Journal of proteome research

Article

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Multitargeted Flavonoid Inhibition of the Pathogenic Bacterium *Staphylococcus aureus*: A Proteomic Characterization

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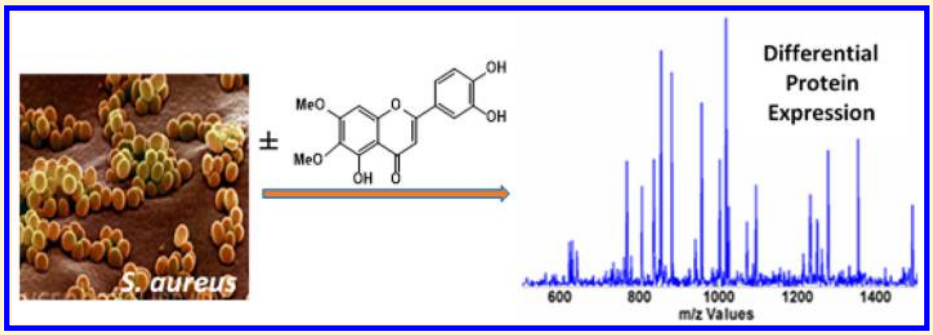
Supporting Information



ABSTRACT: Growth inhibition of the pathogen *Staphylococcus aureus* with currently available antibiotics is problematic in part due to bacterial biofilm protection. Although recently characterized natural products, including 3′,4′,5-trihydroxy-6,7-dimethoxy-flavone [**1**], 3′,4′,5,6,7-pentahydroxy-flavone [**2**], and 5-hydroxy-4′,7-dimethoxy-flavone [**3**], exhibit both antibiotic and biofilm inhibitory activities, the mode of action of such hydroxylated flavonoids with respect to *S. aureus* inhibition is yet to be characterized. Enzymatic digestion and high-resolution MS analysis of differentially expressed proteins from *S. aureus* with and without exposure to antibiotic flavonoids (**1**−**3**) allowed for the characterization of global protein alterations induced by metabolite treatment. A total of 56, 92, and 110 proteins were differentially expressed with bacterial exposure to **1**, **2**, or **3**, respectively. The connectivity of the identified proteins was characterized using a search tool for the retrieval of interacting genes/proteins (STRING) with multitargeted *S. aureus* inhibition of energy metabolism and biosynthesis by the assayed flavonoids. Identifying the mode of action of natural products as antibacterial agents is expected to provide insight into the potential use of flavonoids alone or in combination with known therapeutic agents to effectively control *S. aureus* infection.

KEYWORDS: *Staphylococcus aureus, antibacterial flavonoids, LC−MS/MS proteomics, multitargeted bacterial inhibition*

INTRODUCTION

Staphylococcus aureus is a pathogenic bacterium that is difficult to eradicate and/or control.[1] This Gram-positive species readily forms a protective extracellular polymer matrix[2] that serves as a physical barrier to prevent the penetration of toxic metabolites such as antibiotics.[3] Moreover, *S. aureus* is responsible for a variety of human maladies that range from mild skin irritations to systemic organ failure including bacteremia, osteomyelitis, and infective endocarditis.[4] Under suitable conditions, *S. aureus* can cause serious complications associated with externally introduced medical devices such as implants and catheters.[5] With the combined bacterial protection of antibiotic resistance and biofilm barrier formation, control of such infections is an ever increasing challenge.

Flavonoids are low molecular weight natural products with antibiotic activity against both Gram-positive and -negative bacteria; these plant-derived benzo-γ-pirone metabolites exhibit dual potency because they do not contain a beta-lactam structural motif, highly susceptible to antibiotic resistance by bacterial beta-lactamases, and some such compounds possess biofilm blocking properties. To examine the mode of action of flavonoids as antibiotics against bacterial pathogens, qualitative and quantitative monitoring of bacterial responses to antibiotic exposure is necessary. Since the bacterial proteome is dynamic in nature and quickly responses to antibiotic exposure,[6] identifying and quantifying bacterial protein changes on a global level with sublethal flavonoid antibiotics is expected to provide insight into the mode of action of flavonoids as antibiotics. Indeed, proteomic analysis has already been widely

