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Brief Communication

# A Family of Pyrazinone Natural Products from a Conserved Nonribosomal Peptide Synthetase in *Staphylococcus aureus*

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## SUMMARY

**Each year in the United States, infections by methicillin-resistant *Staphylococcus aureus* (MRSA) are responsible for ~19,000 deaths and result in $3–$4 billion of health care costs. Because skin colonization is a major risk factor for *S. aureus* infection, identifying novel small molecules produced by *S. aureus* can lead to new molecular insights into its ability to colonize and infect the host and new targets for antibacterial intervention. Here, we report that a nonribosomal peptide synthetase conserved across *S. aureus* and other skin-associated staphylococci encodes a family of three pyrazinone natural products. These molecules likely result from the synthesis and release of a dipeptide aldehyde, its spontaneous cyclization to a dihydropyrazinone, and subsequent oxidation to a pyrazinone. As an unexpected family of small molecule natural products from the pathogen *S. aureus*, the pyrazinones may open a new window into the interspecies interactions that underlie the poorly understood process of skin colonization.**

## INTRODUCTION

Each year in the United States, infections by methicillin-resistant *Staphylococcus aureus* (MRSA) are responsible for ~19,000 deaths (Klevens et al., 2007) and result in billions of dollars of health care costs (Klein et al., 2007). A major risk factor for *S. aureus* infection is skin colonization (von Eiff et al., 2001). Although ~20% of people are consistently colonized by *S. aureus* and ~60% are intermittently colonized (Kluytmans et al., 1997), the molecular factors that determine whether an individual is colonized are unknown. One set of factors likely to contribute to colonization state are molecular interactions among skin-associated bacteria and between these bacteria and the host. As a component of our effort to characterize small molecules from the human microflora, we set out to identify new small molecules produced by *S. aureus* that might play a role in colonization-related interspecies interactions.

*S. aureus* produces siderophores (Beasley et al., 2009; Cotton et al., 2009) and the glycosylated terpenoid virulence factor staphyloxanthin (Pelz et al., 2005), but staphylococci are not otherwise known to produce diffusible small molecule natural products. From a bioinformatic search for biosynthetic gene clusters in the genomes of human-associated bacteria, we were intrigued to find an uncharacterized gene cluster that is conserved across all sequenced strains of *S. aureus* and two other skin-associated species, *Staphylococcus epidermidis* and *Staphylococcus capitis*, but is not found in other sequenced staphylococci.

Here, we report that this uncharacterized nonribosomal peptide gene cluster encodes a family of three pyrazinone natural products. One of these molecules, phevalin, was previously discovered from a soil actinomycete and shown to harbor protease inhibitory activity (Alvarez et al., 1995). The other two, tyrvalin and leuvalin, are new molecules. While our manuscript was under review, Magarvey and coworkers published an article reporting similar findings and extending them by showing that these small molecules regulate *S. aureus* virulence factor expression and are necessary for a productive infection in a mouse model of *S. aureus* bacteremia (Wyatt et al., 2010).

## RESULTS AND DISCUSSION

### Bioinformatic Analysis of the *pzn* Gene Cluster

Our bioinformatic search revealed that all sequenced strains of *S. aureus* harbor an uncharacterized biosynthetic gene cluster putatively encoding a nonribosomal peptide synthetase (NRPS) (Fischbach and Walsh, 2006), here termed *pzn*. Further bioinformatic analyses suggest that the first gene, *pznA*, encodes a two-module NRPS in which the first adenylation (A) domain is predicted to insert Val whereas the second is selective for Phe or Tyr. Other two-module NRPSs including the thaxtomin (Loria et al., 2008) and lyngbyatoxin (Edwards and Gerwick, 2004) synthetases are known to produce modified dipeptides. Interestingly, PznA harbors a C-terminal reductase (R) domain in place of the more common thioesterase domain, indicating a 2-electron or 4-electron mode of reductive chain release (Walsh and Fischbach, 2010), as has been observed previously in bacterial (Li et al., 2008; Read and Walsh, 2007) and fungal (Liu and Walsh, 2009) NRPS enzymes. We therefore predicted that PznA would synthesize a dipeptide alcohol or aldehyde. *pznB* encodes a

