

Propionibacterium-Produced Coproporphyrin III Induces *Staphylococcus aureus* Aggregation and Biofilm Formation

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ABSTRACT The majority of bacteria detected in the nostril microbiota of most healthy adults belong to three genera: *Propionibacterium*, *Corynebacterium*, and *Staphylococcus*. Among these staphylococci is the medically important bacterium *Staphylococcus aureus*. Almost nothing is known about interspecies interactions among bacteria in the nostrils. We observed that crude extracts of cell-free conditioned medium from *Propionibacterium* spp. induce *S. aureus* aggregation in culture. Bioassay-guided fractionation implicated coproporphyrin III (CIII), the most abundant extracellular porphyrin produced by human-associated *Propionibacterium* spp., as a cause of *S. aureus* aggregation. This aggregation response depended on the CIII dose and occurred during early stationary-phase growth, and a low pH (~4 to 6) was necessary but was not sufficient for its induction. Additionally, CIII induced plasma-independent *S. aureus* biofilm development on an abiotic surface in multiple *S. aureus* strains. In strain UAMS-1, CIII stimulation of biofilm depended on *sarA*, a key biofilm regulator. This study is one of the first demonstrations of a small-molecule-mediated interaction among medically relevant members of the nostril microbiota and the first description of a role for CIII in bacterial interspecies interactions. Our results indicate that CIII may be an important mediator of *S. aureus* aggregation and/or biofilm formation in the nostril or other sites inhabited by *Propionibacterium* spp. and *S. aureus*.

IMPORTANCE Very little is known about interspecies interactions among the bacteria that inhabit the adult nostril, including *Staphylococcus aureus*, a potential pathogen that colonizes about a quarter of adults. We demonstrated that coproporphyrin III (CIII), a diffusible small molecule excreted by nostril- and skin-associated *Propionibacterium* spp., induces *S. aureus* aggregation in a manner dependent on dose, growth phase, and pH. CIII also induces *S. aureus* to form a plasma-independent surface-attached biofilm. This report is the first description of a role for CIII in bacterial interspecies interactions at any human body site and a novel demonstration that nostril microbiota physiology is influenced by small-molecule-mediated interactions.

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The pathobiont (1) *Staphylococcus aureus* resides primarily in human nostrils and colonizes at least a quarter of the U.S. population (2, 3). (A pathobiont is a member of the microbiota with the capacity to cause significant disease; this distinguishes it from benign or beneficial members lacking virulence potential.) The nostrils (also known as anterior nares) are the openings into the nasal vestibules, which are covered by skin that harbors sweat and pilosebaceous glands and exhibit a characteristically acidic pH (4) (Fig. 1). Henceforth, we refer to the nostrils and nasal vestibules simply as “nostrils.” In contrast to the nostrils, the epithelium of the more posterior nasal cavity, beginning at the limen nasi, produces mucus and has a pH close to neutral (5). Within the body, the nostrils are a unique environment with distinct exposures, environmental characteristics, and microbiota (6–11).

Although generally harmless, *S. aureus* nostril colonization is

associated with an increased risk of *S. aureus* infection (12–15). An estimated 80% of *S. aureus* bloodstream isolates match the patient's nostril strain (16, 17). The true global burden of *S. aureus* infections, which range in severity from mild skin infection to life-threatening invasive disease (e.g., bacteremia, endocarditis, and pneumonia), is unknown. However, from 2005 to 2011, methicillin-resistant *S. aureus* (MRSA) alone is estimated to have caused over 80,000 cases of invasive disease and over 10,000 deaths yearly in the United States (18). To date, *S. aureus* has eluded repeated efforts at vaccine development (19, 20). Thus, a deeper understanding of *S. aureus* and its membership in human nostril microbiota is an important public health issue.

Within the nostril ecosystem, *S. aureus* is part of a mixed-species microbial community, with three genera accounting for the majority of bacteria detected in most adults: *Staphylococcus*,

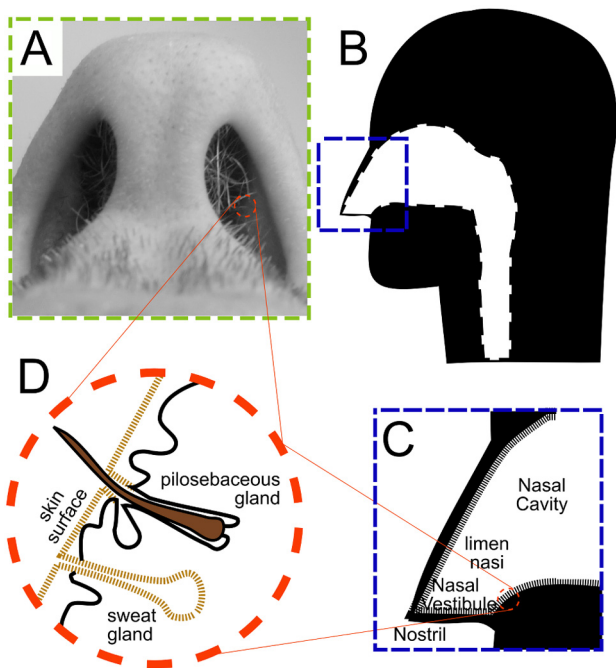


FIG 1 The nostrils (anterior nares) open onto the nasal vestibules, which are lined by skin, complete with sweat and pilosebaceous glands. (A) Inferior view of the nostrils and nasal vestibules; (B) sagittal illustration of the nasal cavity; (C) the limen nasi forms a boundary between the nasal vestibule and posterior aspects of the nasal cavity, which have a mucosal epithelial lining; (D) the nasal vestibule epithelium (skin) contains sweat and pilosebaceous glands and does not secrete mucus.

Corynebacterium, and *Propionibacterium* (6–11). Few studies have examined nostril-associated *Corynebacterium* and *Propionibacterium*. In particular, there is a surprising lack of data about the interactions that can occur among members of these three genera and how these impact the growth and behavior of *S. aureus*.

