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GENETICS AND MOLECULAR BIOLOGY

Methionine Limitation Impairs Pathogen Expansion and Biofilm Formation Capacity

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ABSTRACT Multidrug-resistant bacterial pathogens are becoming increasingly prevalent, and novel strategies to treat bacterial infections caused by these organisms are desperately needed. Bacterial central metabolism is crucial for catabolic processes and provides precursors for anabolic pathways, such as the biosynthesis of essential biomolecules like amino acids or vitamins. However, most essential pathways are not regarded as good targets for antibiotic therapy since their products might be acquired from the environment. This issue raises doubts about the essentiality of such targets during infection. A putative target in bacterial anabolism is the methionine biosynthesis pathway. In contrast to humans, almost all bacteria carry methionine biosynthesis pathways which have often been suggested as putative targets for novel anti-infectives. While the growth of methionine auxotrophic strains can be stimulated by exogenous methionine, the extracellular concentrations required by most bacterial species are unknown. Furthermore, several phenotypic characteristics of methionine auxotrophs are only partly reversed by exogenous methionine. We investigated methionine auxotrophic mutants of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (all differing in methionine biosynthesis enzymes) and found that each needed concentrations of exogenous methionine far exceeding that reported for human serum (~30 μM). Accordingly, these methionine auxotrophs showed a reduced ability to proliferate in human serum. Additionally, *S. aureus* and *P. aeruginosa* methionine auxotrophs were significantly impaired in their ability to form and maintain biofilms. Altogether, our data show intrinsic defects of methionine auxotrophs. This result suggests that the pathway should be considered for further studies validating the therapeutic potential of inhibitors.

IMPORTANCE New antibiotics that attack novel targets are needed to circumvent widespread resistance to conventional drugs. Bacterial anabolic pathways, such as the enzymes for biosynthesis of the essential amino acid methionine, have been proposed as potential targets. However, the eligibility of enzymes in these pathways as drug targets is unclear because metabolites might be acquired from the environment to overcome inhibition. We investigated the nutritional needs of methionine auxotrophs of the pathogens *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. We found that each auxotrophic strain retained a growth disadvantage at methionine concentrations mimicking those available *in vivo* and showed that biofilm biomass was strongly influenced by endogenous methionine biosynthesis. Our experiments suggest that inhibition of the methionine biosynthesis pathway has deleterious effects even in the presence of external methionine. Therefore, additional efforts to validate the effects of methionine biosynthesis inhibitors *in vivo* are warranted.

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The increasing prevalence of multidrug-resistant (MDR) bacterial pathogens and the limited efficacy of available antibiotics create an urgency for the development of alternative antimicrobial agents (1, 2). In particular, *Enterococcus faecium*, *Staphylococcus aureus*, *Clostridium difficile*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae* (ESCAPE pathogens) are of increasing prevalence in clinical practice (3).

Staphylococcus aureus is a major cause of health care-associated infections leading to severe morbidity and mortality along with tremendous costs for health care systems (4). Methicillin-resistant *S. aureus* (MRSA) is resistant to most β-lactam antibiotics and causes a substantial proportion of staphylococcal infections in hospitals and, in the United States and Asia, increasingly in the community. Antibiotics of last resort against MRSA, such as vancomycin and daptomycin, are much less effective than β-lactams. Only a few anti-MRSA drugs are in development pipelines, but most of them do not have the right characteristics to solve the MRSA problem (5). Thus, MRSA will remain a pressing problem if no better preventive and therapeutic options become available. In addition, certain types of staphylococcal infections are particularly difficult to treat. This is the case for infections associated with artificial implants, such as hip and knee joint replacements, or artificial heart valves. Device-associated biofilms are largely insensitive to antibiotics and host defense factors (6). Infected implants usually have to be replaced. This replacement leads to an enormous burden for patients and extra costs for health care systems. *Pseudomonas aeruginosa* forms biofilms within the lungs of cystic fibrosis patients (7, 8) and within lung ventilators of intensive care patients (9–11). *Escherichia coli* is another important ESCAPE pathogen. About 20% of all bacteremia cases in the United Kingdom are caused by *E. coli* (12). The sharp increase in frequency of isolation of MDR ESCAPE pathogens, including those expressing extended-spectrum β-lactamases, seems to be diminished by more careful use of antibiotics (13). Nonetheless, novel routes to treat MDR pathogens or to lower their pathogenic potential, for example by inhibiting biofilm formation, are needed.

The bacterial folate biosynthesis inhibitor trimethoprim combined with sulfamethoxazole is used to treat bacterial infections, and the identification of additional targets in bacterial metabolic pathways has the potential for the development of novel antibiotics (14). The methionine biosynthesis pathway is one option, since it fulfills important criteria. First, humans rely on exogenous methionine in their diet, and no methionine biosynthesis pathway is encoded by the human genome. In contrast, almost all prokaryotes carry methionine biosynthesis pathways, suggesting that inhibitors might have the potential to be broad-spectrum antibiotics. Second, methionine is crucial for bacterial protein biosynthesis and is required both for the initiation and elongation stages of translation. Finally, methionine limitation is expected to have a broad impact on bacterial physiology since methionine is the precursor of the global methyl group donor *S*-adenosyl-L-methionine (SAM), which is required for DNA methylation, protein methylation, and polyamine biosynthesis.

A potential disadvantage of the methionine biosynthesis pathway as a drug target is the ability of bacteria to import exogenous methionine. The concentrations of free methionine in different body fluids vary and might depend on the diet of the host. Human nasal secretions are devoid of methionine and a *S. aureus* methionine auxotroph is attenuated in an animal nasal colonization model (15). Human serum is reported to contain 25 to 48 μM methionine (16, 17). However, the concentrations of methionine required by different pathogens for optimal growth and virulence are mostly unknown.

The methionine biosynthesis pathways of many bacterial species are well characterized and are reviewed in detail elsewhere (18). The common precursor of methionine

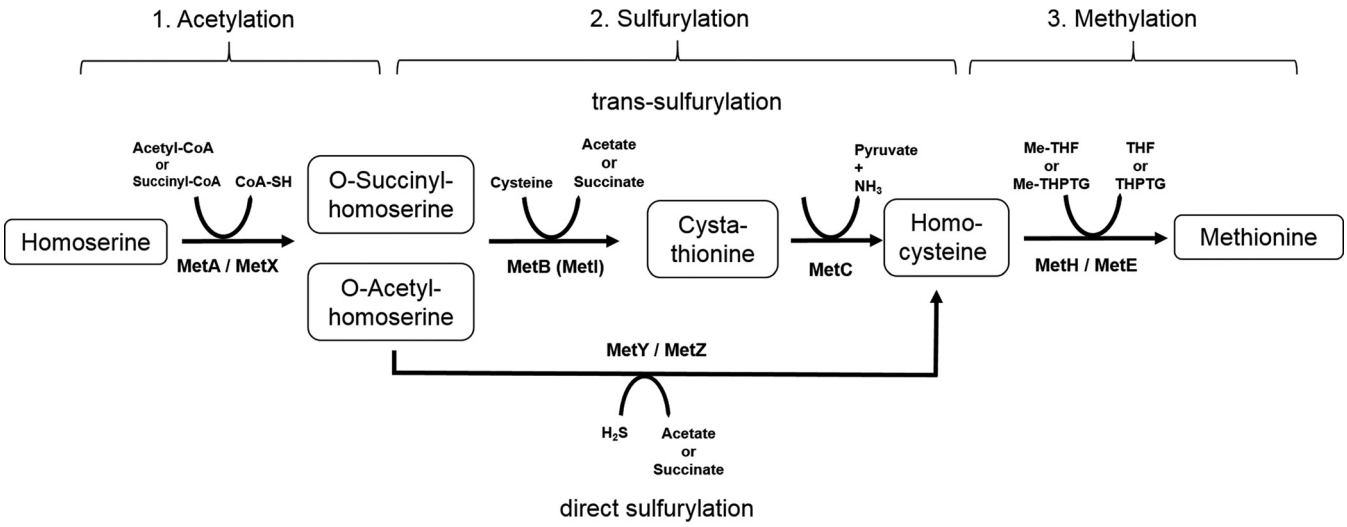

FIG 1 Schematic diagram of the bacterial methionine biosynthesis pathways. Me-THF, 5-methyl-tetrahydrofolate; Me-THPTG, 5-methyl-tetrahydopteroyl tri-L-glutamate.

