**Staphylococcus aureus** Genomes Harbor Only MpsAB-Like Bicarbonate Transporter but Not Carbonic Anhydrase as Dissolved Inorganic Carbon Supply System

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**ABSTRACT** In recent years, it became apparent that not only autotrophic but also most other bacteria require CO$_2$ or bicarbonate for growth. Two systems are available for the acquisition of dissolved inorganic carbon supply (DICS): the cytoplasmic localized carbonic anhydrase (CA) and the more recently described bicarbonate transporter MpsAB (membrane potential generating system). In the pathogenic species *Staphylococcus aureus*, there are contradictions in the literature regarding the presence of a CA or MpsAB. Here, we address these contradictions in detail. We could demonstrate by careful BLASTp analyses with 259 finished and 4,590 unfinished *S. aureus* genomes that *S. aureus* does not contain CA and that the bicarbonate transporter MpsAB is the only DICS system in this species. This finding is further supported by two further pieces of evidence: (i) *mpsAB* deletion mutants in four different *S. aureus* strains failed to grow under atmospheric air, which should not be the case if they possess CAs, since we have previously shown that both CA and MpsAB can substitute for each other, and (ii) *S. aureus* is completely resistant to CA inhibitors, whereas *Staphylococcus carnosus*, which has been shown to have only CA, was inhibited by ethoxyzolamide (EZA). Taken together, we demonstrate beyond doubt that the species *S. aureus* possesses only the bicarbonate transporter MpsAB as its sole DICS system.

**IMPORTANCE** The discrepancies in the current literature and even in NCBI database, which listed some protein sequences annotated as *Staphylococcus aureus* carbonic anhydrase (CA), are misleading. One of the existing problems in publicly available sequence databases is the presence of incorrectly annotated genes, especially if they originated from unfinished genomes. Here, we demonstrate that some of these unfinished genomes are of poor quality and should be interpreted with caution. In the present study, we aimed to address these discrepancies and correct the current literature about *S. aureus* CA, considering the medical relevance of *S. aureus*. If left unchecked, these misleading studies and wrongly annotated genes might lead to a continual propagation of wrong annotation and, consequently, wrong interpretations and wasted time. In addition, we also show that bicarbonate transporter MpsAB-harboring bacteria are resistant to CA inhibitor, suggesting that pathogens possessing both MpsAB and CA are not treatable with CA inhibitors.

**KEYWORDS** carbonic anhydrase, *Staphylococcus carnosus*, Firmicutes, MpsAB, *Staphylococcus aureus*, bicarbonate transporter

Bicarbonate or hydrogen carbonate is a simple carbon molecule which occupies surprisingly crucial roles in various biological processes: for example, the tricarboxylic acid (TCA) cycle, cellular pH and volume regulation, and photosynthesis (1). The biochemistry of bicarbonate is fundamental to nearly all domains of life. For this reason, there are numerous pathways responsible for the fixation and assimilation of dissolved inorganic carbon (DIC), which consists mainly of free CO$_2$ (gas), the bicarbonate ion (HCO$_3^-$), and carbonate ion (CO$_3^{2-}$).
(CO₂⁻) (2). In plants and most autotrophic bacteria, the first reaction of photosynthetic CO₂ fixation is catalyzed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) via the Calvin-Benson-Bassham (CBB) cycle.

That being said, HCO₃⁻ is also equally as important even for nonautotrophic bacteria due to the fact that many metabolic pathways require either HCO₃⁻ or CO₂ as the substrates or as products of metabolism (3, 4). In this regard, these bacteria utilize the enzyme carbonic anhydrase (CA) as a dissolved inorganic carbon supply (DICs) system (3, 5) and the more recently described bicarbonate transporter MpsAB (membrane potential generating system) in Staphylococcus aureus (4). MpsAB is present not only in autotrophic bacteria such as Hydrogenovibrio crunogenus, Nitrobacter winogradskyi (6), and Halothiobacillus neapolitanus (7) but also in many nonautotrophic bacteria, like some strains of Bacillus subtilis, Legionella pneumophila, and Vibrio cholerae (6, 8). MpsAB works alone and/or together with CA function to supply bicarbonate for anaerobic reactions. Although both systems are interchangeable, they rarely coexist in a given species (4).

In our previous work, we showed that Staphylococcus carnosus harbors only a CA gene and confirmed that the protein is functional (8). As there was no St. carnosus-specific CA homolog present in S. aureus, we deduced that MpsAB functions as the sole CO₂/bicarbonate concentration system in S. aureus. Moreover, MpsAB outperforms CA, and the former has an advantage in species where CO₂ diffusion is impeded, for example in mucus biofilm-forming bacteria. As such, our findings are in contradiction with other studies about the presence of CA in S. aureus.