We hypothesized that *Propionibacterium* spp. produce small molecules that mediate interspecies interactions with other

nostril-associated bacteria. Due to its medical importance, we selected *S. aureus* as a candidate interactor. We identified coproporphyrin III (CIII) as an abundant *S. aureus* aggregation-inducing molecule in *Propionibacterium* conditioned medium (CM) and characterized this response. Previous studies establish that CIII is the most abundant porphyrin detected in sebaceous material from human skin, supporting its presence on nostril surfaces (21, 22). Porphyrins include a range of biologically important molecules (e.g., chlorophyll, vitamin B₁₂, hemoglobin), and the porphyrin biosynthetic pathway is highly conserved (23–25) (see Fig. S1 in the supplemental material, adapted from reference 26). Nevertheless, the biological function(s) of some porphyrins, e.g., coproporphyrins, are poorly understood. This work represents the application of an experimental hypothesis generated from microbiome enumeration data to novel observations of the physiology and molecular biology of a clinically relevant bacterium, *S. aureus*.

RESULTS

***Propionibacterium* spp. induce *S. aureus* coaggregation via extracellular CIII.** Members of the *Actinobacteria*, which includes *Propionibacterium*, are well-known producers of bioactive small molecules (27, 28). We hypothesized that cutaneous *Propionibacterium* spp. produce extracellular small molecules affecting *S. aureus*. The significant difference in the rate of growth to stationary phase (1 day for *S. aureus* compared to over 5 days for *Propionibacterium* spp.) limited cocultivation in liquid. Therefore, to test this hypothesis, a crude extract of conditioned medium (CM) from the genome-sequenced strain *Propionibacterium acnes* KPA171202 was concentrated, lyophilized, and resolubilized in dimethylformamide (DMF). This CM extract was added to cultures of two different *S. aureus* strains: a commonly studied clinical isolate, UAMS-1, and a primary nostril isolate, KPL1845 (Table 1). The addition of *P. acnes* KPA171202 CM extract resulted in aggregation of both strains, yielding a significantly lower aggregation ratio (after 390 min) than that of a DMF-only control (Fig. 2A; two-tailed, paired *t* test, $P < 0.001$). (Aggregation ratio is the optical density at 600 nm [OD₆₀₀] of a standing liquid culture at a given time posttreatment addition divided by the original

TABLE 1 Bacteria used in this study, isolation source, and genotypes

Genus and species	Strain/isolate name	Source (human)	Genotype	Reference
<i>Corynebacterium accolens</i>	DSM44278	Cervix	NA ^a	[52]
<i>C. accolens</i>	KPL1855	Nostril	NA	This study
<i>Escherichia coli</i>	MG1655	Feces	NA	[53]
<i>Propionibacterium acnes</i>	KPA171202 (DSM16379)	Skin	NA	[45]
<i>P. acnes</i>	KPL1849	Nostril	NA	This study
<i>Propionibacterium avidum</i>	DSM4901	Feces	NA	[54]
<i>Propionibacterium granulosum</i>	DSM20700	Unknown	NA	[55]
<i>P. granulosum</i>	KPL1844	Nostril	NA	This study
<i>Staphylococcus aureus</i>	UAMS-1	Osteomyelitis	NA	[56]
<i>S. aureus</i>	UAMS-929	NA	<i>sarA::tet</i>	[57]
<i>S. aureus</i>	SC-01 (UAMS-732)	Human	NA	[58, 59]
<i>S. aureus</i>	Newman	Unknown	NA	[60]
<i>S. aureus</i>	HG003	Sepsis	RN1 derivative; <i>rsbU tcaR</i> repaired	[61]
<i>S. aureus</i>	KPL1845	Nostril	NA	This study
<i>S. aureus</i>	JE2	Skin/soft tissue infection	USA300 LAC without p01 and p03	[62]
<i>Staphylococcus epidermidis</i>	DSM20044	Nostril	NA	[63]
<i>S. epidermidis</i>	KPL1815	Nostril	NA	This study

^a NA, not applicable.

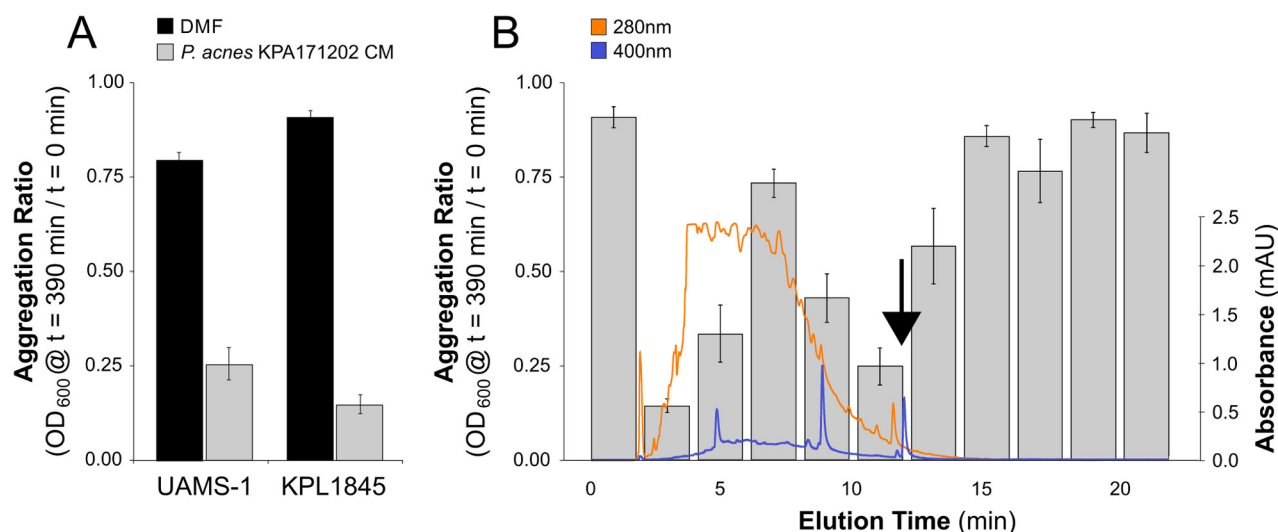


FIG 2 Extracts of *P. acnes* KPA171202 conditioned medium (CM) induce *S. aureus* aggregation. (A) Addition of a crude extract of *P. acnes* KPA171202 CM to stationary-phase cultures of *S. aureus* grown in SSD0Fe induced aggregation; (B) HPLC fractions (denoted by elution time) of *P. acnes* KPA171202 CM were added to *S. aureus* KPL1845 cultures. The 400-nm (blue line) and 280-nm (orange line) absorbance during HPLC elution are shown. The black arrow indicates the 400-nm-absorbing HPLC peak included in the 10- to 12-min fraction. Histogram bars represent the mean *S. aureus* aggregation ratio for each elution fraction or sample. All data are from five independent experiments; error bars are standard errors of the means (SEM).