is homoserine, which is derived from aspartate. Biosynthesis of methionine occurs in three stages (Fig. 1). The first is the acetylation of homoserine. This reaction is performed by two protein families, namely, MetA and MetX, that are unrelated both in amino acid sequence and protein structure. MetA-class enzymes can use either succinyl-coenzyme A (CoA) or acetyl-CoA to produce *O*-succinylhomoserine or *O*-acetylhomoserine, respectively. In contrast, MetX-class enzymes seem to exclusively use acetyl-CoA to produce *O*-acetylhomoserine. The second step is the sulfurylation of acylhomoserine to form the intermediate homocysteine. Sulfurylation reactions involved in methionine biosynthesis are more diverse than acetylation reactions, and yet, two types are most prominent. One pathway (called transsulfurylation) is a two-step reaction using cysteine as a sulfur donor. In this pathway, cystathionine-γ-synthases (MetB/MetI) incorporate cysteine to form the intermediated cystathionine. In a second step, cystathionine-γ-lyases (MetC) create homocysteine. The second prominent type of sulfurylation (direct sulfurylation) uses hydrogen sulfide by MetY- and MetZ-class enzymes, allowing the creation of homocysteine in a single step. The third step in methionine biosynthesis is the methylation of homocysteine. MetH- and MetE-class enzymes use the methyl group donors 5-methyl-tetrahydrofolate (Me-THF) and 5-methyl-tetrahydopteroyl tri-L-glutamate (Me-THPTG), respectively, to form methionine from homocysteine.

It remains unclear whether bacteria that use different methionine biosynthesis pathways have different nutritional requirements and whether some steps of methionine biosynthesis represent more promising targets than others. Answering such questions is important if methionine biosynthesis is to be considered a target for antibiotic development.

RESULTS

Methionine auxotrophs need high concentrations of free methionine to overcome growth defects. We chose three different model organisms, namely, *E. coli*, *S. aureus*, and *P. aeruginosa*, for analysis of physiological effects associated with a lack of methionine biosynthesis. Each organism uses distinct methionine biosynthesis pathways and enzyme classes to perform the reactions (Table 1). *E. coli* performs the acetylation step using succinyl-CoA and a MetA-class enzyme. In contrast, *S. aureus* and *P. aeruginosa* use acetyl-CoA and a MetX-class enzyme. *E. coli* and *S. aureus* employ the transsulfurylation pathway to create homocysteine, while *P. aeruginosa* relies on the direct sulfurylation pathway. Finally, all model organisms perform the methylation of homocysteine using MetE and MetH-class enzymes.

TABLE 1 Methionine biosynthesis pathways of model organisms

Enzyme type by pathway	Enzyme by organism[a]		
	E. coli	*S. aureus*	*P. aeruginosa*
Acetylation			
Cofactor	Succinyl-CoA	Acetyl-CoA	Acetyl-CoA
Product	*O*-Succinylhomoserine	*O*-Acetylhomoserine	*O*-Acetylhomoserine
Encoded	MetA	MetX	MetX
Transsulfurylation			
Encoded	MetB + MetC	MetI + MetC	—
Direct sulfurylation			
Encoded	—	—	MetY/MetZ
Methylation			
Encoded	MetE/MetH	MetE/MetH	MetE/MetH

[a]—, not encoded.

Isogenic mutants defective in key enzymes of the methionine biosynthesis pathways in each organism were obtained. Markerless deletion mutants in *S. aureus* (strains Newman, SH1000, and USA300 LAC) were created by allelic replacement. Transposon insertion mutants of *P. aeruginosa* PA14 as well as *E. coli* BW25113 strains with *met* genes replaced by a kanamycin marker were acquired from mutant libraries (https://cgsc2.biology.yale.edu/, http://pa14.mgh.harvard.edu/cgi-bin/pa14/home.cgi). Each organism carries the two redundant enzyme classes MetE and MetH. Mutants defective in either one alone would not be expected to be auxotrophic for methionine. Indeed, *P. aeruginosa* mutants deficient in either MetE or MetH showed wild-type (WT) levels of growth yield in methionine-dependent growth assays (see Fig. S1 in the supplemental material). Therefore, we focused on the other steps of the pathway.

All mutant strains grew in a similar fashion to the parental strain when cultivated in complex medium (data not shown) but were unable to grow when inoculated into defined medium without methionine, confirming methionine-auxotrophic phenotypes (Fig. 2). Only *E. coli* BW25113 Δ*metC* showed slow growth in the absence of methionine and reached an optical density (OD) of ~0.5 after 50 h of incubation (see Fig. S2 in the supplemental material). However, in our standard experiments (~12 to 15 h of growth), the increase in optical density was hardly detectable. Furthermore, the results showed that the *metY* gene of *P. aeruginosa* was dispensable, while *metZ* was essential for proliferation without external methionine. This result was unexpected since both enzymes are supposedly able to perform the direct sulfurization reaction.

All of the mutants needed large amounts of methionine to reach the same density as the WT. Mutants of *S. aureus* strains Newman, USA300 LAC, and SH1000 needed a concentration of 50 to 100 μM methionine to overcome auxotrophy (Fig. 2; see Fig. S3 in the supplemental material). In contrast, *E. coli* and *P. aeruginosa* needed even higher concentrations (100 to 200 μM). Furthermore, we observed that growth yields of *S. aureus* Newman and *P. aeruginosa* PA14 wild-type strains did not increase when additional methionine was available. This result suggests that autotrophic strains are actively synthesizing endogenous methionine rather than acquiring it form exogenous sources.

The individual methionine auxotrophs of each species showed very similar phenotypic characteristics. This finding makes it unlikely that secondary site mutations or polar effects might be associated with mutagenesis. However, we thought to confirm this in one model organism and complemented the *E. coli* Δ*metA* strain by expression of the *metA* gene from the IPTG inducible vector pME6032. This abolished the observed differences in final bacterial density (see Fig. S4 in the supplemental material).

These experiments indicate that blockage of methionine biosynthesis cannot be easily overcome by using environmentally available methionine and that auxotrophic strains have growth disadvantages at concentrations reported to be available in human serum (25 to 48 μM).

