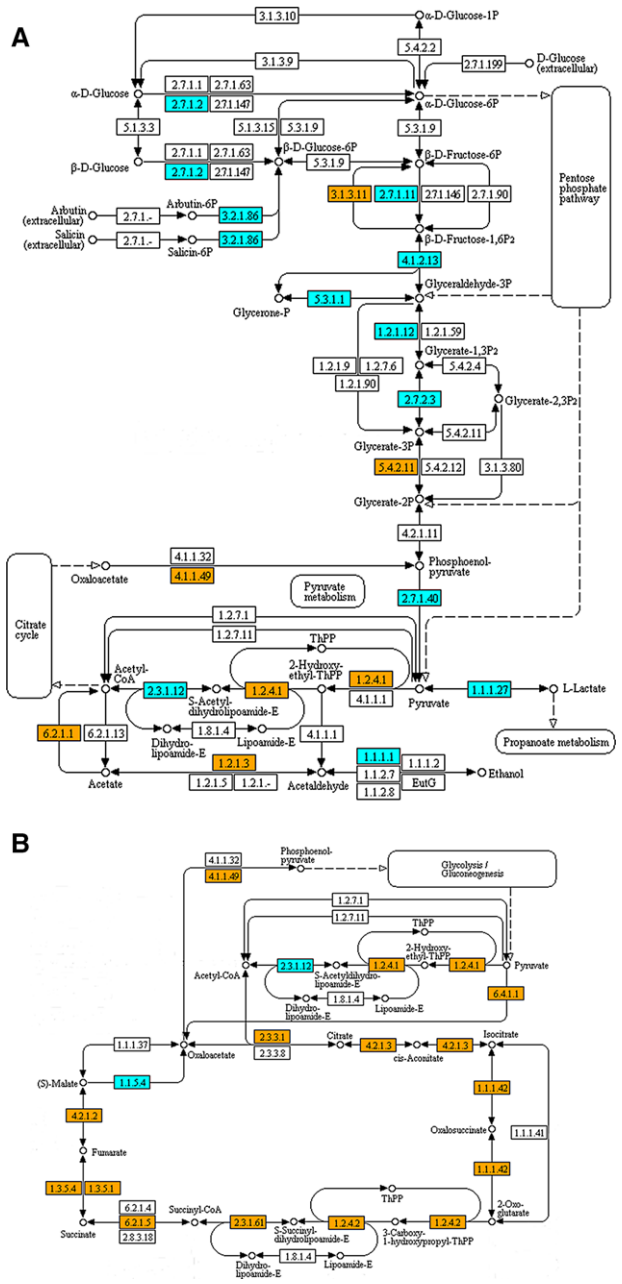

Figure 2. The glycolytic and the citric acid cycling enzymatic pathways in *S. aureus*. Enzymes with increased accumulation from punicalagin are in blue and those with decreased accumulation are in orange. A) Glycolysis. B) The citric acid cycle. The KEGG identifier and abbreviation key is provided in the Supporting Information.

formation of single strand DNA (ssDNA) after cell exposure to ultraviolet (UV) radiation, drugs, or through other natural and unnatural processes that lead to replication stalling at replication forks in bacterial chromosomes.[27] RecA is a major regulator of the SOS response, and we saw increased amounts of RecA as well as RuvX and RuvA, known regulators of RecA.[28] RecA functions to recruit enzymes to promote homologous recombination and repair ssDNA through a select set of low-fidelity DNA polymerases and enzymes while deactivating sets of high-fidelity DNA polymerases.[29] The low-fidelity polymerases insert incorrect nucleotides during polymerization which increases the mutation rate and creates variation that may enable survival under distressing conditions.[30] Indeed, punicalagin-treated cells had decreased amounts of high-fidelity PolA. Other proteins with decreased amounts included other RecA regulators like RecN, RecJ, RecG, and RecX (**Table 2**).[27,31,32] These protein accumulation changes for RecA, RecN, and RecX mirror the gene expression changes in *S. aureus* exposed to ciprofloxacin, another inducer of SOS.[33] In essence, the proteomics results reveal a complicated regulation of SOS in response to punicalagin and that DNA damage, alongside iron starvation and protein synthesis inhibition, likely led to reduced cell growth.