OD₆₀₀ of that culture immediately before treatment addition [$t = 0$]). These data indicate that *P. acnes* KPA171202 secretes into the environment an active principle that induces *S. aureus* aggregation.

To identify the small molecule(s) that induced *S. aureus* aggregation, we fractionated the crude *P. acnes* KPA171202 CM extract by preparative high-performance liquid chromatography (HPLC). Testing each 2-min fraction for induction of *S. aureus* KPL1845 aggregation revealed several active fractions (Fig. 2B). The 10- to 12-min fraction was notable for its strong aggregation-inducing activity and its markedly lower chemical complexity than that of earlier-eluting active fractions. Examination of the different absorption spectra within this 10- to 12-min fraction revealed a relatively abundant peak with an absorption maximum at 400 nm that eluted at the end of the 10- to 12-min fraction, tailing into the less-active 12- to 14-min fraction (Fig. 2B, arrow).

Since porphyrins are well known for having an absorption maximum at 400 nm, we next investigated the potential involvement of extracellular porphyrins as a mediator of the *Propionibacterium*-produced *S. aureus* aggregation-inducing activity. To this end, we performed an additional analysis using a primary nostril isolate of *Propionibacterium* (KPL1844) putatively identified to the species level as *Propionibacterium granulosum* and isolated from the same nostril as *S. aureus* KPL1845. In an effort to enhance the amount of extracellular porphyrins, we added 5-aminolevulinic acid (5-ALA) to the medium; 5-ALA is the first committed precursor in porphyrin biosynthesis (reviewed in reference 26; see also Fig. S1 in the supplemental material) and is known to increase extracellular porphyrin production by *Propionibacterium* spp. (29). The crude CM extract of *Propionibacterium* sp. KPL1844 grown in the presence of 5-ALA also induced aggregation of both *S. aureus* KPL1845 and UAMS-1 (see Fig. S2A in the supplemental material). When this extract was fractionated and assayed, as described above, the 10- to 12-min fraction again exhibited strong aggregation-inducing activity and harbored a

peak with an absorption maximum at 400 nm (see Fig. S2B). Comparison of the absorption spectra from this fractionation with that from *P. acnes* KPA171202 revealed that the 400-nm-absorbing peak was one of the most abundant common peaks in the 10- to 12-min fractions. All these data led us to hypothesize that a *Propionibacterium*-produced extracellular porphyrin might mediate *S. aureus* aggregation activity.

To determine if the compound with an absorption maximum at 400 nm in the 10- to 12-min fractions was a porphyrin, we developed an analytical HPLC method for separation of four commonly occurring porphyrins (see Fig. S3A in the supplemental material). This method was optimized to enable the separation of the regioisomers CIII and CI, as a previous study reported that CIII and CI are the most abundant porphyrins present in 403-nm-absorbing *P. acnes* and *P. granulosum* CM extracts (29). The 10- to 12-min *P. acnes* KPA171202 fraction was analyzed with this HPLC method and found to contain a doublet of peaks with absorption maxima at 400 nm with the same retention time as that of CI and CIII (see Fig. S3B). These peaks were consistent with CI/CIII by mass spectrometry analysis (observed $[M + H]^+ = 655.2$; monoisotopic molecular weight for CI/CIII = 654.27; see Fig. S3C). To determine which regioisomer was most abundantly produced by representatives of common nostril bacteria, we used the analytical HPLC method to separate and quantify extracellular CI and CIII from crude extracts of cell-free CM (CFCM) of representative strains of nostril bacteria. Production of extracellular CIII per milligram of dry cell weight exceeded CI levels at least 3-fold for all analyzed strains (Fig. 3, gray [CIII] and black [CI] bars). *Propionibacterium* spp. (*P. acnes* and *P. granulosum*) were the most prominent coporphyrin producers, yielding amounts that were at least 2-fold greater than those produced by *Corynebacterium accolens* (Fig. 3). Although no CI production was detected for the *Staphylococcus* spp. (*S. aureus* and *Staphylococcus epidermidis*), they did produce low levels of CIII (ranging from

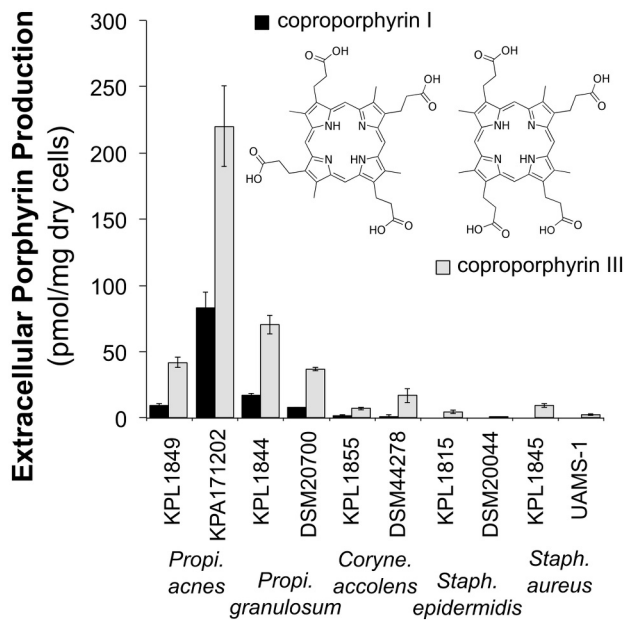


FIG 3 Production of extracellular coproporphyrin I and III by primary nostril isolates (KPL) and standard strains (inset, chemical structures). Extracellular coproporphyrin production estimated from HPLC fractionations. Identification to the species level of the primary nostril isolates (KPL1815, KPL1844, KPL1845, KPL1849, and KPL1855) is described in Text S1, Table S1, and Fig. S7 in the supplemental material. Histogram bars represent the means from three biological replicates; error bars are SEM.