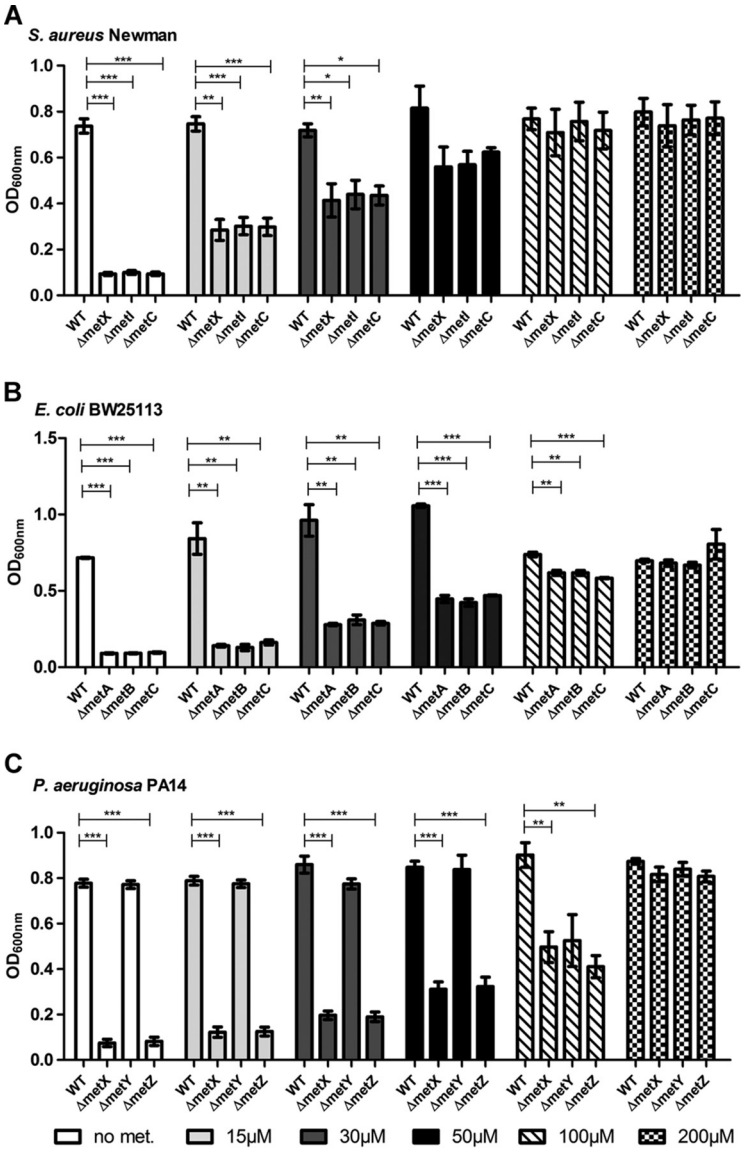

FIG 2 Growth of model organisms with defined amounts of methionine. In a 48-well microtiter plate, defined medium (SNM20 for *S. aureus* and M9 for *E. coli* and *P. aeruginosa*) was inoculated and supplemented with increasing concentrations of methionine. OD_{600} was monitored over 48 h of growth. Optical densities when strains entered the stationary phase are shown. The mean and standard error of the mean (SEM) of three independent experiments are shown. Statistical analysis was performed using Student's unpaired *t* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

Human serum contains an insufficient source of methionine. The addition of 30 μM methionine to defined medium was insufficient to restore a WT phenotype to methionine auxotrophs of all three organisms. However, it remained unclear whether this finding would apply *in vivo* as human serum is a complex mixture of free amino acids, proteins, and peptides that might be used as a source of methionine by invading microorganisms. Therefore, we investigated the potential of heat-inactivated pooled human serum to permit the growth of methionine auxotrophic mutants. For each species, we chose a representative mutant defective in the first step of methionine biosynthesis (acetylation). Therefore, free methionine as well as all methionine precursors downstream of the acetylation reaction (compare in Fig. 1) should be able to serve to bypass the blockage. Since all strains grew poorly in pooled human serum (data not shown), we supplemented human serum with methionine-free defined medium and compared the number of CFUs after 24 h of incubation. As shown in Fig. 3, the mutants

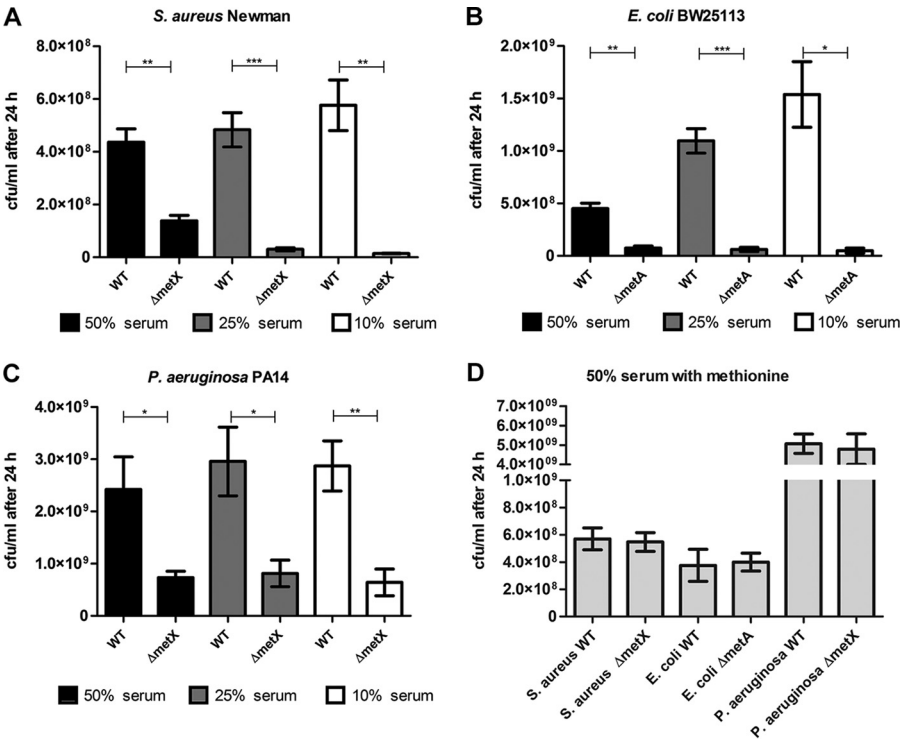

FIG 3 Growth yields of model organisms with pooled human serum as a source of methionine. (A, B, and C) Pooled human serum was diluted with defined medium (SNM20 for *S. aureus* and M9 for *E. coli* and *P. aeruginosa*) and inoculated to 5×10^6 CFU/ml. CFUs were enumerated after 24 h of growth. (D) Pooled human serum was diluted with defined medium (SNM20 for *S. aureus* and M9 for *E. coli* and *P. aeruginosa*) and supplemented with 30 μM methionine (*S. aureus*) or 100 μM methionine (*E. coli* and *P. aeruginosa*). The serum was inoculated to 5×10^6 CFU/ml. CFUs were enumerated after 24 h of incubation. Shown are the mean and SEM of at least three independent experiments. Statistical analysis was performed using Student's unpaired *t* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

retained a distinct growth disadvantage over the entire range of serum concentrations tested and even 50% pooled human serum did not allow them to reach WT levels of growth yield. Importantly, the addition of free methionine to human serum abolished the differences between the WT and mutant strains (Fig. 3D), indicating that methionine limitation was responsible for the observed defects.

These data indicate that the combined sources of methionine present in human serum are insufficient to restore a WT phenotype in mutant strains, suggesting that methionine biosynthesis might be a valid target for new antimicrobials.

Methionine auxotrophy reduces protein-dependent biofilm formation by staphylococci. Both staphylococci and pseudomonads form clinically relevant biofilm, for instance, in catheter- and joint replacement-associated infections (19). *S. aureus* SH1000 biofilm relies predominantly on proteins (20). Therefore, we speculated that the capacity to form biofilm might depend on the ability to synthesize methionine and investigated this using the methionine biosynthesis inhibitor propargylglycine (PG). PG specifically inhibits cystathionine-γ-synthase (21) which is well conserved among staphylococcal species. Indeed, in the absence of free methionine, PG reduced the growth yields of *S. aureus* SH1000 (Fig. 4A). PG concentrations reducing growth yields only partially had profound effects on biofilm formation of the strain (Fig. 4A and B). To exclude that this effect reflected the decreased final growth yields in the presence of PG, we analyzed the protein levels in the cell wall of SH1000. Cell wall-anchored proteins, such as the fibronectin-binding proteins (22) or SasG (23), are associated with biofilm formation in different staphylococcal lineages. Therefore, we assumed that cell wall-associated proteins might also contribute to biofilm formation under our experimental conditions. We investigated this using cell wall extracts of SH1000 grown under