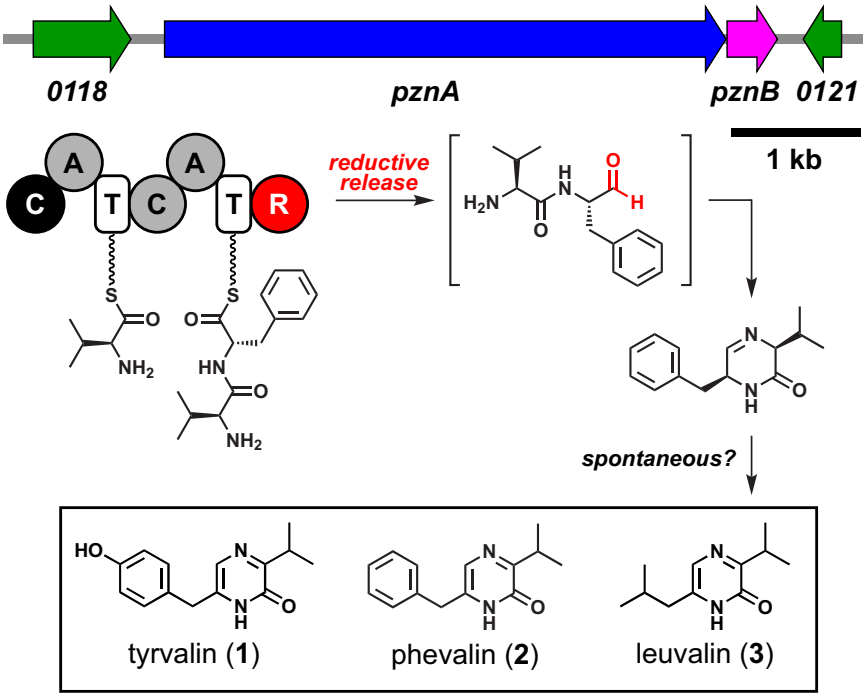


**Figure 1. Schematic of the *S. aureus* Pyrazinone (*pzn*) Gene Cluster and Proposed Biosynthetic Pathway for the *Staphylococcus* Pyrazinones**

A: adenylation domain; C: condensation domain; T: thiolation domain; R: reductase domain.

4′-phosphopantetheinyltransferase (Lambalot et al., 1996), which likely catalyzes the posttranslational priming of both thiolation domains in PznA (Figure 1). Bioinformatic analyses of the genes flanking *pznA* and *pznB* predict functions unrelated to small molecule synthesis, regulation, or transport, suggesting that *pznAB* is a self-contained two-gene cluster. The *pzn* gene cluster is found in all sequenced strains of *S. aureus*, *S. epidermidis*, *S. capitis*, and *Staphylococcus lugdunensis*, but not in other sequenced *Staphylococcus* species.

## Production and Characterization of a *pzn* Knockout Strain

To identify the product of the *pzn* gene cluster, we used an allelic replacement strategy (Arnaud et al., 2004) to generate a Δ*pznA* mutant of *S. aureus* RN4220 (Figure 2). Comparative LC-MS analysis of culture extracts from the wild-type and Δ*pznA* strains revealed three peaks that were present in the wild-type extract but not the mutant extract (Figure 3). The peaks showed similar ultraviolet/visible (UV/Vis) absorption spectra with three maxima at 204, 229, and 325 nm (Figure 3, inset B). Identical peaks were present in culture extracts from two *S. epidermidis* strains that harbor the *pzn* gene cluster, American Type Culture Collection (ATCC) 12228 and ATCC 35984, but not *Staphylococcus saprophyticus* S-41, which does not harbor the *pzn* cluster (Figure 3). Growth curves of the wild-type and Δ*pznA* strains were comparable, suggesting that cell growth and viability are not directly affected by the loss of the *pzn* gene cluster (data not shown). The time course of the phevalin production (Figure 3, inset A) links the biosynthetic activity of the *pzn*-encoded enzymes to the time period between 24 and 48 hr of batch cultivation, which corresponds to the stationary growth phase under the culture conditions we used for small molecule production.