0.7 to 9.4 pmol/mg dry cells, Fig. 3). High-level production of extracellular CIII was specific to *Propionibacterium* spp.

Purified exogenous CIII induces *S. aureus* aggregation in a dose-dependent manner. We proceeded to focus on determining the effect of CIII on *S. aureus* for two reasons. First, the above-described data indicated that CIII is the most abundant coproporphyrin produced by cutaneous *Propionibacterium* spp. Second, CIII is the most abundant porphyrin detected in material from pilosebaceous glands on human skin (21, 22), which indicates that CIII is likely the most abundant porphyrin on skin, including in the nostrils.

Addition of authentic CIII at 50 μ M induced aggregation of both *S. aureus* KPL1845 and UAMS-1 (Fig. 4A, gray symbols) compared to that of a solvent-only control (Fig. 4A, black symbols). A solution of 50 μ M CIII in DMF is colored pink; 4 h after adding CIII to our *S. aureus* aggregation assay, the majority of the pink coloration was associated with aggregated cells at the bottom of the cuvette, leaving the medium clear. From this observation, we hypothesized that *S. aureus* coaggregates with CIII. Microscopic examination of *S. aureus* UAMS-1 cells showed groups of two and four cells in DMF-treated culture (Fig. 4B), in contrast to larger multicellular aggregates in the presence of CIII (Fig. 4C). *S. aureus*-CIII coaggregation was dose dependent in terms of both the rate and total amount of aggregation (see Fig. S4 in the supplemental material). In addition to UAMS-1 and KPL1845, we tested other *S. aureus* strains (e.g., Newman); however, the aggregation assay was unable to detect increased aggregation in the presence of CIII for these strains due to a high level of self-aggregation in the presence of solvent alone.

High concentrations of some porphyrins (e.g., heme) are toxic

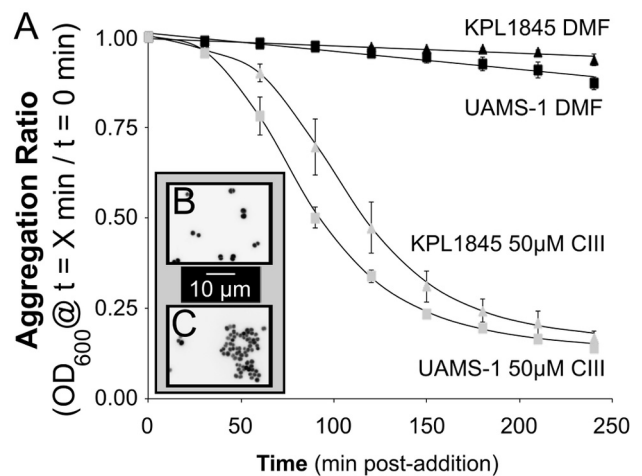


FIG 4 Coproporphyrin III induces *S. aureus* aggregation in culture. (A) DMF (black symbols) does not induce aggregation; 50 μ M CIII (gray symbols) induces aggregation. Data points are the means from five biological replicates of aggregation assays done after 19 h of growth. Error bars are SEM. (Inset) Epifluorescence microscopy of Syto-9-stained UAMS-1 cultures 4 h after addition illustrates lack of aggregation with only DMF (B) and aggregation into multicellular clusters with 50 μ M CIII (C).

to *S. aureus* (30). Examination of *S. aureus* cells using live-dead staining showed no statistical difference in the percentage of dead cells between solvent-only (DMF) and CIII-treated cultures (Fig. S5; two-tailed, paired *t* test, $P > 0.3$). These live-dead staining data indicate that exogenous CIII at the concentrations utilized here did not have a significantly deleterious effect on *S. aureus* survival.

CIII-mediated *S. aureus* coaggregation occurs in early stationary phase. We combined a 24-h growth curve with the aggregation assay to better understand the relationship between growth phase of *S. aureus* UAMS-1 in the semidefined, low-iron medium SSD0Fe and aggregation potential in response to exogenous CIII. In SSD0Fe, *S. aureus* UAMS-1 exhibited diauxic growth, which was modeled using a published equation (31) (Fig. 5, open squares). *S. aureus* did not detectably coaggregate with CIII until early stationary phase (Fig. 5, gray squares). Onset of aggregation correlated with the concomitant drop in the culture pH to a range of 6 to 6.5 (Fig. 5, top).

CIII aggregation in *S. aureus* CFCM requires both acidic pH and an additional *S. aureus* product(s). Because some porphyrins self-aggregate at acidic pH (32), the observed correlation between the drop in culture pH and CIII-*S. aureus* coaggregation led us to question the role of pH in this response. To study CIII self-aggregation in SSD0Fe, we tested the impact of pH on CIII aggregation kinetics without *S. aureus* addition. SSD0Fe was either adjusted to the pH detected in *S. aureus* cultures at a point in stationary phase corresponding to the maximal aggregation response (i.e., pH of ~4.5 to 5.0 per Fig. 5) or left at its original pH (~8 to 8.5). CIII did not self-aggregate in fresh SSD0Fe at a pH of 5 at concentrations and time scales used in our assays (Fig. 6 and Fig. S6 in the supplemental material, open squares).