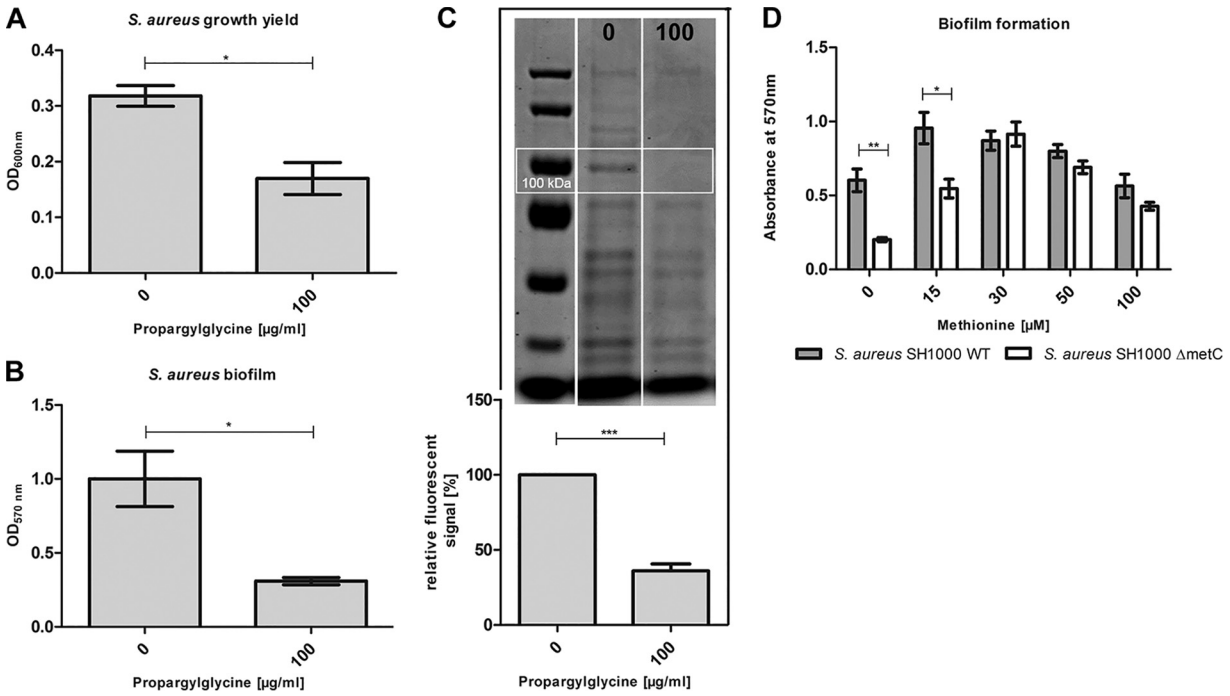

FIG 4 Impact of PG on proteinaceous biofilms formed by *S. aureus* SH1000. (A) Growth inhibition by PG. In 96 well plates, *S. aureus* SH1000 was allowed to form biofilm in SNM3 with or without 100 μg/ml PG for 24 h. OD_{600} was quantified prior to biofilm staining. Values represent the mean and SEM of three independent experiments. Statistical analysis was performed using Student's unpaired *t* test. (B) Inhibition of biofilm formation by PG. In 96-well plates, *S. aureus* SH1000 was allowed to form biofilm in SNM3 with or without 100 μg/ml PG for 24 h. Biofilm was stained with crystal violet, and the absorbance at 570 nm was measured. Values represent the mean and SEM of three independent experiments. Statistical analysis was performed using Student's unpaired *t* test. (C) Protein abundance within the *S. aureus* cell wall. *S. aureus* SH1000 was grown in SNM3 with or without 100 μg/ml PG for 24 h. Cell wall extracts were isolated by digestion of the peptidoglycan in the presence of 0.5 M sucrose to protect protoplasts. Protoplasts were removed and cell wall extracts were analyzed by SDS-PAGE. Gels were stained with Coomassie blue (a strong 700-nm fluorophore), and bands were quantified using infrared fluorescent imaging. The upper picture shows a representative gel. The lower picture shows quantification of the prominent protein band indicated. The values represent mean and SEM of four independent experiments. Statistical analysis was performed using Student's paired *t* test. (D) Biofilm formation of *S. aureus* Δ*metC*. In 96-well plates, *S. aureus* SH1000 was allowed to form biofilm in SNM3 containing different concentrations of methionine for 24 h. Biofilm was stained and quantified as in (B). Values represent the mean and SEM of three independent experiments. Statistical analysis was performed using Student's unpaired *t* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

methionine-limiting conditions in the presence or absence of PG. The extracts were analyzed by infrared quantification of proteins separated by SDS-PAGE and stained with Coomassie blue. PG treatment led to a decrease in cell wall protein content (Fig. 4C), supporting the idea that reduced protein amounts in the cell wall decreased the capacity to form proteinaceous biofilms.

We also created a Δ*metC* deletion mutant in the SH1000 background and tested the mutant for biofilm formation. Similar to *S. aureus* Newman mutants described above, SH1000 Δ*metC* was auxotrophic for methionine and needed 50 μM methionine to reach the same growth yields as the WT (Fig. S3 in the supplemental material). The auxotrophic mutant also showed a reduced biofilm formation, and WT levels of biofilm were restored by the addition of 30 to 50 μM methionine (Fig. 4D).

Next, we investigated whether methionine limitation also affects the formation of polysaccharide-dependent biofilms. *S. epidermidis* RP62A produces a biofilm dependent on the polysaccharide intracellular adhesion (PIA) (24). Similar to the inhibition of SH1000, PG reduced growth yields of *S. epidermidis* RP62A in methionine-deficient medium (Fig. 5A). However, the biomass of RP62A biofilms remained unaltered (Fig. 5B), suggesting that biosynthesis of the PIA matrix was not dependent on methionine biosynthesis. To validate this finding, we isolated the biofilm matrix of *S. epidermidis* RP62A grown in methionine-deficient medium in the presence and absence of PG and assessed PIA semiquantitatively. Indeed, we found that the amount of PIA was not reduced by PG. In contrast, the amount of PIA seemed slightly increased in the

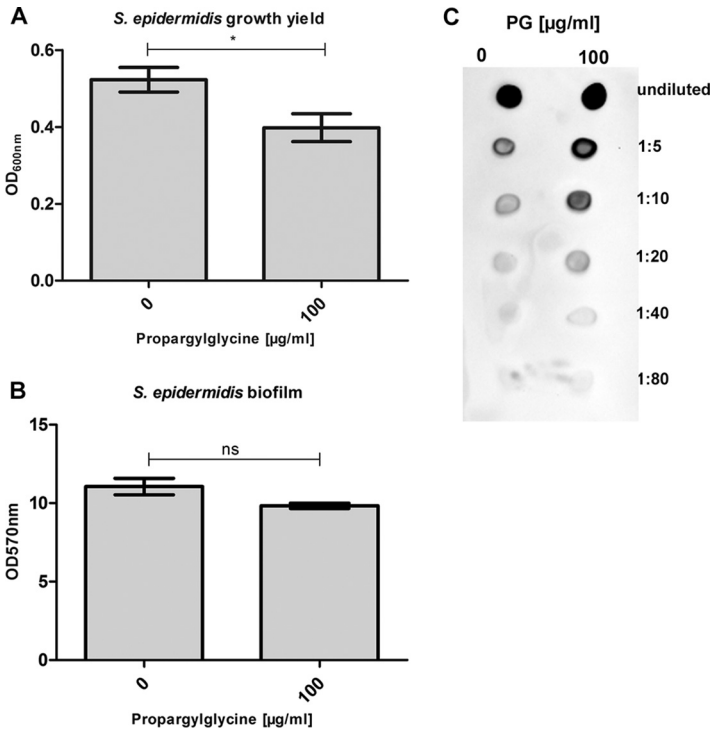

FIG 5 Impact of PG on polysaccharide biofilms formed by *S. epidermidis* RP62A. (A) Growth inhibition by PG. In 96-well plates, *S. epidermidis* RP62A was allowed to form biofilm in methionine-deficient mTMS with or without 100 μg/ml PG for 24 h. OD_{600} was quantified prior to biofilm staining. (B) Inhibition of biofilm formation by PG. In 96-well plates, *S. epidermidis* RP62A was allowed to form biofilm in methionine-deficient mTMS with or without 100 μg/ml PG for 24 h. Biofilm was stained with crystal violet, and the absorbance at 570 nm was measured. (C) PIA in *S. epidermidis* biofilms. In 96-well plates, *S. epidermidis* RP62A was allowed to form biofilm in methionine-deficient mTMS with or without 100 μg/ml PG for 24 h. The biofilm matrix was isolated, serial dilutions were dotted on a nitrocellulose membrane, and PIA was detected using wheat germ agglutinin coupled to horseradish peroxidase. The experiment was carried out three times with similar results. Values represent the mean and SEM of three independent experiments. Statistical analysis was performed using Student's paired *t* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$.

presence of PG (Fig. 5C). Of note, no PIA was detected when an *icaA*-deficient mutant was used, confirming the specificity of this assay (data not shown).

In conclusion, these data indicate that in contrast to polysaccharide biofilms, proteinaceous staphylococcal biofilm formation depends on the availability of sufficient amounts of methionine.