Since there are several publications in which an S. aureus-specific CA has been described and also studied (9–12), we investigated the question of whether a CA actually exists in S. aureus. Using BLASTp analyses, phenotypic characterization of mpsAB mutants, and the resistance studies to CA inhibitors, we demonstrate that there is no CA present in S. aureus.

RESULTS

All finished S. aureus genomes contain no CA-related Pfam motifs. To enable a quick and systematic search for the presence of CA in S. aureus, we screened for the occurrence of protein families (Pfam) motifs (PFam00484, PFam00194, and PFam10563 for prokaryotic-type CAs, eukaryotic-type CAs, and putative CA-like domain, respectively) using the database from Integrated Microbial Genomes and Microbiomes (IMG/M) (13). We used this database instead of NCBI because it is more organized to perform searches using the database from Integrated Microbial Genomes and Microbiomes (IMG/M) (13). Since there are several publications in which an S. aureus-specific CA has been described and also studied (9–12), we investigated the question of whether a CA actually exists in S. aureus. Using BLASTp analyses, phenotypic characterization of mpsAB mutants, and the resistance studies to CA inhibitors, we demonstrate that there is no CA present in S. aureus.

To demonstrate the reliability of Pfam motifs, we performed protein-protein Basic Local Alignment Search Tool search (BLASTp) of the protein sequence of an experimentally confirmed CA from Staphylococcus carnosus (8) against fully sequenced (finished) representative genomes from the genus Staphylococcus. The presence of CAs based on Pfam motif correlated with the high percentage of protein identity from S. carnosus CA (Table 1). No significant protein identity was detected when there was no Pfam motif present, such as in S. aureus, Staphylococcus haemolyticus, and Staphylococcus lugdunensis. In addition, a search in all the 259 finished S. aureus genomes in IMG/G and also AureoWiki (14), which is manually curated, revealed that no protein is annotated as CA or putative CA.

BLASTp showed no protein similarity of α-, β-, and γ-CAs in S. aureus. Given that S. aureus and S. carnosus are from the same genus, they should share protein homology and more similarity with each other than with any bacteria from other genera. Therefore, the protein sequence of S. carnosus CA, which is from the class of β-CAs, was subjected to BLASTp search against all finished S. aureus genomes in IMG/M, but no similarity was found. As not all the microbial genomes might be integrated in IMG/M yet, we also performed the same BLASTp against S. aureus (taxonomy ID [taxid]: 1280) in NCBI database. We found two hits: NCBI accession numbers SPZ78436.1 and SPZ78435.1 (Table 2). As both the proteins are found in only one strain and based on the data in Table 2, most likely the genomes samples sequenced belonged to other staphylococcal species or the genes were wrongly annotated. Therefore, we concluded that there is no β-CA in S. aureus.
Since the different classes of CAs have independent evolutionary origins (3), we also searched for the presence of α- and γ-CAs in *S. aureus*. We selected some bacteria whose CAs were experimentally proven, and these protein sequences were subjected to BLASTp search in *S. aureus*, as well as two CA-harboring species, *S. carnosus* and *Staphylococcus pseudintermedius*, as controls (Table 3 and 4). As shown in Table 3, there was no similarity among these CAs with *S. aureus*, *S. carnosus*, and *S. pseudintermedius* except for two cases. First, BLASTp of two human CAs resulted in two hits with proteins annotated as *S. aureus* CA (Table 3). Considering that they are found in only two unfinished *S. aureus* genomes (Table 3) and the errors observed in these sequences (Table 2), the genome samples were most likely contaminated. For the same reason as that mentioned previously, we deduced that there are no α- and γ-CAs in *S. aureus* (Table 3 and 4).

**TABLE 1** The presence of CAs inferred from Pfam motif correlates with the CA protein identity of *S. carnosus* in selected finished *Staphylococcus* genomes