3.6. In Vitro Tests to Evaluate Iron Chelation and the SOS Response

Because of the proteomic observations, we were interested in whether the punicalagin-induced iron-deficiency and SOS responses were similar to iron-deficiency and SOS responses induced by other chemicals. First, we found that extracts of sublethal punicalagin-treated cells had ten times less free iron in them (**Figure 3**A). Then, by supplementing lethal media (0.5 mg mL^{-1} punicalagin) with 7.4 μM ferric chloride, we restored *S. aureus* growth to 64% of controls (Figure 3B). Together, these results confirmed that punicalagin-induced *S. aureus* growth inhibition was at least partly due to iron starvation. Next, we evaluated RNA expression for genes for several proteins that exhibited changes in response to punicalagin. For the marker genes for iron-deficiency response (*SbnG*, *IsdB*, *IsdI*, *SbnF*, *IsdG*, *Fur*, *DtxR*, *FtnA*), the transcriptional responses to punicalagin agreed with the observed proteomic responses to punicalagin with the exception of *IsdG* and *DtxR* which followed posttranscriptional regulation models (Figure 3C). For the SOS markers (*RecA*, *RuvA*, *HolB*), *HolB* transcriptional and proteomic responses to punicalagin agreed with each other, but there was no transcriptional change for *Rec*A, although this does not contradict the known posttranslational control of RecA during the SOS response.[27] The *Ruv*A transcriptional decrease was opposite of the proteomic increase during punicalagin treatment, but gene expression might not sufficiently explain the complicated proteomic regulation of RecA by RuvA during an SOS response.[34] Nevertheless, we took these data to describe the transcriptional response to punicalagin, and we used these for the comparison to transcriptional responses to EDTA, a chelator, and ciprofloxacin, an SOS inducer. We found that the transcriptional responses to EDTA were weaker than the responses to punicalagin (Figure 3C). This suggests that punicalagin sequesters iron or induces an iron-scarcity response better than EDTA. Surprisingly, ciprofloxacin also increased the expression of iron-regulated *SbnG*, *IsdB*, *IsdI*, *SbnF*, and *IsdG*, and did so to greater levels than EDTA. Also, ciprofloxacin increased the expression of these genes while increasing the expression of Fur and DtxR instead of decreasing them like punicalagin did. This suggests that ciprofloxacin induces an

Table 2. Proteins related to SOS response with significantly altered accumulation after punicalagin treatment.

Accession	Description	Log_2 fold change (punicalagin/control)
685632380	Recombinase RecA	0.75
685632797	Holliday junction resolvase RuvX	0.75
685632823	ATP-dependent DNA helicase RuvA	0.75
685632552	5′-3′ exonuclease	0.45
685633086	ATP-dependent DNA helicase PcrA/UvrD	−0.52
685632866	DNA polymerase I	−0.53
685631218	DNA gyrase subunit B	−0.54
685632702	DNA recombination protein RecN	−0.67
685631214	Chromosomal replication initiation protein DnaA	−0.67
685633022	DNA repair exonuclease	−0.68
685631681	DNA repair protein RadA	−0.73
685632852	DNA mismatch repair protein MutT	−0.79
685632808	Recombinase RarA	−0.86
685632817	Single-stranded DNA exonuclease RecJ	−0.86
685631215	DNA polymerase III subunit beta DnaN	−0.98
685633619	DNA mismatch repair protein MutT	−1.1
685632583	DNA-binding protein HupB	−1.2
685633335	ATP-dependent DNA helicase RecG	−1.2
685633051	RecX-family transcriptional regulator	−1.4
685632466	DNA gyrase subunit A	−1.4
685631617	DNA mismatch repair protein MutT	−1.6
685631634	DNA polymerase III subunit delta′ HolB	−2.0

iron-scavenging response that may be disconnected from Fur and DtxR. It also appeared that the SOS response regulation caused by ciprofloxacin was different from that caused by punicalagin. Ciprofloxacin induced much greater expression for RecA than did punicalagin. Hence, punicalagin treatment may not induce SOS in *S. aureus* in exactly the same way that ciprofloxacin does.

4. Discussion

Punicalagin, a natural compound from pomegranate, inhibits the growth of *S. aureus*, a human, food-borne pathogen, and significantly alters the proteomes of cells. Changes include repression of a Fur homolog transcriptional regulator and an induction of iron-sequestration proteins. There also was a decrease in accumulation of citric acid cycle enzymes along with an increase in glycolysis proteins, an iron-deficiency response to shift ATP production to glycolysis as the citric acid cycle lost efficiency from the lack of iron (Figure 2). These findings reveal the proteomic effect of punicalagin on cellular iron homeostasis and agree with prior observations that punicalagin chelates iron.[2] Iron also is necessary for abscess formation during pathogenesis[35]; thus, punicalagin could be effective in reducing host colonization, although the in vivo mechanism of action for the latter is yet to be elucidated.