Received: March 9, 2017

DOI: 10.1021/acs.jproteome.7b00137
J. Proteome Res. XXXX, XXX, XXX−XXX

implemented to study the molecular mechanisms of antibacterial agents as well as the cytotoxicity of biomaterials.[7−11]

Enzymatic digestion of total extracted protein combined with high-resolution mass spectral analysis allows for the identification and quantification of differentially expressed proteins from a given biological system with a certain treatment. Elucidating the targets and mechanism of action of antibiotic is central to an activity-based research approach for antibiotic development.[12] Proteomic analysis explicitly identifies changes in protein expression, interactions, cellular localization, and post-translational modifications. In combination with protein changes, computational modeling can be employed to predict and characterize dynamic properties that can be part of a complex signaling network. With such systems biology analyses, proteins that exhibit up- or downregulation with a given treatment can be identified as potential drug targets for therapy.[13] In this study, a proteomics approach combined with systems biology techniques is utilized to examine the expression of proteins in biofilm cells grown with and without the presence of flavonoid antibiotics. The results indicate that the mechanism of action of antibiosis in *S. aureus* can be attributed to a multi-inhibitory response that includes energy metabolism and amino acid, protein, DNA, ATP, and fatty acid biosyntheses.

EXPERIMENTAL PROCEDURES

Chemicals

Dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), ethylene-bis(oxyethylene nitrile)tetraacetic acid (EGTA), dimethyl sulfoxide (DMSO), and MS-grade formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride and disodium phosphate were purchased from Mallinckrodt (St Louis, MO). Tris-(hydroxymethyl)aminomethane (Tris) was obtained from Shelton Scientific, Inc. (Peosta, IA), and ethylenedinitrilotetraacetic acid (EDTA) was purchased from Fisher Scientific (Waltham, MA). HPLC-grade water was from Mallinckrodt Chemicals (Phillipsburg, NJ), and HPLC-grade acetonitrile was from J.T. Baker (Phillipsburg, NJ). Mass-spectrometry grade trypsin was obtained from Promega (Madison, WI).

Flavonoids

Compounds **1**−**3** were isolated from *Teucrium polium* and identified based on standard spectroscopic techniques as described previously.[14,15]

Bacterial Biofilm Induction

The biofilm prevention assay was performed as described previously[15] with slight modifications. Compounds **1**−**3** (Figure 1) were added at 0.01 mM, which is a concentration below their biofilm inhibitory concentration. Briefly, *S. aureus* strain AH133-GFP was grown overnight in Luria−Bertani (LB) broth and diluted to ca. 1×10^8 CFU/mL. The cultures were serially diluted 10-fold to obtain inoculums of ca. 1×10^5 CFU/mL, and the utilized flavonoids were prepared at 0.01 mM in DMSO. A 10 μL sample of the diluted culture was applied to a 6 mm cellulose disc (Becton Dickinson), and 10 μL of the test compound, DMSO, and/or ampicillin was added. Plates were incubated for 24 h at 37 °C. The biofilm was quantified by counting the colony forming units (CFUs) per disc.[16]

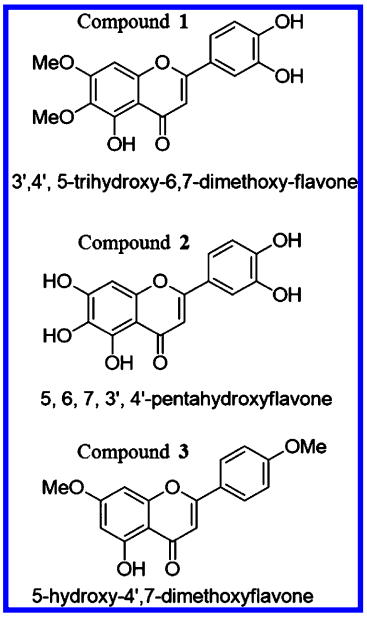


Figure 1. Structures of the tested compounds.