Next, we explored whether growth medium conditioned by *S. aureus* would induce CIII aggregation in the absence of cells. The addition of CIII to stationary-phase *S. aureus* CFCM (pH 4.5 to 5) resulted in visible CIII aggregation within 3 h (Fig. 6 and

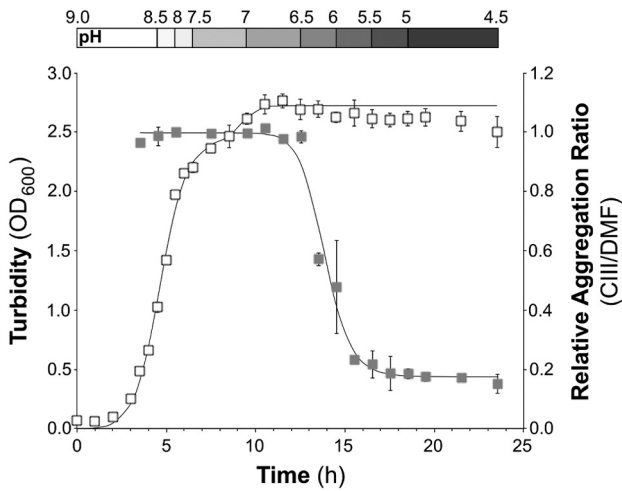


FIG 5 *S. aureus*-CIII coaggregation is growth phase dependent. *S. aureus* UAMS-1 exhibited diauxic growth in SSD0Fe over 24 h (white squares). At each time point, cells were harvested to assess the response to CIII addition; “relative aggregation ratio” (gray squares) is the aggregation ratio with 50 μ M CIII over the aggregation ratio with DMF alone 4 h postaddition. The measured pH range of the culture at each time point is depicted above the graph. All values are the means from three biological replicates; error bars are SEM.

Fig. S6 in the supplemental material, black circles). Raising the pH of this stationary-phase CFCM from its acidic baseline (4.5 to 5.0) up to 8.0 to 8.5 abrogated CIII aggregation (Fig. 6 and Fig. S6 in the supplemental material, gray circles). In fact, CIII did not aggregate within 7 days in high-pH *S. aureus* CFCM, low-pH SSD0Fe, or normal-pH SSD0Fe. From all of these data, we concluded that although a low pH was necessary for CIII aggregation, it was not sufficient in this assay. Similarly, although *S. aureus* conditioning of the medium was necessary to induce CIII aggregation,

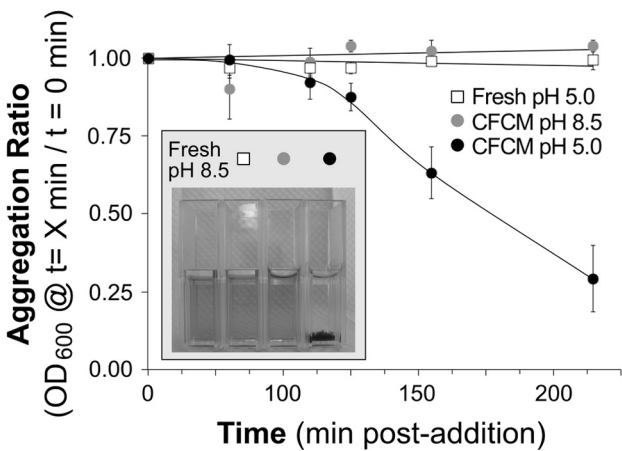


FIG 6 *S. aureus* UAMS-1 cell-free conditioned medium induces CIII aggregation at a low pH. CM from a 19-h UAMS-1 culture was filter sterilized; then the pH was either left unadjusted (black circles) or adjusted to 8.5 with NaOH (gray circles). Fresh SSD0Fe was adjusted to pH 5.0 with HCl (white squares). CIII (FC of 100 μ M) was added to each condition and aggregation was measured. Graph points represent the mean aggregation ratio from three experiments, each using medium prepared on a different date; error bars are SEM (inset). A photo taken 72 h post-CIII addition shows CIII visibly aggregated at the bottom of the cuvette with CFCM pH 5.0 but remained in solution in the other three conditions.

it too was not sufficient in this assay. Rather, the induction of aggregation required both acidic pH and an additional *S. aureus* product(s).

Purified exogenous CIII also induces *S. aureus* surface-attached biofilm formation. *S. aureus* is known for its ability to both self-aggregate and form surface-attached biofilms (33, 34). Aggregation does not guarantee biofilm formation on a surface, as this also requires surface attachment. When assayed, exogenous CIII (Fig. 7A, gray) induced *S. aureus* biofilm formation on an abiotic surface, compared to DMF alone (Fig. 7A, black). UAMS-1 is known to require plasma pre-coating of plastic surfaces to form robust biofilm (35). So it was striking that CIII-induced UAMS-1 surface-attached biofilm formation did not require pre-coating of the plastic surface with plasma. We intentionally did not pre-coat wells for this assay, as plasma proteins are not expected to be present on intact nostril and skin surfaces. Using this surface-attached biofilm assay, we observed an effect of CIII addition on a number of *S. aureus* strains that could not be effectively studied by the aggregation assay, due to robust baseline self-aggregation (Fig. 7A and B). Although the majority of strains tested exhibited a clear increase in biofilm formation in response to CIII, a few did not (SC-01 and Newman), demonstrating strain-to-strain variation in the *S. aureus* response to CIII. *Escherichia coli* MG1655, a human-associated bacterium that is not indigenous to the nostril, also grew in and acidified SSD0Fe but did not form biofilm either in the absence or presence of CIII (Fig. 7), providing support that low pH, which is a characteristic of human skin surfaces, is not alone sufficient to cause coaggregation of CIII with a different bacterial species.

CIII-mediated biofilm formation requires *S. aureus* components needed for the general biofilm response. The *sarA* gene encodes a key transcriptional regulator of biofilm formation in some *S. aureus* strains, including UAMS-1 (35–37). As expected, a UAMS-1 *sarA*-deficient mutant was defective in surface-associated biofilm formation compared to the wild type. The addition of CIII did not alter the *sarA* mutant biofilm deficit (Fig. 7C). This demonstrates that CIII-induced biofilm formation requires *sarA*-regulated cellular factors that are part of the standard biofilm response.

DISCUSSION

This work breaks new ground by demonstrating that a small molecule produced by human-associated *Propionibacterium* spp. can impact the behavior of the medically important pathobiont *S. aureus*. Specifically, we demonstrated that, in culture, *Propionibacterium*-produced coproporphyrin III (CIII) induces *S. aureus* coaggregation and biofilm formation. To our knowledge, this is the first demonstration of a *Propionibacterium*-produced small molecule altering the behavior of *S. aureus* (or any other *Staphylococcus* spp.) and one of only a few reports of *S. aureus*-*Propionibacterium* interaction (38, 39).