## Isolation and Structure Elucidation of the *Staphylococcus* pyrazinones

We cultured *S. aureus* in a minimal medium, modified MSgg (Branda et al., 2001), and purified multimilligram quantities of each compound by preparative HPLC; the yields of each compound were 1 mg/L for phevalin and tyrvalin and 0.3 mg/L for leuvalin. Using a combination of high-resolution mass spectrometry (MS) and two-dimensional nuclear magnetic resonance (2D NMR) experiments (Table 1; see Table S1 and Figures S1–S4 available online), the *pzn*-encoded natural products were determined to be a family of three pyrazinones—tyrvalin (**1**), phevalin (**2**), and leuvalin (**3**)—that differ in the identity of one of their constituent amino acid side chains (Figure 1; Figures S1–S4). Interestingly, **2** was previously isolated from *Streptomyces sp*. SC433, a free-living bacterium unrelated to *Staphylococcus* that was obtained from a soil sample collected in Taiwan (Alvarez et al., 1995). **1** and **3** have not previously been described. To directly compare the chemical structure of **2** to the reported compound its $^{1}$H-NMR spectrum was recorded under comparable experimental conditions using $CDCl_3$, as described by Alvarez et al. (1995) (Table S2).

The structures of **1**–**3** are consistent with the proposed biosynthetic scheme shown in Figure 1. It is often possible to predict the structure and biosynthetic route of the small molecule product of an NRPS from the order of its constituent domains (Fischbach and Walsh, 2006). Our bioinformatic analysis of the domain structure of PznA is consistent with the following predicted pathway: the two NRPS modules of PznA would generate a nascent dipeptide, which would undergo two-electron reductive release by the R domain. The C-terminal aldehyde would likely condense with the N-terminal amino group spontaneously, yielding a 6-membered dihydropyrazinone intermediate that would undergo a two-electron oxidation to become **1**–**3** (Figure 1). Given the absence of additional oxidoreductase-encoding genes in the *pzn* gene cluster, we presume that this final 2-electron oxidation is either spontaneous (assisted by the driving force of aromatization) or is catalyzed by the PznA R domain operating in reverse as an oxidoreductase/dehydrogenase. However, we cannot rule out the participation of an oxidase encoded elsewhere in the genome.

## Insights into *Staphylococcus* Pyrazinone Biosynthesis from Feeding Studies

*S. aureus* produces **1** and **2** in a ~1:1 ratio, with **3** as a minor product (Figure 3). Although the production of low-level side