Protein Extraction and Enzymatic Digestion

The cellulose discs (*S. aureus* AH133, ca. 1×10^5−10^6 CFU/mL) were removed from the LB plates, and the biofilm was disrupted by vortexing in 1 mL of PBS. Each cell pellet was lysed in 250 μL of extraction buffer (150 mM NaCl, 20 mM Tris, 2.5 mM Na_2HPO_4, 1 mM EDTA, 1 mM EGTA, 0.5% w/v CHAPS). Samples were then sonicated for 45 min at 4 °C prior to centrifugation for 10 min at 14 800 rpm with the centrifuge held at 4 °C. The supernatant was collected in separate containers. The buffer of the extracted proteins was exchanged into 50 mM ammonium bicarbonate using Amicon Ultra 0.5 mL centrifugal filters (3K nominal molecular weight limit; EMD Millipore Ltd., Billericam, MA). This buffer was utilized for efficient tryptic digestion.

A 10 μg aliquot of each sample, determined by bicinchoninic acid (BCA) protein assay (Thermo Scientific/Pierce, Rockford, IL), was diluted to a 50 μL final volume using 50 mM ammonium bicarbonate. Thermal denaturation was performed at 65 °C for 10 min. The protein was reduced to 5 mM at 60 °C for 45 min and alkylated with 20 mM IAA at 37.5 °C for 45 min in the dark. Excess IAA was consumed by the addition of DTT to a final concentration of 10 mM and incubated at 37.5 °C for 30 min. The tryptic digestion was performed at 37.5 °C for 18 h followed by microwave digestion at 45 °C and 50 W for 30 min. A 0.5 μL aliquot of neat formic acid was added to quench the digestion. The resulting peptides were vacuum-dried and stored at −20 °C until LC−MS/MS analysis.

LC−MS/MS Analysis

Each digested sample, corresponding to 10 μg of protein, was resuspended in 60 μL of HPLC water with 0.1% formic acid. A 6 μL aliquot of each sample, which corresponds to 1 μg of protein was subjected to LC−MS/MS analysis. LC−MS/MS was acquired using a Dionex 3000 ultimate nano-LC system (Dionex, Sunnyvale, CA) interfaced to an LTQ Orbitrap Velos (Thermo Scientific, San Jose, CA) equipped with nano-ESI source. The separation was attained using an Acclaim PepMap RSLC column (75 μm I.D. × 15 cm, 2 μm particle size, 100 Å pore size; Dionex, Sunnyvale, CA) with a flow rate of 350 nL/min. During the separation, the column compartment was