The relevance of our findings to the *in vivo* ecology of *Propionibacterium* and *S. aureus* is supported by reports that CIII is the predominant porphyrin present in sebaceous material collected from inflamed pilosebaceous follicles, i.e., acne lesions, in which *Propionibacterium* spp. are abundant (21, 22). Additionally, the CIII response observed in culture occurs in a pH range (4.5 to 6.5) that is physiologically relevant in the habitats where *S. aureus* is naturally most abundant; the pH at various skin sites is acidic, generally ranging from 4.5 to 6, with the nostril surface pH around

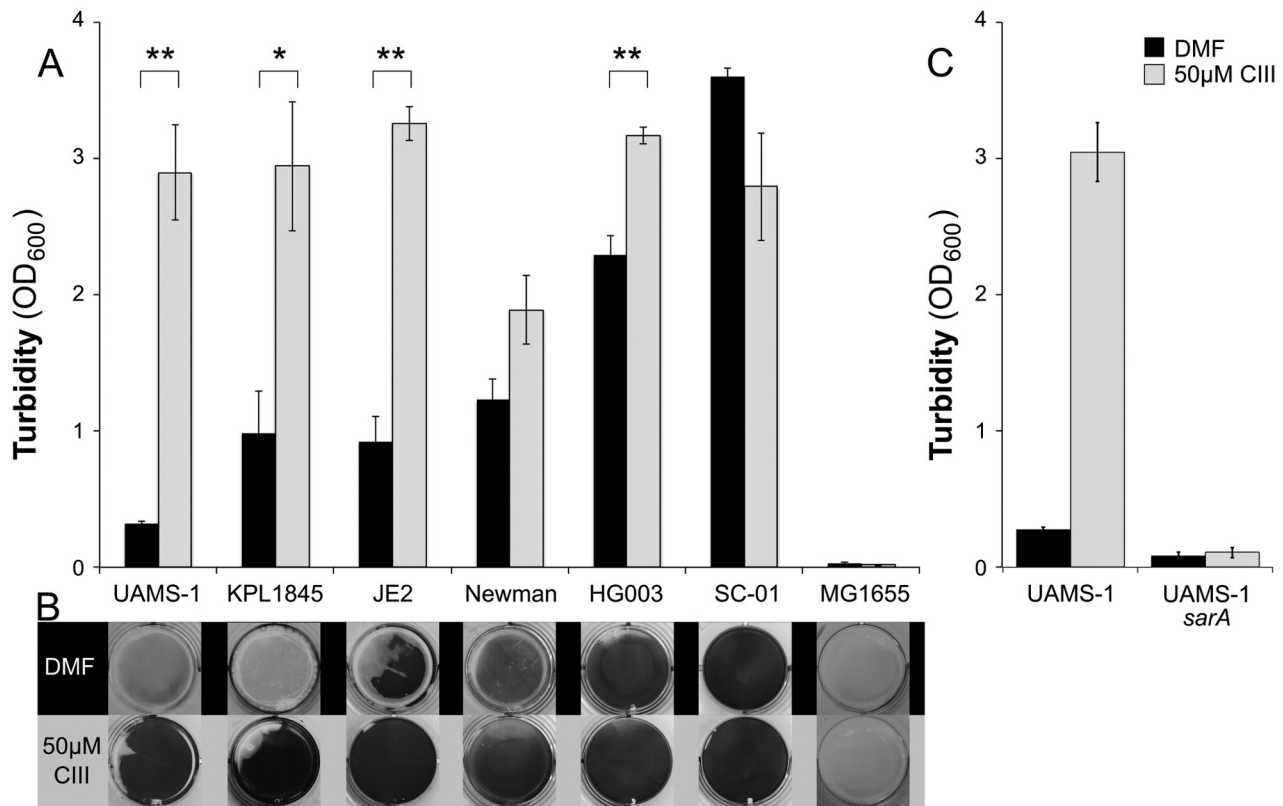


FIG 7 CIII induces *S. aureus* biofilm formation in culture; in strain UAMS-1, this induction requires *sarA*. (A) For four *S. aureus* isolates, cell density (biofilm formation) was significantly greater in the presence of 50 μM CIII (gray bars; two-tailed, paired *t* test with Bonferroni correction: **, $P < 0.05$; *, $P < 0.06$) compared to DMF alone (black bars). *E. coli* MG1655 did not form biofilm under these assay conditions. Data are the means from five biological replicates; error bars represent SEM. (B) Representative photos taken of individual wells after 20 min of 1% crystal violet staining. (C) *S. aureus* UAMS-1 formed a biofilm in response to culture with SSD0Fe and 50 μM CIII (gray bars) but was unable to form a biofilm in identical conditions when the *sarA* gene was interrupted. Data represent the means from three biological replicates; error bars are SEM.

5.5 (4). Stationary-phase *S. aureus* cells responded to CIII-induced coaggregation. This is consistent with the requirement in strain UAMS-1 for a SarA-regulated factor(s) and raises the question of whether early stationary phase in batch culture is similar to the physiological state of bacteria on skin/nostril surfaces. Surprisingly, CIII-mediated induction of *Staphylococcus* biofilm formation in culture was independent of precoating plastic with plasma free of plasma, such as skin. This finding also indicates that in the presence of *Propionibacterium* spp., *S. aureus* biofilm formation does not require binding of plasma proteins by surface-expressed proteins.