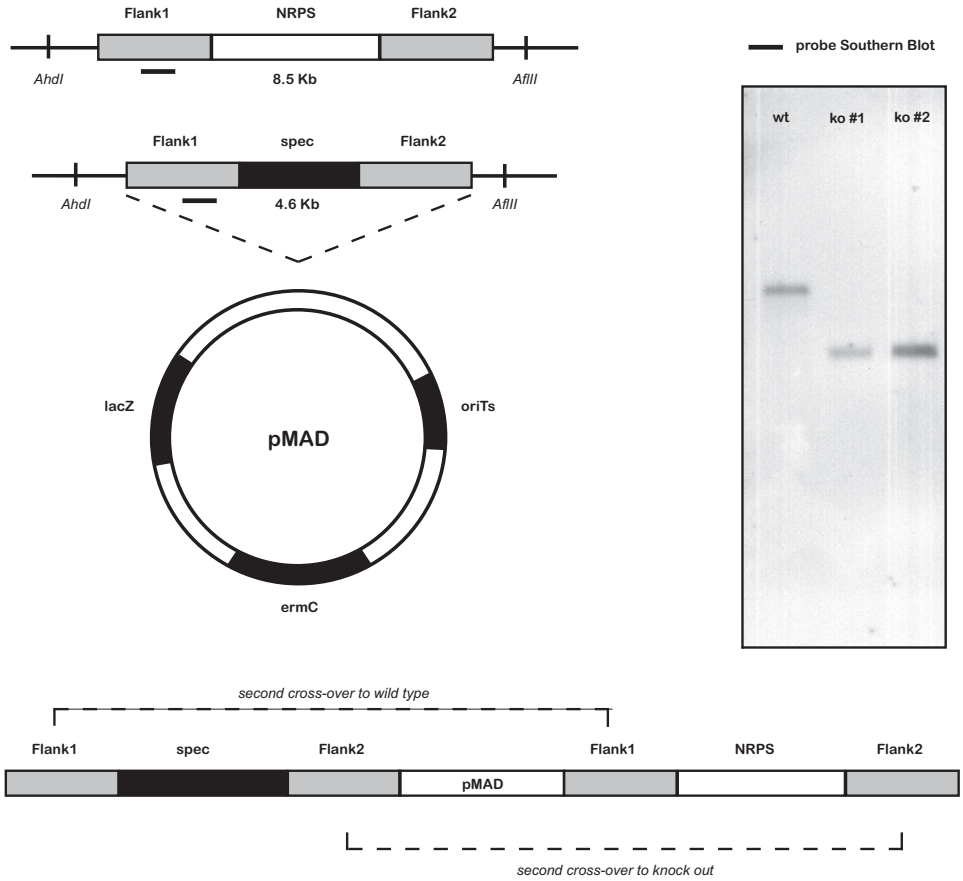


**Figure 2. Strategy for the Allelic Replacement of *pznA* in *S. aureus* RN4220**

After integration of the plasmid by the first genomic crossover event, the second crossover can either lead to the desired knockout strain or reversion to the wild-type strain. Successful allelic replacement is demonstrated by Southern blot. Wt corresponds to the wild-type strain of *S. aureus* RN4220. Ko#1 and #2 correspond to independently generated and selected knockout strains for the NRPS gene *pznA*. Scheme adapted from Horsburgh et al. (1999).

products is common in NRPS systems, the production of two major products is unusual, so we investigated their biosynthetic origins. Such a mixture of products could derive from the relaxed substrate specificity of the second adenylation domain in PznA, or it could result from the partial post-release hydroxylation of **2** to **1** by an unidentified oxygenase. To distinguish between these possibilities, we carried out feeding experiments with $^{2}$H-labeled amino acids to track their incorporation into **1**–**3**. Supplementation of modified MSgg and subsequent LC-MS analysis of culture extracts revealed roughly equivalent levels of incorporation of [ring-$D_5$]-L-phenylalanine and [ring-3,5-$D_2$]-L-tyrosine, supporting A domain promiscuity as the explanation for the coproduction of **1** and **2**. Interestingly, *S. epidermidis* ATCC 35984 produces more **1** than **2**, suggesting that the degree of PznA-A2 promiscuity may vary between species of *Staphylococcus*. A domain promiscuity could also explain the fact that **3** is produced at a lower level than **1** and **2**, because Leu would presumably be accepted at lower efficiency than Phe and Tyr.