DOI: 10.1021/acs.jproteome.7b00137
J. Proteome Res. XXXX, XXX, XXX−XXX

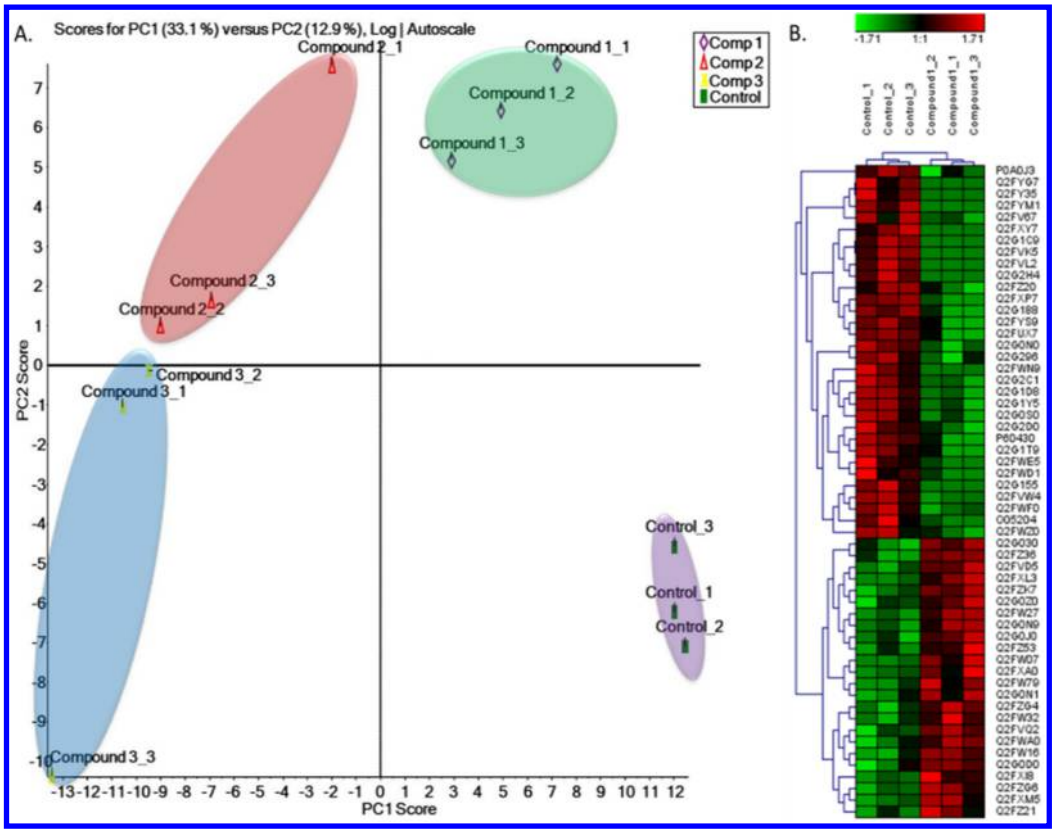


Figure 2. Multivariate statistics of proteins identified from segregated groups. (A) Principal component analysis of the whole spectral count dataset derived from the identified proteins in each group. Each cluster is highlighted in a different color. (B) Hierarchical clustering of significantly altered proteins from the comparison between the control and compound **1** treated groups. The heat map was constructed using *Z*-score values (number of standard deviations from the mean) of normalized spectral counts.

maintained at 29.5 °C. The LC elution gradient of solvent B used in both LC−MS/MS analyses was 5% over 10 min, 5−20% over 55 min, 20−30% over 25 min, 30−50% over 20 min, 50−80% over 1 min, 80% over 4 min, 80−5% over 1 min, and 5% over 4 min. Solvent B consisted of 100% ACN with 0.1% formic acid, and solvent A was composed of 2% ACN with 0.1% formic acid.

The LTQ Orbitrap Velos mass spectrometer was operated in positive mode with the ESI voltage set to 1500 V. Data-dependent acquisition was employed to achieve two scan events. The first scan event was a full MS scan of 400−2000 m/z at a mass resolution of 15 000. The second scan event was the CID MS/MS of parent ions selected from the first scan event with an isolation width of 3.0 m/z, at a normalized collision energy (CE) of 35%, and an activation Q value of 0.250. The CID MS/MS scans were performed on the 10 most intense ions observed in the MS scan event. The dynamic exclusion was set to have a repeat count of 2, repeat duration of 30 s, exclusion list size of 200, and exclusion duration of 90 s.

Data Analysis

LC−ESI−MS/MS data was transformed to a mascot generic format file (*.mgf) by Proteome Discover version 1.2 software (Thermo Scientific, San Jose, CA) and then searched using the UniProt database (2014_08, taxonomy *Staphylococcus aureus*, strain NCTC 8325, 2896 entries) in MASCOT version 2.4 (Matrix Science Inc., Boston, MA). IAA modification of cysteine was set as a fixed modification, and oxidation of methionine was set as a variable modification. The MS and MS/MS m/z tolerance was set to 6 ppm and 0.8 Da, respectively. The maximum number of missed cleavages was set to 2. MASCOT search results were validated and quantified through spectral counting by Scaffold (version 3.6.3, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm.[17] Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by a Protein Prophet algorithm.[18] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The normalized spectral count was used to quantify the relative abundance of each identified protein among different samples. An independent *t*-test was performed to evaluate the statistical significance of the data. Proteins showing a significant difference (a systems biology analysis at $p < 0.05$) in spectral counts were used in quantitative assessments. Hierarchical cluster analysis was performed using normalized spectral-count values with proteins whose abundance was significantly changed after compound treatment (Figures 2B and S1). A functional genomic strategy was employed to identify bacterial proteins differentially regulated upon antimicrobial natural product exposure (Figures S2−S6).