In addition to its relevance to *S. aureus* research, we have addressed a long-standing question about *Propionibacterium* physiology. More than three decades after reports that human-associated *Propionibacterium* spp. produce extracellular coproporphyrins (29, 40), this work establishes a role for one of these molecules, coproporphyrin III, as a mediator of interspecies interactions among members of the nostril and skin microbiota. Although it has long been known that *Propionibacterium* spp. produce and excrete porphyrins, the biological roles of extracellular porphyrins and the mechanisms by which these act remain poorly understood. Schaller and colleagues implicated *Propionibacterium*-produced CIII in stimulation of interleukin 8

(IL-8) production by keratinocytes (21). Possibly, CIII secretion could be involved in divalent metal ion scavenging, similar to its proposed Cu^{II} acquisition function in the soil bacterium *Paracoccus denitrificans* (41). Alternatively, extracellular porphyrins could play a role in electron shuttling, analogous to the secretion of flavins or the system cell surface decaheme cytochrome nanowires used by *Shewanella* spp. (42–44). A genomic analysis of *P. acnes* porphyrin biosynthesis reveals that the coproporphyrinogen III oxidase *hemN* (PPA0911 in *P. acnes* KPA171202) is not clustered with the rest of the heme biosynthetic operon (*hemABCDEHLY*, PPA0301 to PPA0310) (45). This gene organization suggests that the porphyrin production phenotype might be adaptive and could provide *P. acnes* with opportunities for differential regulation of its porphyrin biosynthesis pathways (see Fig. S1 in the supplemental material).

In addition, suitable hypotheses are necessary to address the evolutionary significance of CIII-mediated *S. aureus* aggregation and biofilm formation. One possibility is that increased coaggregation with CIII might benefit *S. aureus*, as biofilm development has been demonstrated to provide biofilm-associated bacteria with increased survival in hostile environments. For example, bacterial biofilms show greater resistance to antibiotic exposure, extracellular enzymes (e.g., those produced by neighboring bacteria), and the adaptive immune response (33, 46–48). Additionally,

the relationship between *S. aureus* and *Propionibacterium* might not be simply antagonistic. For example, rather than just inducing single-species *S. aureus* microcolonies, CIII might promote mixed biofilm formation with *Propionibacterium* spp. and/or other nostril/skin community members.

Future research is needed to elucidate the mechanism by which CIII coaggregates with *S. aureus* cells. We have demonstrated that this interaction requires *S. aureus* factors that are regulated, at least in UAMS-1, by the transcription factor SarA. In UAMS-1, SarA is critical for biofilm formation, suggesting that the CIII interactor(s) also plays a general role in UAMS-1 biofilm formation. The CIII interaction with *S. aureus* requires both *S. aureus*-produced factors and an acidic pH in the normal range of nostril and skin pH. This acidic pH requirement could indicate that CIII, which should be negatively charged under these conditions, must be in a particular state to undergo a specific interaction with an *S. aureus* factor(s) or that CIII might interact nonspecifically with positively charged cell surface-expressed proteins on *S. aureus* (but not on *E. coli*). Future experiments will be directed at determining the molecular mechanisms of the CIII-*S. aureus* interaction.

Approaching each human body site as an ecosystem implies the existence of a network of interspecies interactions, many of which could be mediated by small molecules. We speculate that *S. aureus* responds with altered behavior/physiology to a number of bacterium-produced small molecules that are excreted by its microbial neighbors at its primary sites of colonization: nostrils, skin, and oropharynx. Understanding the molecular mechanisms of this network of interactions, and the ramifications for the local community of removing or adding species, is likely to be essential to developing an ecologically sound approach for managing the composition of nostril microbiota to promote long-term health, e.g., a “parks management” approach (49).

MATERIALS AND METHODS

Bacterial strains, cultures, and media. Bacteria were streaked on agar medium from frozen stocks 1 to 7 days before each assay’s start depending on the species (Table 1). *Staphylococcus* spp. were grown aerobically using tryptic soy broth (TSB); *Corynebacterium* spp. were grown aerobically using brain heart infusion (BHI) with 1% Tween 80 (BHIT); *Propionibacterium* spp. were grown anaerobically using BHI. Complex media were BD brand (Becton, Dickinson, and Co.). Low-iron medium (SSD0Fe) used to grow *S. aureus* for aggregation and biofilm assays was prepared using published recipes (50, 51) as detailed in Text S1 in the supplemental material. Routine culture of antibiotic-requiring strains was with 10 $\mu\text{g}/\text{ml}$ erythromycin.

Ethics statement. Primary nostril isolates were collected as part of an ongoing protocol to study the bacterial composition of adult nostril microbiota that was initially approved by the Harvard Medical School Committee on Human Studies (8) and is currently approved by the Forsyth Institute Institutional Review Board (IRB). All participants were adults, 21 years or older, who, after receiving both a verbal and a written explanation of the study, provided verbal informed consent prior to nostril swabbing.

Preparation of *Propionibacterium* sp. conditioned medium crude extract and HPLC fractionation. The conditioned medium (CM) of 2 liters of BHI (pH 6.1) *Propionibacterium* sp. cultures (37°C for 90 h, anaerobic) was extracted with Diaion HP-20 beads (Sigma-Aldrich) by being stirred in a light-protected environment. The beads were washed with distilled H₂O (dH₂O) and extracted 3 times with MeOH, and combined extracts were dried down. The resulting dry crude extract was dissolved in 10 ml MeOH, and a 1-ml aliquot was filtered and separated by preparative

HPLC (1200 series; Agilent Technologies) on a semipreparative C₁₈ column (Phenomenex; Luna 5- μM C₁₈, 250 by 10 mm) at a flow rate of 7 ml/min, using a linear 20-min gradient of 10% to 100% acetonitrile in water (both solvents containing 0.1% trifluoroacetic acid [TFA]). For further details regarding the extraction and separation, see Text S1 in the supplemental material.

Analytical HPLC porphyrin quantitation from cell-free conditioned medium. For each strain, 5-ml cultures were grown at 37°C to stationary phase under the following conditions: *S. aureus* and *S. epidermidis*, TSB, 18 h, aerobic; *C. accolens*, BHIT, 30 h, aerobic; *P. granulosum* and *P. acnes*, BHI, 50 h, anaerobic. CM and cell pellets were separated by centrifugation (3,250 \times g, 15 min, 4°C). Cell pellets were washed with dH₂O and then lyophilized to determine the dry cell mass for standardization of the production results. Intracellular porphyrin levels were determined by combining the three lyophilized cell pellets of each strain and homogenizing them in 5 ml ethyl acetate and acetic acid (3:1, vol/vol). Each CM was filtered (0.22- μm pore size) and extracted with an equal volume of ethyl acetate and acetic acid (3:1, vol/vol). The organic phase from the above-described cell pellet or CM extractions was collected, all solvent was evaporated, and the dried residue was resuspended in 250 μl acetone and MeOH (1:1, vol/vol).