## Initial Efforts to Characterize the Biological Activities of the *Staphylococcus* Pyrazinones

As cyclized dipeptide natural products, **1**–**3** are relatives of diketopiperazines (DKPs), a large and diverse family of natural products with a wide range of biological activities (Holden et al., 1999; Niku-Paavola et al., 1999; Strom et al., 2002). Because many DKPs, including the structurally related (but biosynthetically unrelated) molecule albonoursin, (Lautru et al., 2002) have antibacterial activity, we assayed **1** against *Escherichia coli*, *Bacillus subtilis*, and the skin-associated actinomycetes *Propionibacterium acnes*, *Corynebacterium accolens*, *Corynebacterium jeikeium*, and *Corynebacterium urealyticum*. However, we observed no growth inhibition, suggesting that the *Staphylococcus* pyrazinones have a distinct activity. The recent report from Magarvey and coworkers suggests that these compounds play a role in regulating *S. aureus* virulence factor expression (Wyatt et al., 2010). Notably, **2** was originally isolated from a screen for calpain inhibitors (Alvarez et al., 1995), indicating that one of the biologically relevant activities of **1**–**3** could be protease inhibition. However, the modest activity of **2** against calpain ($IC_{50}$ = 1.3 μm) suggests that if **2** is indeed a protease inhibitor, its primary target may be a different protease. Given that the *pzn* gene cluster is only found in skin-associated staphylococci and host proteases play an important role in the skin barrier function (Leyvraz et al., 2005), characterizing the protease inhibitory activity of the *Staphylococcus* pyrazinones will be one starting point for

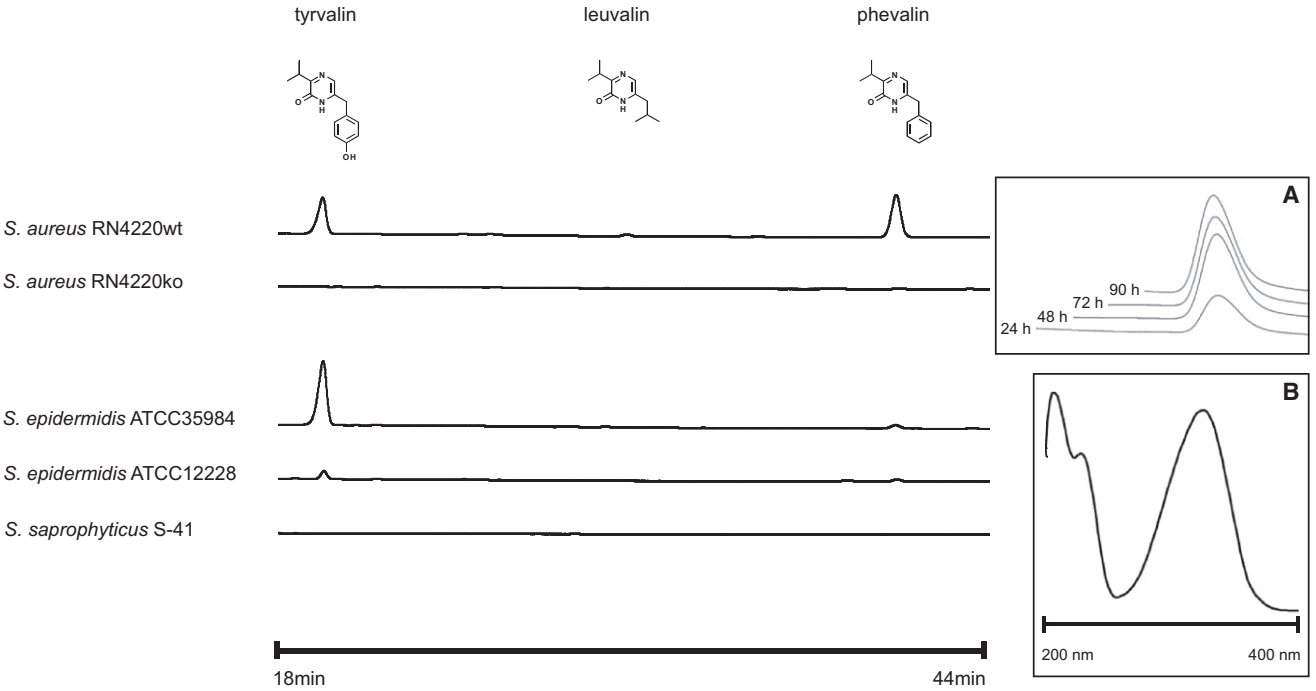


**Figure 3. Comparison of Pyrazinone Production across *Staphylococcus* Species**

RP-HPLC analysis (325 nm) of methanol extracts of culture supernatants after 48 hr are shown. RN42220ko corresponds to the Δ*pznA S. aureus* strain. Both *S. epidermidis* strains harbor the pzn gene cluster, whereas *S. saprophyticus* S-41 does not. Inset A: Time course for the production of phevalin by *S. aureus* RN4220wt. Inset B: Ultraviolet/visible absorption spectrum of phevalin.

future efforts to investigate the role of **1–3** in colonization-related interspecies interactions.