Bioinformatic Analysis

The lists of altered proteins due to flavonoid treatment of *S. aureus* AH1331 were subjected to bioinformatics analysis using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING v. 9.1, http://www.string-db.org) to disclose func-

DOI: 10.1021/acs.jproteome.7b00137
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Table 1. List of Proteins Showing a Significant Change in Response to Metabolites 1−3

pathway	gene ontology (GO) ID	proteins	accession no.	fold change %[a] 1	2	3
fatty acid metabolism	sao00071	fadB/SAOUHSC_00196	Q2G1C9	100	100	100
Staphylococcus aureus infection	sao05150	sbi	Q2FVK5	100	100	100
DNA replication	sao03030	dnaN	Q2G2H4	100	100	100
purine metabolism	sao00230	rpoZ	Q2G1T9	82	100	100
pyrimidine metabolism	sao00240	rpoZ	Q2G1T9	82	100	100
pyruvate metabolism	sao00620	SAOUHSC_01064	Q2G2C1	84	100	100
	sao00620	ldh2	Q2G1Y5	34	---	61
RNA polymerase	sao03020	rpoZ	Q2G1T9	82	100	100
arginine and proline metabolism	sao00330	arcA	Q2FUX7	81	100	100
oxidative phosphorylation	sao00190	atpD	Q2FWF0	51	---	73
	sao00190	SAOUHSC_00878	Q2FZV7	---	100	100
fatty acid biosynthesis	sao00061	fabD	Q2FZ54	---	100	100

[a]Fold change zero (100): complete inhibition of protein expression in the treated sample compared to nontreated control. Fold change < 100: the percentage of the protein of interest in the treated sample compared to nontreated control. (---) indicates that the protein of interest was not significantly modified in the treated sample.

tional interactions between altered proteins. This database has the capability of performing multiple sequence analyses for predefined organisms. We chose *S. aureus* 8325 strain as the reference for our analysis since *S. aureus* AH1331 is derived from this *S. aureus* 8325 lineage. Each protein is represented by a node, and protein−protein interactions are represented by a colored line/edge. Systems biology analysis was implemented by STRING to categorize the altered proteins per their biological processes, cellular localizations, molecular functions, or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. KEGG pathways were used to provide insight into the effectiveness of a given treatment against *S. aureus*.

RESULTS AND DISCUSSION

S. aureus AH133 was used to study cellular responses under a biofilm subinhibitory concentration of 0.1 μmol/disc with compounds **1**, **2**, or **3** (Figure 1) or with a control treated with DMSO alone. A total of 1485 unique polypeptides were identified upon antibiotic exposure. These amino acid sequences corresponded to 287 proteins at a 99% minimum protein identification probability with a minimum of 2 peptides per protein. Among ca. 260 quantified proteins 56, 92, and 110 proteins met the significantly different threshold ($p < 0.05$) for compounds **1**−**3**, respectively. The entire lists of quantified proteins, normalized spectral count values, Student's *t*-test *p*-values, protein fold changes, and protein regulations are given in Tables S1−S3. With each antibiotic treatment, fewer proteins were observed to be upregulated than downregulated. Specifically for **1**−**3**, 24 versus 32, 20 versus 72, and 10 versus 100 polypeptides were up- versus downregulated, respectively. Per principal component analysis (PCA) (Figure 2A), the control group and each different compound treated group were clearly resolved from each other. This observation suggests that each compound has variable effects on AH133.