Methionine auxotrophy leads to the dispersal of *P. aeruginosa* biofilms. The capacity to form biofilms is key for *P. aeruginosa* to cause ventilator-associated pneumonia, catheter-associated urinary tract infections, and chronic lung infections in cystic fibrosis (CF) patients (9–11). Its biofilm consists of exopolysaccharides, such as galactose and mannose-rich polysaccharide Psl (25), the glucose-rich polysaccharide (Pel) (26), and alginate (27). In addition, extracellular DNA (eDNA) and proteins are present (28). Biofilm formation by the *P. aeruginosa* PA14 WT strain and the isogenic methionine auxotrophic mutants was compared. Defined medium was supplemented with 100 μM methionine, and biofilm formation was measured at the liquid/air interface using the crystal violet microtiter plate method (29) which allows staining and quantification of biofilm mass comprising embedded cells and surrounding matrix. Only a minor difference in growth yield between the WT and the mutant strains was observed. (Fig. 6A). However, all mutants produced a significantly reduced biofilm mass under these conditions. Also, the *P. aeruginosa* Δ*metY* mutant, which did not show any growth deficit compared with the parental strain, exhibited impaired biofilm formation (Fig. 6B).

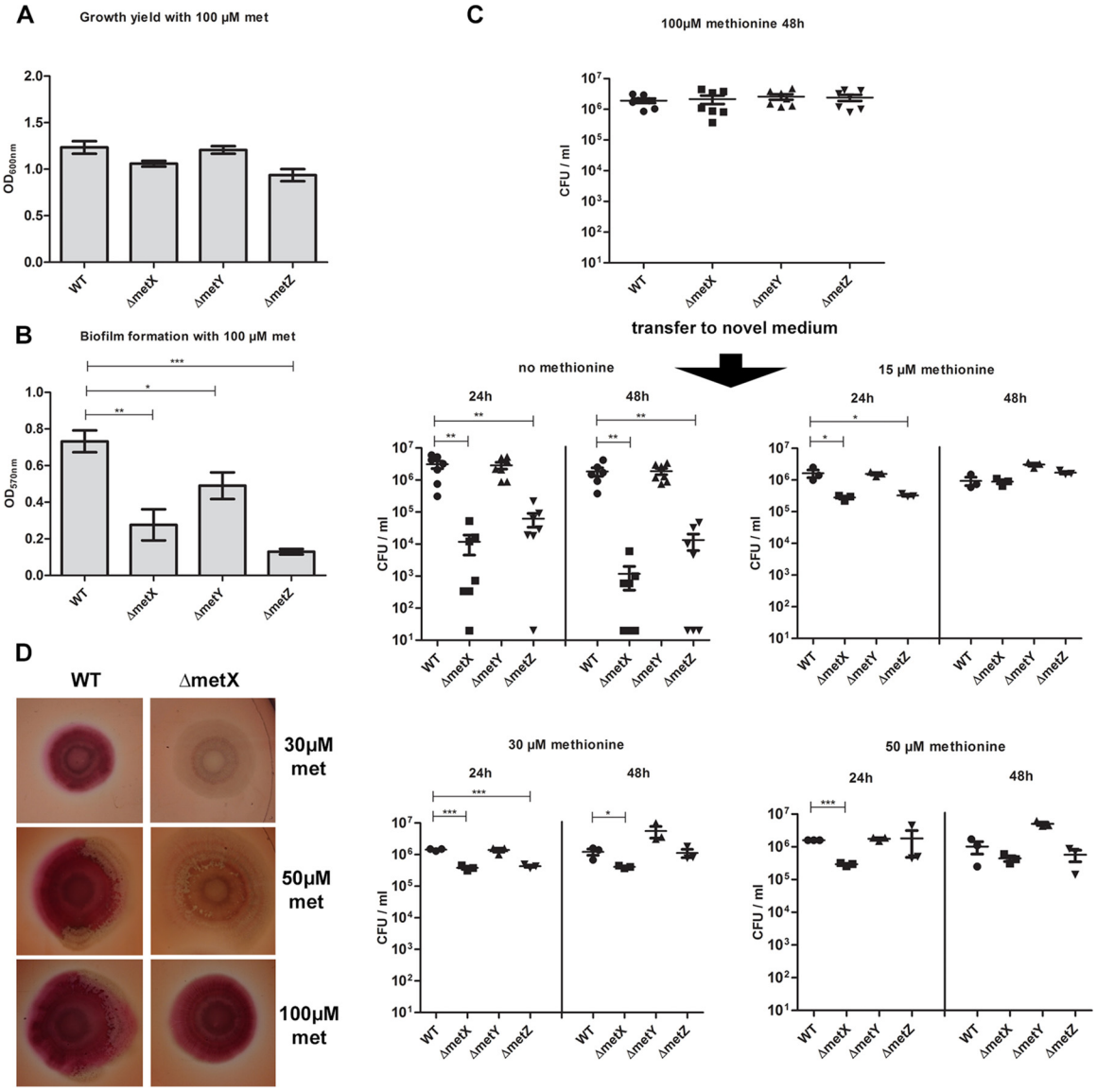

FIG 6 *Pseudomonas* biofilm formation and maintenance. (A) Growth yields of *P. aeruginosa* under methionine limitation. Strains were cultivated for 24 h in 96-well plates containing M9 medium (100 μM methionine). OD_{600} was quantified prior to biofilm staining. Values represent the mean and SEM of triplicate wells. The experiment was carried out three times with similar results. Statistical analysis was performed using Student's unpaired *t* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$. (B) Biofilm formation by auxotrophic *P. aeruginosa* strains. Strains were cultivated for 24 h in 96-well plates containing M9 medium (100 μM methionine). Biofilm was stained with crystal violet, and the absorbance at 570 nm was quantified. Values represent the mean and SEM of triplicate wells. The experiment was carried out three times with similar results. Statistical analysis was performed using Student's unpaired *t* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$. (C) Biofilm formation at the peg plates. *P. aeruginosa* biofilms were allowed to form on the lid of peg plates in M9 medium containing 100 μM methionine. Biofilms were then transferred to medium with varying concentrations of methionine and incubated for an additional 24 to 48 h. Biofilms were disassociated at different time points, and living cell counts were enumerated. CFUs of at least three independent experiments are shown. Statistical analysis was performed using Student's unpaired *t* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$. (D) Congo red staining. *P. aeruginosa* strains were applied onto M9 agar plates containing Congo red and varying concentrations of methionine. The color of colonies was evaluated after 24 h of incubation at 37°C, followed by 24 h of incubation at room temperature. The experiment was repeated thrice with similar results. A representative picture is shown.

Given the strong effects of methionine auxotrophy on *P. aeruginosa* biofilm formation, we asked whether this reflects differences in the production of the extracellular matrix or within the number of bacterial cells. To investigate this question, the Calgary biofilm device was used consisting of a 96-well plate filled with defined medium and a lid containing pegs that reach into the medium (30). The biofilm formed on the pegs was dissociated to determine viable counts of bacteria located within the biofilm (31). In media containing 100 μM methionine, biofilms of methionine auxotrophs and the

autotrophic parental strain harbored similar numbers of CFU (Fig. 6C, top), suggesting that differences between WT and mutants in the crystal violet assay were due to different quantities of extracellular matrix. This result opened the question as to whether methionine biosynthesis is relevant for the initial formation of the biofilm or whether it is also important for biofilm growth and maintenance. To investigate this question, *P. aeruginosa* biofilm was allowed to form on pegs in the presence of 100 μM methionine. After 48 h, the pegs were transferred to fresh medium containing different concentrations of methionine and incubated for 24 to 48 h, after which CFUs were enumerated. The WT and the Δ*metY* mutant were able to maintain a constant number of CFUs from 48 to 96 h in medium lacking methionine. In contrast, the viable counts of Δ*metX* and Δ*metZ* mutants decreased by 4 to 5 orders of magnitude in the same time frame. In half of the experiments, no viable bacteria were recovered from biofilms of the Δ*metX* and Δ*metZ* mutants (Fig. 6C). This effect was abolished when extracellular methionine (10 to 50 μM) was present in the medium. However, significantly fewer Δ*metX* bacteria were recovered from the biofilm than from the WT even in the presence of 30 to 50 μM methionine.