## SIGNIFICANCE

**Our results demonstrate the power of genome mining (Challis, 2008; McAlpine, 2009) for the discovery of natural products from human-associated bacteria. Using this approach, we identified an NRPS-encoding gene cluster conserved across all sequenced *S. aureus* strains. A combination of bacterial genetics and chemical analysis revealed that this gene cluster encodes three pyrazinone natural products, one of which was previously discovered from a soil actinomycete and characterized as a protease inhibitor, whereas the others are novel molecules. These molecules do not have detectable antibacterial activity; discovering the biological activities of these molecules could lend insights into the role of chemical interactions between *S. aureus* and the host—or other skin microbes—in the process of skin colonization.**

## EXPERIMENTAL PROCEDURES

### Production and Characterization of *S. aureus* Knockout Strain

Plasmid pMAD was used to construct the Δ*pznA* strain of *S. aureus* as previously described (Arnaud et al., 2004). Genomic DNA of *S. aureus* strain RN4220 was isolated by phenol/chloroform extraction. Briefly, tryptic soy broth (TSB; Difco) was inoculated 1:100 with an overnight culture of *S. aureus* RN4220 in the same culture medium. After 5 hr of incubation at 37°C with shaking at 220 rpm, bacterial cells were pelleted from a 5 ml culture aliquot and incubated in 700 μl lysis buffer (50 mM EDTA, pH 8; 100 mM NaCl) supplemented with 0.3 mg/mL RNase A (Invitrogen), 2.5 mg/mL lysozyme from chicken egg white (Sigma), and 0.2 mg/mL proteinase K (Invitrogen). After incubating for 10 min at 37°C, 0.15 volumes of sodium N-lauroylsarcosine

**Table 1. Biosynthetic Incorporation of Isotopically-Labeled Amino Acids**

| Compound | Calculated Mass | HR-MS | L-Val (D8)[a] | L-Tyr (Ring-3,5-D2) | L-Phe (Ring-D5) | L-Leu (5,5,5-D3) |
|---|---|---|---|---|---|---|
| Tyrvalin (**1**) | 245.1285 | 245.1234 | 252.4 (+7) | 247.4 (+2) | | |
| Phevalin (**2**) | 229.1335 | 229.1329 | 236.4 (+7) | | 234.4 (+5) | |
| Leuvalin (**3**) | 195.1497 | 195.1490 | 202.4 (+7) | | | 198.4 (+3) |

HR-MS: High resolution mass spectra. Incorporation of amino acid precursors into *Staphylococcus* pyrazinones. HR-MS were recorded by Fourier transform mass spectrometry and the theoretical masses were calculated based on the molecular formula. The values in brackets indicate the gain of mass caused by the corresponding deuterated amino acid. All results are indicated in daltons. All values were recorded in positive ionization mode and represent $(M + H)^+$.

[a] α-Carbon labeling, proton lost during aromatization.

(20%) (Sigma) were added and two phenol/chloroform extractions were carried out followed by an ethanol precipitation of the DNA after acidification with 0.1 volumes of sodium acetate (3 M, pH 5.5).

DNA fragments corresponding to the 3′ and 5′ region of *pznA* (nt: 9–1005 and 6136–7130, respectively) were polymerase chain reaction (PCR)-amplified using Taq polymerase (Roche) with the primer pairs MZ-021/022 and MZ-023/024, respectively (Table S3). After treatment with the restriction enzymes (NEB) indicated in Table S3, the two fragments were sequentially inserted into pMAD between the restriction sites BamHI/EcoRI and EcoRI/BglII, respectively. Finally, a 1.3 kb DNA fragment bearing the spectinomycin resistance gene was amplified from plasmid pKM079 (provided by David Rudner, Harvard Medical School) with the primer pair MZ-019/020 (Table S3) and introduced into EcoRI between the two fragments of *pznA* (Figure 2).