Several proteins associated with bacterial biofilm assemblage were downregulated with antibiotic treatment including arginine deiminase (arcA) (Table 1), a hydrolase functioning in energy generation and biofilm pH homeostasis.[19] This enzyme catalyzes arginine catabolism to L-citruline and ammonia with subsequent citruline conversion to L-ornithine, ATP, and CO_2.[20] Through the arcA pathway, bacteria generate energy as well as reduce acidic conditions with the production of the nitrogenous base ammonia. Since arginine deiminase functions to protect bacteria against the damaging effects of acidic environments,[21,22] enzyme downregulation may be associated with decreased bacterial pathogenicity. arcA is targeted by the C-18 lipid linoleic acid, which is considered to be the mode of action for inhibiting bacterial virulence and pathogenicity.[23] In fact, the development of complex multispecies communities such as biofilms is controlled by interbacterial communication systems, and arcA serves as a signal that initiates such intergeneric communication.[24] In one study, an arcA-deficient mutant of *Stipecampus cristatus* was unable to communicate with *Porphyromonas gingivalis*, inhibiting dental biofilm formation.[24] Moreover, genes such as *arcA*, are also inhibited by rhein, an anthraquinone natural product obtained from rhubarb also known as cassic acid. Rhein has been reported to have potential *in vitro* antimicrobial activity against more than 20 *S. aureus* strains.[25]

Defective biofilm formation with reduced sliding motility is associated with downregulation of the RNA polymerase (RNAP) omega (ω) subunit (*rpoZ*),[26] and levels of this *rpoZ* subunit are observed to be downregulated by **1** (Figures 3B and S4B). In the case where a deletion mutation for *rpoZ* is present in *Mycobacterium smegmatis*, biofilm formation is blocked and a decline in growth rate and loss of colony morphology[26,27] are observed. Specifically, this mutant line fails to synthesize short-chain mycolic acids characteristic of biofilm growth in *M. smegmatis*.[26] Clofazimine and the natural products myxopyronin, corallopyronin, ripostatin, and lipiarmycin inhibit *Staphylococcal* RNAP and exhibit broad-spectrum antibacterial activity.[28,29] Bacterial RNAPs are an attractive and previously utilized target for antibiotics because they are highly conserved among bacteria while being distinct enough from eukaryotic RNAPs to confer therapeutic selectivity.[29,30]

DNA polymerase III subunit B (dnaN), which is responsible for most replicative synthesis as well as 3′ to 5′ exonuclease activity in bacteria, can be targeted by multiple unrelated phage polypeptides, suggesting its vulnerability to inhibition.[31] Indeed, the β sliding clamp is predicted to be a potential antibiotic target because it is essential for cell viability and at the same time shares no sequence homology with the eukaryotic PCNA clamp.[32,33] This polypeptide (dnaN) is indeed downregulated by **1** (Figures 3B and S2A).

Another factor that reduces bacterial survival and is central to *S. aureus* pathogenicity is the avoidance of host immunity.[34]