The biofilm matrix of *P. aeruginosa* PA14 binds Congo red, giving WT strain colonies a distinct red color in the presence of this dye. In contrast, Δ*pel* mutants that are deficient in the carbohydrate-processing Pel proteins fail to stain with Congo red (26). *P. aeruginosa* WT and the Δ*metX* mutants were grown on defined M9 agar plates containing 30 to 100 μM methionine and Congo red. All strains formed comparably sized colonies. The PA14 WT strain showed an intense red color on all concentrations of methionine, indicating the formation of a glycocalyx. In contrast, the color of Δ*metX* colonies was dependent on the methionine concentration. The strain remained virtually colorless in the presence of 30 to 50 μM methionine, strongly resembling the reported phenotype of Pel-deficient mutants. This phenotype was reversed by increasing the concentration of methionine (Fig. 6D).

These data suggest that the availability of sufficient levels of methionine is critical for both the development and maintenance of the biofilm, as well as for glycocalyx formation.

DISCUSSION

Antibiotic-resistant pathogens have become more and more prevalent. Notably, members of the ESCAPE group, such as *P. aeruginosa*, *E. coli*, *S. aureus*, *K. pneumoniae*, and *A. baumannii*, are frequently highly resistant to multiple classes of antibiotics, making the development of alternative treatment strategies a pressing challenge (32). It has become more and more difficult to find new compounds active against the small number of known antibacterial targets, and previous strategies for the identification of new targets has had only limited success. Therefore, new routes for the identification of novel antibiotics or, as a first step, the identification of new targets are desperately needed. Such targets might exist within bacterial central metabolism or within biosynthetic pathways for essential biomolecules, such as amino acids or vitamins. However, conventional antibiotic screening programs rely on the use of complex growth media. These media contain an excess of essential nutrients, which may have to be synthesized endogenously during *in vivo* growth and infection. The methionine biosynthetic pathway represents an interesting target, as many aspects suggest it could be a valid target for novel anti-infectives (33, 34). However, whether the host can provide sufficient methionine to overcome auxotrophy is unclear.

Methionine auxotrophy and the regulation of methionine biosynthesis have been studied for decades (35–38), and important characteristics of auxotrophs are known. For auxotrophs of *E. coli*, the external methionine concentration governs the growth rate, suggesting that this molecule is of central importance (39). Furthermore, it was reported that methionine limitation abolished chemotaxic migration (40, 41). However, knowledge regarding the roles of methionine and the phenotypic consequences of limitation in different species is incomplete.

S. aureus, *P. aeruginosa*, and *E. coli* each differ in discrete steps in methionine

biosynthesis and the enzyme classes employed. They represent the major pathways of methionine biosynthesis found among most bacterial pathogens. We found that methionine auxotrophs of all three species needed higher levels of exogenous methionine than those that occur in human serum to reach WT levels of growth yield. The defect was most prominent in *E. coli* and *P. aeruginosa* where >100 μM methionine was needed. In contrast, *S. aureus* needed 50 to 100 μM methionine to restore full growth. Human serum is reported to contain only between 25.6 and 48 μM methionine (16, 17, 42, 43). Such variations are most likely due to differences in dietary intake. Additionally, diseases, such as multiple sclerosis and liver failure, are associated with increased levels of serum methionine (16, 44).

Pooled human serum was used to determine whether an average concentration of methionine in serum supports bacterial growth. In these experiments, we used mutants deficient in the first steps of methionine biosynthesis (acetylation) to allow any downstream intermediates, such as acylhomoserine, cystathionine, and homocysteine, that might be available in human serum to serve as potential methionine precursors. A level of homocysteine in human serum of ~10.3 μM was measured (16), a concentration that needs to be considered when interpreting results. However, we found that human serum did not supply adequate methionine, as it did not allow any of the organisms tested to overcome the growth disadvantage associated with methionine auxotrophy. This observation correlates with the finding that methionine auxotrophs of *Salmonella enterica* serovar Typhimurium and of the fungus *Cryptococcus neoformans* are attenuated during invasive disease (45, 46). Interestingly, bronchoalveolar fluid (47) was found to be devoid of methionine, and accordingly, transposon insertion sequencing identified methionine biosynthesis as important during lung infection by *K. pneumoniae* (48) and *A. baumannii* (49). Also, human nasal secretions are devoid of methionine, and *S. aureus* methionine auxotrophs are attenuated in the cotton rat model of nasal colonization (15).

As such, methionine biosynthesis inhibitors might hold the potential to treat nasal colonization of methicillin-resistant *S. aureus* or to treat systemic infections. Several inhibitors of the individual steps of methionine biosynthesis are known. The clavams (50) and microcin 15m (51) inhibit acetylation reactions, while propargylglycine and several other inhibitors of the sulfurylation reaction are available (52, 53). Yet, to our knowledge, none of these inhibitors has been tested for toxic effects on eukaryotic cells or for the potential to eliminate infections caused by MDR pathogens. The potential value of inhibiting the anabolic processes should not be underestimated. For instance, trimethoprim and sulfamethoxazole inhibit the biosynthesis of folate and are used for decades to treat bacterial infection.

The failure to produce methionine endogenously also impacts bacterial phenotypes that are not directly associated with the growth rate. Formyl-methionine is used by all bacteria for the initiation of translation. Furthermore, polypeptide elongation needs additional methionine if the mRNA possesses methionine codons. Methionine limitation will, therefore, reduce the translation rate and most likely reduce the amount of protein synthesized. Many bacterial species produce biofilms where bacterial cells are embedded in an extracellular matrix consisting of polysaccharides, eDNA, and in many cases vast amounts of secreted and cell surface-associated proteins (6). Staphylococci classically produce two forms of biofilm. The first form is produced by *S. epidermidis* and many methicillin-sensitive *S. aureus* strains and depends on the polysaccharide PIA (54). However, proteins are still important for the formation of the biofilm, as they allow primary attachment to surfaces and an accumulation of cells (i.e., the accumulation-associated protein Aap [55]). Most MRSA strains produce proteinaceous biofilms that are independent of polysaccharides, while proteins mediate the cross-linking of cells and allow formation of the extracellular matrix (i.e., the fibronectin-binding proteins FnbpA/B [22], the extracellular matrix binding protein Embp [56], and the biofilm associated protein Bap [57]). We found that inhibition of methionine biosynthesis by PG impacted biofilm formation of *S. aureus* SH1000, a strain known to form proteinaceous biofilms. In contrast, PG had no impact on the biofilm formed by *S. epidermidis* RP62a,

which forms a PIA-dependent biofilm. These experiments suggest that PG-mediated methionine limitation reduces the amount of produced and secreted proteins, thereby reducing the biofilm quantity. Indeed, we discovered that the abundance of cell wall-associated proteins decreased after PG treatment of *S. aureus*, while the amount of PIA produced by *S. epidermidis* was not.

P. aeruginosa produces a biofilm that consist of various exopolysaccharides as well as eDNA and proteins (28). We found that methionine limitation had a profound impact on biofilm formation of *P. aeruginosa*. Even with 200 μM exogenous methionine, the biofilm formed by the auxotrophic mutants showed a significantly reduced biomass compared with the biofilm formed by the WT. Interestingly, we found that the number of viable cells within the biofilms did not differ between the WT and the individual mutants. However, when the biofilms were transferred to methionine-deficient conditions, the biofilms formed by the mutants dissolved over a period of 2 days, leading to a rapid decrease of live bacterial numbers. The mechanisms underlying the dependence of *P. aeruginosa* biofilms on methionine availability have yet to be investigated. However, we hypothesize that membrane-associated proteins responsible for the processing of polysaccharides might contribute to the effects observed in our models. Under methionine-limiting conditions, methionine auxotrophic strains failed to incorporate Congo red stain into the surface of colonies. This phenotype is a hallmark of Pel-deficient strains that cannot process carbohydrates to form biofilms (26). We suggest that methionine limitation might reduce the abundance of Psl/Pel proteins, thereby reducing biofilm production. However, this hypothesis needs further experimental evidence to be validated and other explanations are possible. For example, the 220-kDa protein CdrA has been described to bind to Psl polysaccharides and to tether them to the cell envelope of *P. aeruginosa*, thereby strengthening and reinforcing biofilm integrity (58). It seems possible that a lack of methionine leads to insufficient biosynthesis of CdrA or similar biofilm-scaffolding proteins, thereby preventing appropriate biofilm formation or restructuring.