The increased amounts of RecA, a protein that regulates the repair of ssDNA during the SOS response, support the hypothesis that punicalagin created DNA damage (Table 2). It is not known whether punicalagin independently created DNA damage or if the DNA damage was linked to iron stress. Transcriptional measurements of marker genes in the presence of EDTA, another chelator, and ciprofloxacin, another SOS inducer, suggested that punicalagin induced a stronger iron-deficiency response but that ciprofloxacin can induce an iron-scarcity response as well (Figure 3). These differences may be rooted in how the cells fine tune their SOS responses to best suit their survival for these different chemicals.[27]

This study is the first to describe the proteomic effects of punicalagin on *S. aureus*. The observed changes reflect a combination of punicalagin's direct action on the cell and the cellular response to survive. These two responses are not easily distinguishable from the data and will need to be resolved through additional investigation. Nonetheless, the results show that punicalagin can induce the SOS response, a cellular survival mechanism that allows DNA mutation to create variation. Hence, if scientists are interested in using punicalagin as a natural antibiotic to reduce disease incidence, the rate at which *S. aureus* may be able to develop tolerance to punicalagin should be studied, as should the potential for punicalagin to induce the formation of ssDNA in microbial and eukaryotic cells alike.

Abbreviations

AGC, automatic gain control; Fur, ferric uptake regulator; ms, millisecond; PSM, peptide-spectrum matches; S/N, signal-to-noise; ss, single strand; TMT, tandem mass tag; TSA, tryptic soy agar; UV, ultraviolet

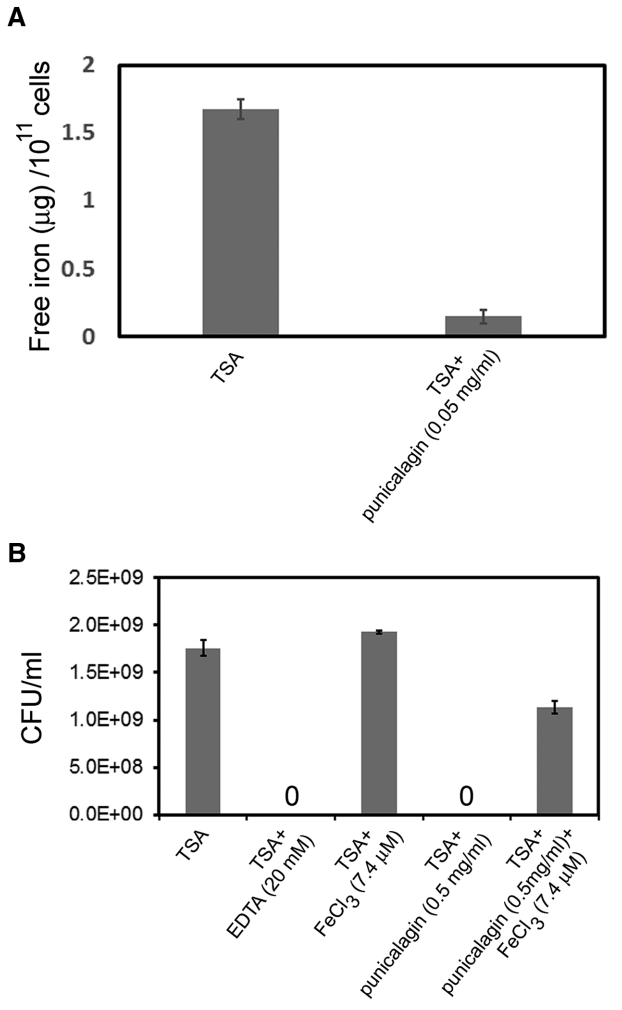

Figure 3. The effects of punicalagin, EDTA, and ciprofloxacin on iron accumulation, cell growth, and gene expression in *S. aureus*. A) Free Fe^{2+} and Fe^{3+} in cells grown in the presence of a sublethal dose of punicalagin compared to controls. B) *S. aureus* grown in the presence of a lethal dose of punicalagin, EDTA, and punicalagin + ferric chloride. C) Marker gene expression changes (LogRQ) of *S. aureus* exposed to punicalagin, EDTA, and ciprofloxacin. Standard deviation from three replicate experiments is shown.

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.

Keywords

Iron homeostasis, Pathogens, Punicalagin, SOS response

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