Fifty microliters of each suspension was injected into analytical HPLC equipment (1200 series; Agilent Technologies) on a C₁₈ column (Phenomenex; Luna 5- μM C₁₈, 100 by 4.6 mm) with detection at $\lambda = 400$ nm. Mobile phase A was 0.1% TFA in dH₂O; mobile phase B was 0.1% TFA in acetonitrile. Separation was obtained at a flow rate of 1 ml/min with a gradient program that started at 10% B, changing to 25% B in 3 min, followed by an increase to 45% B over 40 min. After this, the solvent composition was increased to 100% B over an additional 7 min followed by a 5-min wash at 100% B. Porphyrin levels of CM or cell pellets (below detection limit) were quantified based on a standard curve that was generated using a dilution series of known porphyrin stock solutions, as described in detail in Text S1 in the supplemental material.

***S. aureus* aggregation assay.** Frozen stocks were streaked on TSB agar and grown aerobically at 37°C for 24 to 48 h. Colonies were picked and resuspended in 1 ml SSD0Fe to an optical density at 600 nm (OD₆₀₀) of ≈ 1 . For each experimental replicate/condition/culture, 50 μl from this resuspension was mixed with 950 μl of fresh SSD0Fe and placed into a sterile 1.7-ml microcentrifuge tube. Culture tubes were incubated at 37°C for 19 h with shaking at 200 rpm. After incubation, 10 μl of either a concentrated coproporphyrin III dihydrochloride solution (CIII; C654-3; Frontier Scientific) solubilized in dimethylformamide (DMF; Sigma-Aldrich) or DMF alone was mixed into each tube. The DMF final concentration (FC) was always 1% (vol/vol). Absorbance readings were made at OD₆₀₀ over a defined period of time with an LED-based spectrophotometer (Amersham Biosciences Ultrospec 10). Each aggregation datum at time x was calculated as a ratio: OD₆₀₀ at time x /OD₆₀₀ at time 0 min (compound addition). All aggregation assay data presented are averages from at least three biological replicates. Aggregation trends were either fit to the four-parameter nonlinear regression model $y = d + [(a - d)/(1 + (x/c)^b)]$ by minimizing the mean squared error (MSE) between the real and model values using the “Solver” tool in Microsoft Excel (Frontline Systems Inc.) or fit using a linear model.

***S. aureus* UAMS-1 growth, aggregation, and pH measurements.** *S. aureus* UAMS-1 colonies were grown as described above. Colonies were picked and resuspended in 2 ml of SSD0Fe to an OD₆₀₀ of ≈ 1 . This was mixed with 38 ml of SSD0Fe and 1-ml aliquots dispensed into 40 sterile, 1.7-ml microcentrifuge tubes prior to incubation at 37°C for up to 23.5 h with shaking at 200 rpm. At intervals during growth, one or more tubes were removed and the OD₆₀₀ was measured; if the OD₆₀₀ was ≥ 1 , the culture was diluted in fresh SSD0Fe to permit measurement in the linear range of the spectrophotometer. The growth curve of *S. aureus* UAMS-1 in SSD0Fe was fit with the diauxic model $y = a_\alpha b_\alpha + a_\beta b_\beta$, where $a_\alpha = (1 - e^{-x/c})/(1 - e^{-x/c} + e^{-x/d})$ and $a_\beta = (1 - e^{-x/d})/(1 - e^{-x/c} + e^{-x/d})$;

additionally, $b_{\alpha} + b_{\beta} = 1$ (31). As described above, the “Solver” tool was used to minimize the MSE between observed and predicted values.

At each time point where OD₆₀₀ was measured, pH was also measured using strips of both pH 2 to 9 (BDH, VWR) and pH 4 to 7 (Merck KGaA). Aggregation assays were started periodically throughout the growth curve. Similar to that described above, 1% (vol/vol) DMF or CIII (FC of 50 μ M) in DMF was mixed into the culture and the OD₆₀₀ was measured. For each time point on the growth curve, the relative aggregation ratio was calculated as the aggregation ratio of the CIII-exposed culture after 4 h divided by the aggregation ratio of the DMF-exposed culture after 4 h. The relative aggregation curve for UAMS-1 was fit using a four-parameter nonlinear regression model as described above.

S. aureus biofilm assay. Each well of a polystyrene, flat-bottom, tissue culture-treated, 12-well plate (BD Biosciences) was filled with 1.9 ml fresh SSD0Fe, 0.1 ml of bacterial culture at an OD₆₀₀ of ≈ 1 prepared as described above, and 10 μ l of either a 10 mM CIII solution in DMF (FC of 50 μ M) or DMF alone. Each plate was incubated standing at 37°C for 24 h; then, the plate was agitated by hand for 30 s to resuspend unattached, sedimented cells. Medium and planktonic cells were removed, and each well was washed with 0.5 ml of fresh SSD0Fe (pH adjusted to 4 to 5 with 1 N HCl) and agitated by hand for 30 s. The liquid was again removed, and each well was again washed and agitated and the liquid was removed as described before. After two washes, 1 ml of fresh SSD0Fe (pH of ≈ 8) was added to each well. Wells were scraped with a sterile plastic spatula or pipette tip to remove all surface-adhered cells. The cell mix from each well was transferred to a plastic cuvette, and the OD₆₀₀ was measured.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01286-14/-DCSupplemental>.

Text S1, PDF file, 0.1 MB.
Figure S1, PDF file, 0.4 MB.
Figure S2, PDF file, 0.2 MB.
Figure S3, PDF file, 0.2 MB.
Figure S4, PDF file, 0.2 MB.
Figure S5, PDF file, 0.1 MB.
Figure S6, PDF file, 0.3 MB.
Figure S7, PDF file, 0.4 MB.
Table S1, PDF file, 0.1 MB.

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