Our results show that promising antimicrobial effects can be achieved by targeting bacterial methionine biosynthesis *in vitro*. Not only proliferation but also biofilm formation is affected when endogenous methionine biosynthesis is prevented, suggesting this biosynthesis pathway as an interesting target for antibiotic intervention. However, whether methionine biosynthesis inhibitors do indeed hold therapeutic potential *in vivo* is still unclear, and further experiments using methionine biosynthesis blockers to treat infections *in vivo* will be required to answer this question.

MATERIALS AND METHODS

As long as not stated otherwise, materials were purchased from Sigma-Aldrich.

Bacterial strains and growth conditions. All bacterial strains used in this study are listed in Table 2. If not specified otherwise, *S. aureus* strains were grown at 37°C in tryptic soy broth (TSB; Oxoid) or on tryptic soy agar (TSA). *E. coli* and *P. aeruginosa* strains were grown at 37°C in lysogeny broth (LB) or on lysogeny agar (LA) (Oxoid).

All liquid overnight cultures for the experiments described below were grown in 20-ml volumes in 100-ml flasks containing one baffle. Cultures were incubated at 37°C with 140 rpm agitation in an Innova 44 incubator (New Brunswick Scientific). CO_2 content was not controlled.

Creation of markerless deletion mutants in *S. aureus*. Targeted mutagenesis of *S. aureus* was performed using the thermosensitive plasmid pIMAY and allelic exchange. The procedure is described in detail elsewhere (59). In brief, 500-bp DNA fragments upstream and downstream of the genes of interest were amplified by PCR (oligonucleotides are summarized in Table 3). A sequence overlap was integrated into the fragments to allow fusion and created an ATG-TAA scar in the mutant allele. The 1-kb deletion fragments were created using spliced extension overlap PCR and cloned into pIMAY. The deletion plasmids were used to transform *S. aureus* target strains using a standard procedure at 30°C. Integration of the plasmid was selected at 37°C, and excision was promoted by growing the strains in liquid culture overnight at 30°C. Loss of the plasmid was selected on plates containing 1 μg/ml anhydrotetracycline, and the sensitivity of strains toward chloramphenicol was confirmed. Strains carrying the mutant allele were identified by colony PCR.

Construction of pME6032::Meta. The *metA* gene was amplified from the chromosome of *E. coli* BW25113 by PCR (Table 3). The PCR product was purified and treated with EcoRI and BglII. After purification, the DNA fragment was cloned into pME3032 treated with the same endonucleases as mentioned previously. The recombinant plasmid was used to transform *E. coli* XL1-Blue using standard techniques. The plasmid was validated by Sanger sequencing and transferred to *E. coli* BW25113Δ*metA*.

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype(s) or description(s)	Source[a]
Bacterial strains		
E. coli K12 BW25113	Wild type	CGSC (no. 7636)
E. coli K12 BW25113	Δ*metA*	CGSC (no. 10856)
E. coli K12 BW25113	Δ*metB*	CGSC (no. 10824)
E. coli K12 BW25113	Δ*metC*	CGSC (no. 10286)
E. coli K12 BW25113	Δ*metA*, pME6032	This study
E. coli K12 BW25113	Δ*metA*, pME6032::*metA*	This study
P. aeruginosa PA14	Wild type	PA14 transposon insertion mutant library
P. aeruginosa PA14	Δ*metX*	PA14 transposon insertion mutant library (ID 55735)
P. aeruginosa PA14	Δ*metY*	PA14 transposon insertion mutant library (ID 40250)
P. aeruginosa PA14	Δ*metZ*	PA14 transposon insertion mutant library (ID 31011)
P. aeruginosa PA14	Δ*metE*	PA14 transposon insertion mutant library
P. aeruginosa PA14	Δ*metH*	PA14 transposon insertion mutant library
S. aureus Newman	Wild type	61
S. aureus Newman	Δ*metC*	This study
S. aureus Newman	Δ*metI*	15
S. aureus Newman	Δ*metX*	This study
S. aureus SH1000	Wild type	62
S. aureus SH1000	Δ*metC*	This study
S. aureus USA300 LAC	Wild type	63
S. aureus USA300 LAC	Δ*metI*	15
S. epidermidis RP62A	Wild type	64
S. epidermidis RP62A/3	Δ*icaA*	65
Plasmids		
pIMAY	Thermosensitive vector for allelic exchange	59
pIMAY::Δ*metX*		This study
pIMAY::Δ*metC*		This study
pME6032	IPTG inducible expression plasmid	66
pME6032::*metA*	Complementation of *metA* in *E. coli*	This study

[a]CGSC, Coli Genetic Stock Center; ID, identifier.

Bacterial growth in methionine-limited media. For overnight cultivation, *S. aureus* was grown in synthetic nasal medium 3 (SNM3) (15) containing 30 μM methionine. *E. coli* and *P. aeruginosa* strains were grown in M9 medium containing 100 μM methionine. Cells were harvested by centrifugation (10 min, 5,000 $\times$ *g*), washed with phosphate-buffered saline (PBS), and adjusted to an OD at 600 nm (OD_{600}) of 1. In a 48-well plate, (Nunclon Delta surface) 500-μl volumes of defined medium (SNM20 for *S. aureus* and M9 for *E. coli* and *P. aeruginosa*) containing different methionine concentrations were inoculated to an OD_{600} of 0.01. The plates were sealed with adhesive covers (Excel Scientific) and incubated at 37°C in an Epoch 2 microplate reader (BioTek) for 20 h while agitating (807 rpm). The OD_{600} was measured every 15 min.

For complementation of *E. coli* Δ*metA*, the overnight cultures were grown and harvested as described above. For the growth assay in the microtiter plate, M9 medium without methionine was supplemented with 1 mM IPTG. The plate was incubated as described above.

Growth in human serum. For overnight cultivation, staphylococci were grown in synthetic nasal medium 3 (SNM3) (15) containing 30 μM methionine. *E. coli* and *P. aeruginosa* strains were grown in synthetic M9 medium containing 100 μM methionine. Cells were harvested by centrifugation (10 min,

TABLE 3 Oligonucleotides used in this study

Name	5′–3′ sequence	Purpose
metC_A	AGGGAACAAAAGCTGGGTACCACTAGCAGGTGTCGTAACCGTC	Cloning of the *metC* deletion fragment for *S. aureus*
metC_B	CATACAATCTCTCCAATCTGAGC	Cloning of the *metC* deletion fragment for *S. aureus*
metC_C	AGATTGGAGAGATTGTATGTAATGTTTTAGTAGCTGATGGCG	Cloning of the *metC* deletion fragment for *S. aureus*
metC_D	CACTATAGGGCGAATTGGAGCTCAAGTAATTTGTGTTTGAAGCGGTTA	Cloning of the *metC* deletion fragment for *S. aureus*
metC_Scr.F	TTATCGACAATACTTTTTTAACACC	Screening of the *metA* deletion fragment for *S. aureus*
metC_Scr.R	GTTGTTTTAATCCTTCATTGATTGC	Screening of the *metA* deletion fragment for *S. aureus*
metX_A	GGAACAAAAGCTGGGTACCCATCCCTTACATTATCGCGCTC	Cloning of the *metX* deletion fragment for *S. aureus*
metX_B	CATTGTTCTTTCCTCCTTAAAC	Cloning of the *metX* deletion fragment for *S. aureus*
metX_C	GGAGGAAAGAACAATGTAATAATATATATTTTCAAAGAATGAAGCC	Cloning of the *metX* deletion fragment for *S. aureus*
metX_D	CACTATAGGGCGAATTGGAGCTCGTTGTTATAATTACTGTAATAAGTGC	Cloning of the *metX* deletion fragment for *S. aureus*
metX_Scr.F	TGTCATTGACAGCATTATTCAACAG	Screening of the *metX* deletion fragment for *S. aureus*
metX_Scr.R	CTAATATGATGGCACTTAAAACGAAAG	Screening of the *metX* deletion fragment for *S. aureus*
metA_F	TAATGAATTCATGCCGATTCGTGTG	Cloning of the *metA* complementation fragment
metA_R	CGATCGAAGATCTCAGAAGATTAATCCAG	Cloning of the *metA* complementation fragment

5,000 × *g*), washed with PBS, and adjusted to an OD_{600} of 0.1. Heat-inactivated pooled human serum derived from healthy males (Sigma-Aldrich) was diluted in either SNM20 or M9 media to a final concentration of 50%, 25%, or 10%, and 200-μl volumes were transferred to a 96-well plate (Greiner). Each well was inoculated with 15 μl of cells (OD_{600}, 0.1; final concentration, ~5×10^6 CFU/ml), sealed with adhesive covers, and incubated at 37°C with shaking (140 rpm) for 24 h in an Innova 44 incubator. To enumerate CFUs, samples were taken and serial dilutions were plated on TSB agar plates.