Allelic replacement was verified by PCR amplification of the crossover junctions (primer pairs MZ-019/034 and MZ-032/033; Table S3), confirmation of susceptibility to erythromycin and resistance to spectinomycin, blue-white screening (β-galactosidase assay with bromo-chloro-indolyl-galactopyranoside), and Southern blotting.

For Southern blotting, genomic DNA of *S. aureus* RN4220wt, RN4220ko#1 and RN4220ko#2 (two independently generated knockout strains) was isolated as described above. A total of 750 ng of DNA was digested overnight by *Afl*II and *Ahd*I (NEB) at 37°C in a total reaction volume of 30 μL. The resulting fragments were separated by agarose gel (1%) electrophoresis (1 × TAE), denatured by alkaline treatment (0.4 N NaOH; 0.6 M NaCl; regeneration in 1.5 M NaCl; 0.5 Tris-HCl, pH 7.5), and subsequently transferred onto a nylon membrane (Roche Diagnostics) with 20 × SCS (3 M NaCl; 0.3 M sodium citrate). Fixation was carried out by UV-cross-linking. A digoxigenin (DIG)-labeled probe was produced by random PCR priming (PCR DIG probe synthesis kit; Roche Diagnostics) of a 291 nt-long PCR-product (primer pair MZ047/048; Table S3), specific for the upstream flanking region of the DNA construct for the allelic replacement (Figure 2). For hybridization and immunodetection the DIG Easy Hyb wash and block buffer set and the CDP-Star solution (Roche Diagnostics) were used according to the manufacturer's protocol.

### Metabolic Comparison of *S. aureus* WT and KO and other *Staphylococcus* Strains

A total of 150 ml of TSB was inoculated with a single colony of either the wild-type or Δ*pznA* strain of *S. aureus* RN4220. After 24, 48, 72, and 90 hr, a 25 ml aliquot was centrifuged at 5000 × g for 30 min, and the resulting pellet and supernatant were lyophilized. The dried samples were extracted by methanol, filtered, evaporated under vacuum and redissolved in 1 ml methanol. One hundred microliters of this sample was analyzed by RP-HPLC (System Gold, Beckman) on a C18(2) Luna 5 μm 100A 250 × 10.00 mm column (Phenomenex) with a linear gradient from 20% to 40% solvent B (acetonitrile) in solvent A (0.1% trifluoroacetic acid/water) over 50 min, with a flow rate of 3.5 mL/min, monitored at 200–600 nm (Figure 3). *S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 35984, and *S. saprophyticus* S41 were obtained from the ATCC and analyzed in the same manner as *S. aureus* (Figure 3).

### Isolation and Structure Elucidation of the *Staphylococcus* Pyrazinones

TSB (4 × 1 L) was inoculated with 1 ml of an overnight culture (in TSB) of *S. aureus* RN4220 WT and incubated at 37°C and 220 RPM for 48 hr. The culture supernatant was subjected to solid-phase extraction using four 10-g C18 SepPak columns (Waters). The columns were washed with two column volumes (CVs) of water and bound molecules were eluted with three CVs of 60% solvent B (acetonitrile) in solvent A (0.1% trifluoroacetic acid/water). The eluted fraction was lyophilized, resolubilized in methanol, and chromatographed using the RP-HPLC conditions described above. The peak at 42 min corresponding to **2** was collected (Figure 3), lyophilized, and further purified by the following isocratic RP-HPLC conditions: C18(2) Luna 5 μm 100A 250 × 10.00 mm column (Phenomenex), 28% solvent B (acetonitrile) in solvent A (0.1% trifluoroacetic acid/water), flow rate 3.5 mL/min.