DOI: 10.1021/acs.jproteome.7b00137
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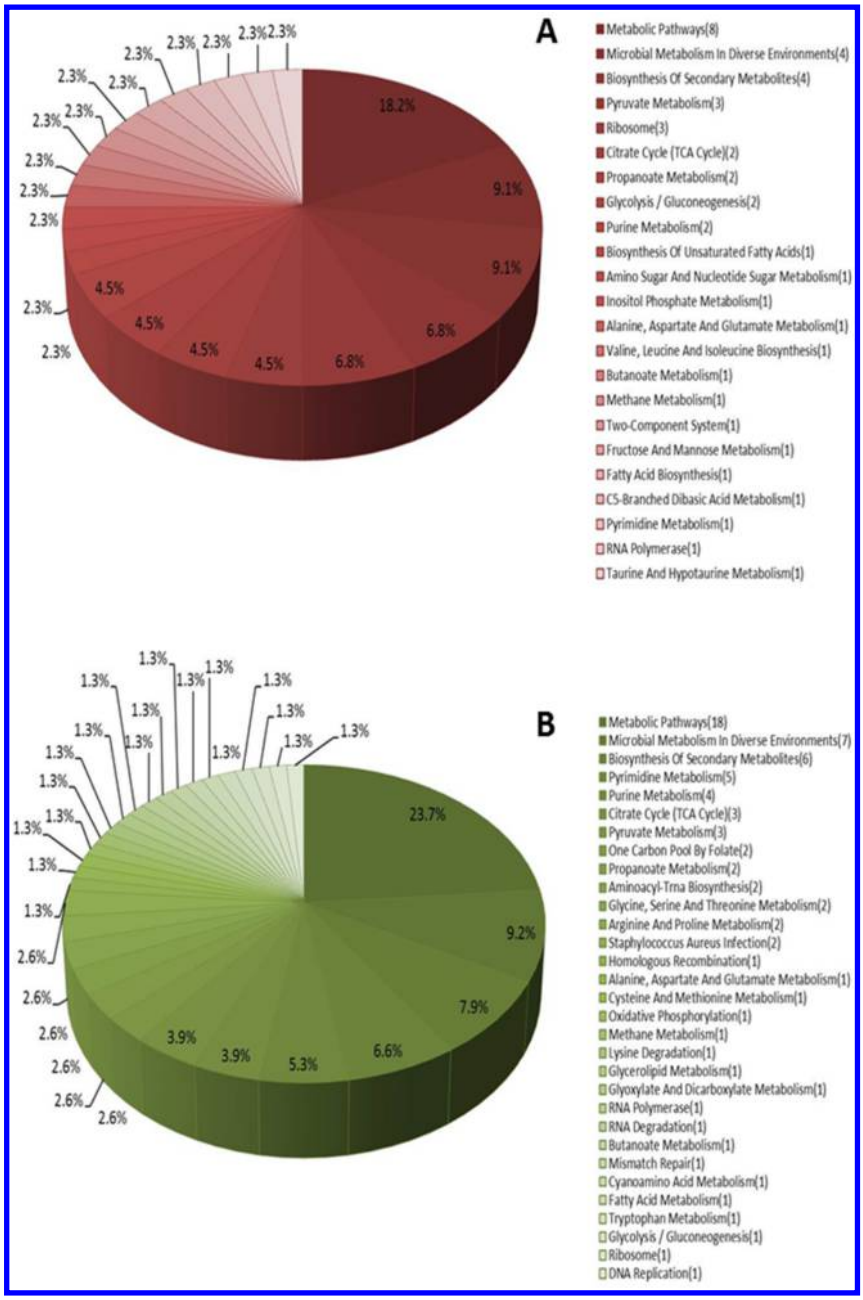

Figure 3. Distributions of the KEGG pathways that are up- (A) and downregulated (B) by **1**. The percentages indicate relative proteins among different pathway categories. The functional classes of the identified proteins were searched by using STRING (http://string-db.org) and KEGG (http://www.genome.jp/kegg).

The IgG-binding protein Sbi is a multifunctional immune evasion factor normally operative in *S. aureus*, although is downregulated with bacterial exposure to **1**. In *S. aureus* strains, Sbi is present in the culture supernatant and is also associated with the cell envelope; both of these Sbi locations contribute to immune avoidance.[35] Following cefoxitin treatment at sub-inhibitory concentrations, Sbi is measurably downregulated.[36] Since the downregulation of Sbi decreases *S. aureus* pathogenicity, Sbi is likely to be an effective protein target for antibiotic effectiveness (Figures 3B and S2A).

Biofilm formation can also be arrested indirectly by a decline in overall metabolism. Reduced ATP availability may be a target, which can be affected by F-type ATP synthase (atpD) that participates in the catalysis of ATP synthesis via oxidative phosphorylation;[37,38] interestingly, atpD is downregulated in bacteria exposed to **1**. This atpD downregulation is also observed with the antibiotic norfloxacin and salicylate.[39] Gold nanoparticles also exhibit both antibacterial and reduced ATPase activities by downregulating F_0F_1 ATP synthase levels in *Escherichia coli*.[40] The upstream metabolism involved in oxidative phosphorylation, e.g., pyruvate carboxylase (PC) that converts pyruvate into oxaloacetate and can provide a substrate for gluconeogenesis, lipogenesis, and biofilm formation,[41,42] is also downregulated by **1**.