Crystal violet assay for quantification of bacterial biofilms. *S. aureus* was grown overnight in SNM3 (15) containing 30 μM methionine. *S. epidermidis* was grown in modified Tris minimal succinate (mTMS) medium containing 30 μM methionine. mTMS was prepared according to a previous report (60), with the exception that casamino acids were replaced with proteinogenic amino acids (A, 990 μM; R, 660 μM; C, 66 μM; E, 660 μM; G, 990 μM; H, 330 μM; L, 1,980 μM; K, 990 μM; F, 990 μM; P, 990 μM; S, 792 μM; T, 1,320 μM; W, 132 μM; and V, 660 μM) and ornithine-HCl (660 μM). After overnight growth, cells were harvested by centrifugation (10 min, 5,000 × *g*) and adjusted to OD_{600} of 1 in SNM3/mTMS containing 1% glucose. This suspension was diluted 1:200 in SNM3/mTMS containing 1% glucose. For *S. aureus* experiments, 96-well Nunclon Δ surface microtiter plates (Thermo Fisher) were coated overnight at 4°C with 100 μl of 0.045 μg/ml fibrinogen (Calbiochem) to increase biofilm formation. After the plates were washed three times with PBS, 200 μl of the inoculated defined medium was added to the wells. For inhibition experiments, a final concentration of 100 μg/ml propargylglycine was added. The plate was incubated statically for 24 h at 37°C. The plates were washed thrice with PBS, stained with 100 μl of 2.3% crystal violet (Sigma-Aldrich), and washed five times with 300 μl PBS. Retained color was extracted in 100 μl 5% acetic acid. OD at 570 nm (OD_{570}) was quantified using a CLARIOstar device (BMG Labtech).

For *P. aeruginosa* biofilms, bacteria were grown overnight in M9 medium containing 100 μM methionine. Overnight cultures were harvested, adjusted to an OD_{600} of 1 in M9 medium (100 μM methionine), and used to inoculate (1:200) M9 medium with appropriate concentrations of methionine. A total of 200 μl was transferred to 96-well plates and grown statically at 37°C for 48 h. The 96-well plate was washed and stained with crystal violet as described above.

Isolation of *S. aureus* cell wall extracts. *S. aureus* was grown overnight in SNM3 containing 30 μM methionine. Cells were harvested by centrifugation (10 min, 5,000 × *g*), and washed with SNM3 without methionine. A 20-ml volume of SNM3 was inoculated to an OD_{600} of 0.1, and PG (0 to 100 μg/ml) was added. The culture was incubated with agitation for 24 h. Cells were collected (10 min, 5,000 × *g*) and washed once with wash buffer (WB; 10 mM Tris-HCl [pH 7] and 10 mM $MgCl_2$). A total of 1 ml WB was adjusted to an OD_{600} of 2. Cells were collected and resuspended in 1 ml digestion buffer (10 mM Tris-HCl [pH 7], 10 mM MgCl, 500 mM sucrose, 0.3 mg/ml lysostaphin, 250 U/ml mutanolysin, 30 μl protease inhibitor cocktail [Roche; 1 complete mini tablet dissolved in 1 ml H_2O], and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and incubated at 37°C for 1 h. The protoplasts were removed by centrifugation (8,000 × *g* for 10 min at 4°C). The supernatant (containing the cell wall fraction) was collected, and 15 μl was used for SDS-PAGE analysis using standard techniques. Next, 7.5% SDS gels were stained with Coomassie brilliant blue G 250 (Serva). As Coomassie blue protein stain is a strong 700-nm fluorophore, the protein amount within individual bands was quantified using an Odyssey CLx device (LI-COR) which allows detection and accurate quantification of fluorescent signals in the near infrared spectrum.

Isolation of *S. epidermidis* biofilm matrix. A 10-ml bacterial culture (mTMS, 30 μM methionine) was grown overnight in a 50-ml flask containing one baffle at 37°C. Bacteria were harvested and washed once with PBS. Next, 2.5 ml methionine-free mTMS was inoculated to an OD_{600} of 0.01, and 0 to 100 μg/ml PG was added. A total of 200-μl volumes of the inoculated medium were dispersed to 12 wells of a 96-well plate (Nunclon Delta surface). The plate was incubated at 37°C for 24 h. The plate was incubated for 30 min in an ultrasonic bath (Bandelin SONOREX RK 100). The biofilm was scratched off the wells and centrifuged at 3,000 × *g* for 10 min. The pellet was resuspended in 200 μl of 0.1 M EDTA (pH 8). The suspension was incubated for 30 min in an ultrasonic bath and centrifuged at 8,000 × *g* for 10 min. The supernatant was used for immunoblot analysis. Serial dilutions were prepared, and 5 μl was spotted on a nitrocellulose membrane (LI-COR). The membrane was blocked for 20 min with blocking buffer (Thermo Scientific) and incubated for 20 min with 130 ng/ml wheat germ agglutinin coupled to horseradish peroxidase (Biotium). The blot was washed twice with Tris-buffered saline with Tween 20 (TBST) and once with Tris-buffered saline (TBS). Detection was carried out using the WesternSure premium chemiluminescent substrate (LI-COR) and the Bio-Rad ChemiDoc XRS imaging system.

Peg plate assay of biofilm formation and detachment of *P. aeruginosa* PA14. *P. aeruginosa* strains were grown overnight in M9 medium with 100 μM methionine. Overnight cultures were diluted to an OD_{600} of 0.1 in M9 medium (with 100 μM methionine), and 200 μl was transferred to a 96-well plate (Thermo Scientific) with a transferable 96-peg solid-phase (TSP) plate on top (Thermo Scientific). The pegs of the TSP plate reached into the inoculated medium and allowed initial bacterial adhesion. The plates were incubated statically for 2 h at 37°C. The TSP plate was transferred to a new 96-well plate filled with 200 μl of sterile M9 medium containing 100 μM methionine and incubated statically for 48 h at 37°C. If needed, the TSP was subsequently transferred to a new microtiter plate containing sterile M9 medium with various concentrations of methionine and incubated for an additional 24 to 48 h. For enumeration of living cells within the biofilms, TSP plates were transferred onto a 96-well plate containing 200 μl 0.1 M EDTA and 0.1% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) and incubated for 1 h on a rocking table. CFUs were enumerated by plating serial dilutions of the bacterial suspensions onto LB agar plates.

Congo red assay to detect *P. aeruginosa* glycocalyx. *P. aeruginosa* strains were grown overnight in M9 medium containing 100-μM methionine. Cells were harvested, washed with methionine-free M9

medium and adjusted to an OD_{600} of 0.025 in the same medium. A total of 5 μl of the bacterial suspension was spotted on defined M9 agar plates containing 30 to 100 μM methionine as well as 40 μg/ml Congo red and 20 μg/ml Coomassie brilliant blue G-250. The color of arising colonies was assessed after incubation at 37°C for 24 h followed by further incubation at room temperature for 24 h.

Statistics. Statistical analysis was performed by using GraphPad Prism.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00177-19.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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