For the production of **1** and **3**, the biomass from TSB cultures was resuspended in 0.5 volumes of modified minimal medium MSgg (100 mM MOPS, pH 7.0; 0.5% L-glutamate; 0.5% glycerol; 2.5 mM $KH_2PO_4$; 2.5 $K_2HPO_4$; 50 μM $FeCl_3$; 2 mM $MgCl_2$; 50 μM $MnCl_2$; 1 μM $ZnSO_4$; 0.7 mM $CaCl_2$; 2 μM thiamine), supplemented with valine and either tyrosine or leucine (each at 1 mg/mL). After incubation for 36 hr at 37°C and 220 rpm the culture supernatant was extracted three times with 0.6 volumes of ethyl acetate. The solvent was evaporated under vacuum and the dry extract was resolubilized in methanol for further separation by RP-HPLC as described above. The peaks at 22 and 32 min, corresponding to **1** and **3**, respectively, were collected (Figure 3). The leuvalin structure (**3**) was further purified using the same protocol as **2**, whereas the contents of acetonitrile was decreased to 20% for the isocratic purification of the more polar **1**.

High resolution masses of the isolated compounds were recorded by FT-MS in the mass spectroscopy service lab of the Keck Foundation at Yale University. $H^1$-NMR spectra and 2D-experiments (gCOSY, gHSQC, and gHMBC) were recorded following the standard protocols on a Varian 600 MHz NMR spectrometer. Data analysis was carried out with the VNMRJ software suite. DMSO-$d_6$ and $CDCl_3$ (Cambridge Isotope Laboratories) were used as solvents.

### Biosynthetic Analysis of the *Staphylococcus* Pyrazinones

The protocol described for the production of the *Staphylococcus* pyrazinones in modified MSgg medium was applied for their labeling with stable isotopes. L-valine ($D_8$), L-phenylalanine (Ring-$D_5$), L-tyrosine (Ring-3,5-$D_2$), and L-leucine were purchased from Cambridge Isotope Laboratories. The total culture volume was 100 ml and the compounds were extracted as described above. The isolated peaks were analyzed by LC-MS (1100 Series Agilent and esquire 3000 plus Bruker). The used column was a ZOROBAX 3.5 μm Extend C18 2.1 × 50 mm (Agilent). A linear gradient from 1% to 99% acetonitrile with 0.1% formic acid was developed over 20 min. The chromatography was monitored at 325 nm and masses between 50 and 2000 Da were recorded.

### Bioactivity Assay

*Corynebacterium accolens* ATCC 49725 and *Corynebacterium urealyticum* ATCC 43042 were obtained from the ATCC, *Corynebacterium jeikeium* NCTC 11915 from the National Collection of Type Cultures (NCTC) and *Propionibacterium acnes* (*P. acnes*) DSM 16379 from the German Collection of Microorganisms and Cell Cultures (DSMZ). *S. aureus* UAMS1 was kindly provided by Katherine Lemon and Roberto Kolter (Harvard Medical School). *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) were from lab stocks. *Staphylococcus* spp., *E. coli*, and *B. subtilis* were cultured in TSB. *Corynebacterium* spp. and *P. acnes* were cultured in Brain Heart Infusion supplemented with 1% Tween 80 (BHIT80).

Ten microliters of a stock solution of tyrvalin in methanol at a concentration of 2.8 mg/mL was spotted on standard filter discs for antibiotic inhibition assays (Waters). Erythromycin (1 mg/mL) in methanol served as a positive control, and pure methanol was used as a negative control.

The tested strains were grown as overnight cultures at 37°C in liquid culture media (the corresponding solid growth medium without the addition of agar). *P. acnes* was grown anaerobically for 5 days. All cultures were diluted to $10^{-4}$ and 100 μl were streaked on solid culture media with the aid of 3 mm glass beads (Fisher brand). The air-dried filter papers were transferred on top of the bacterial lawn, followed by an incubation at 37°C and (under anaerobic conditions for *P. acnes* using a glove box by Coy Laboratories, MI).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three tables, and five figures, and can be found with this article online at doi:10.1016/j.chembiol.2010.08.006.

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