Bacterial fatty acid synthesis is another metabolic process being actively pursued by several research groups to develop anti-staphylococcal agents. Bacterial fatty acid synthesis inhibitors such as isoniazid and triclosan have fueled the development of some promising new inhibitors through structure-based drug design that targets drug-resistant *S. aureus*

DOI: 10.1021/acs.jproteome.7b00137
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infections.[43] The acyl carrier protein transacylases (MCATs) that include fabD belong to a family of acyltransferase transferring units other than aminoacyl groups that regulate fatty acid biosynthesis (Figures S7 and S8). FabD is downregulated by **2** and **3**. MCATs are considered to be critical enzymes in bacterial fatty acid biosynthesis (FASII), generating phospholipid components, and as such are promising drug targets to block growth.[44] In fact, cerulenin, thiolactomycin, and platensin are previously identified natural products that also target FASII, and recent efforts have focused on optimizing natural and synthetic compounds to block FASII machinery.[43]

Bacteria have been observed to respond to cytotoxic agents by upregulating protein synthesis (Figure S2B);[45] protein components upregulated by **1** include 50S ribosomal protein L10 component (rplJ), an elongation factor G component (fusA), and a ribosome-recycling factor (rrf). This ribosome-recycling factor is responsible for the release of ribosomes from mRNA during protein biosynthesis termination, allowing for ribosome recycling, and increased rrf levels are associated with aberrant stationary-phase events including enhanced protein biosynthesis.[46]

To examine whether responses observed were specific to flavonoid treatment or a nonspecific response due to a general chemical perturbation, *S. aureus* AH133 was also treated with the sesquiterpene 4b,5b-epoxy-7a*H*-germacr-10(14)-en,1b-hydroperoxy-l,6b-ol (EGHP); EGHP was also isolated from *T. polium* and exhibited antibacterial activity against the development of *S. aureus* biofilms.[14] Per PCA (Figure S9), EGHP and flavonoids were clearly resolved from each other even though their chemical exposure protocols were identical. This observation suggests that each compound has variable effects on AH133. Several proteins were downregulated by EGHP (Figure S10) including arcA, dnaN, and SAOUHSC_01064, with fold changes of 91, 100 and 65, respectively (Table S4). Unlike **1**−**3**, EGHP did not show anti-biofilm activities through downregulation of fadB/SAOUHSC_00196, Sbi, dnaN, rpoZ, rpoZ, ldh2, rpoZ, atpD, SAOUHSC_00878, and fabD proteins. On this basis, the flavonoids exhibited inhibitory action against *S. aureus* through multiple protein perturbation mechanisms that are altogether diverse from those demonstrated by EGHP (Figure S11). These data support the observation that different classes of natural products exert unique inhibitory effects against *S. aureus* biofilm development.

CONCLUSION

This study identified a small subset of bacterial proteins that accumulate differentially with flavonoid treatment and can be linked to the inhibition of biofilm formation and bacterial growth. This investigation is consistent with other such proteomic studies that have utilized small molecules or natural products to identify proteins differentially produced by *S. aureus* in response to antibiotic treatment.[31,47] Future work will examine the role of other bacterial proteins differentially regulated with antibiotic flavonoid treatment.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00137.

Identified proteins with **1**−**3** versus control; protein expression in response to metabolites **1**−**3** and EGHP; hierarchical clustering of significantly altered proteins in **2** and **3** treated groups; molecular function, cellular localization, and biological process distributions with **1**; Data Network analysis and protein−protein interactions with **2** and **3**; KEGG, biological process, and cellular localization distributions with **2** and **3**; hierarchical clustering of altered proteins for **1**−**3** and EGHP; Data Network analysis of downregulated of target protein−protein interactions due to EGHP; and KEGG pathways downregulated by EGHP (PDF)

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Notes

The authors declare no competing financial interest.
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository[48] with the dataset identifier PXD006030.

ACKNOWLEDGMENTS

This research was supported in part by the Robert Welch Foundation (D-1478), NSF equipment grant CHE-1048553, and the NSF CRIF program.

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