| Genome                        | CA* (based on Pfam) | Identity (%) (amino acids aligned based on BLASTp) |
|-------------------------------|---------------------|---------------------------------------------------|
| *Staphylococcus agnetis* 908  | +–                  | 73 (135/186)                                      |
| *Staphylococcus argenteus* BN75 | ––                  |                                                   |
| *Staphylococcus aureus* aureus MSHR1132 | ––                  |                                                   |
| *Staphylococcus aureus* aureus USA300_FPR3757 | ––                  |                                                   |
| *Staphylococcus capitis* AYP1020 | ––                  |                                                   |
| *Staphylococcus carnosus* LTH 3730 | +–                  | 100 (192/192)                                    |
| *Staphylococcus cohnii* SNUDS-2 | ––                  |                                                   |
| *Staphylococcus condimenti* DSM 11674 | +–                  | 97 (187/192)                                    |
| *Staphylococcus epidermidis* RP62A | ––                  |                                                   |
| *Staphylococcus equorum* K51039 | ––                  |                                                   |
| *Staphylococcus felis* ATCC 49168 | +–                  | 73 (135/186)                                    |
| *Staphylococcus haemolyticus* JCSC1435 | ––                  |                                                   |
| *Staphylococcus hominis* hominis K1 | ––                  |                                                   |
| *Staphylococcus hyicus* ATCC 11249 | +–                  | 72 (134/185)                                    |
| *Staphylococcus lugdunensis* C_33 | ––                  |                                                   |
| *Staphylococcus lutrae* ATCC 700373 | +–                  | 70 (133/191)                                    |
| *Staphylococcus muscae* NCTC 13833 | +–                  | 70 (130/188)                                    |
| *Staphylococcus nepalensis* JS1 | ––                  |                                                   |
| *Staphylococcus pasteurii* SP1 | ––                  |                                                   |
| *Staphylococcus pettenkofleri* FDAARGOS_288 | +–                  | 75 (140/192)                                    |
| *Staphylococcus piscifermentans* NCTC 13836 | +–                  | 96 (185/192)                                    |
| *Staphylococcus pseudintermedius* ED99 | +–                  | 70 (131/188)                                    |
| *Staphylococcus saprophyticus* 883 | ––                  |                                                   |
| *Staphylococcus schleiferi* 1360-13 | +–                  | 73 (132/185)                                    |
| *Staphylococcus sciuri* SNUSD-18 | +–                  | 67 (126/188)                                    |
| *Staphylococcus simulans* SIMI 13838 | ––                  |                                                   |
| *Staphylococcus simulans* FDAARGOS_124 | +–                  | 87 (167/192)                                    |
| *Staphylococcus stepanovicii* NCTC 13839 | +–                  | 66 (125/190)                                    |
| *Staphylococcus succinus* 14BME20 | ––                  |                                                   |
| *Staphylococcus warneri* SG1 | ––                  |                                                   |
| *Staphylococcus xylosus* SMO121 | ––                  |                                                   |

*The presence of the proteins was inferred based on the following protein families (Pfam) domains search from finished bacterial genomes in the Integrated Microbial Genomes and Microbiomes (IMG/M) database: prokaryotic type-carbonic anhydrase (CA) (pro) (PFam00484), eukaryotic-type CA (euk) (PFam00194), and PFam10563 for putative CA-like domain. The symbols + and – indicate the presence or absence of the protein domains. Identity refers to identical residues shared with CA from *S. carnosus* (WP_015900702.1) using protein-protein Basic Local Alignment Search Tool (BLASTp).*
these genomes are often unreliable and should be interpreted with caution. To prove our point, we extended the same BLASTp search in 4,590 unfinished genomes. Results similar to those found with finished genomes were found, and all other hits were found in assemblies which were marked as contaminated by NCBI (Table 5). One particular strain, C0673, showed multiple hits for NGG14433.1, NGB42162.1, SPZ78435.1, SPZ78436.1, and WP_094666538.1.

According to NCBI, C0673 is an unfinished genome with 89 contigs where the taxonomy check is inconclusive. Although this strain is annotated as \textit{S. aureus} C0673 in NCBI database, it is highly questionable. Thus, we downloaded the genome sequence and checked it against public databases for molecular typing and microbial genome

### TABLE 2 Analysis of proteins wrongly annotated as \textit{S. aureus} CA in NCBI

| NCBI accession no./ length (amino acids) | Annotation in NCBI | Source (strain) | Comment |
|-----------------------------------------|-------------------|----------------|---------|
| MBO8619751.1 (64) | Carboxyl anhydrase family protein, partial \textit{(Staphylococcus aureus)} | \textit{S. aureus} strain IHMA68, unfinished genome with 268 contigs$^b$ | When this sequence was subjected to BLASTp search in NCBI, there was only one hit against its own sequence (100% identity). The rest of the hits were from multiple organisms with the highest identity from one \textit{Homo sapiens} and other primates such as \textit{Hylobates} moloch, \textit{Pan troglodytes}, and \textit{Pongo abelii} (86–91% identity). |
| MBO8666615.1 (77) | Carboxyl anhydrase family protein, partial \textit{(Staphylococcus aureus)} | \textit{S. aureus} strain IHMA56, unfinished genome with 680 contigs$^b$ | When this sequence was subjected to BLASTp search in NCBI, there was only one hit against its own sequence (100% identity). The rest of the hits were from multiple organisms with the highest identity from \textit{Homo sapiens} (100% identity, 100% protein coverage). |
| MVW54107.1 (151) | Carboxyl anhydrase, partial \textit{(Staphylococcus aureus)} | \textit{S. aureus} strain mecC 165 PE, unfinished genome with 37 contigs | When this sequence was subjected to BLASTp search in NCBI, there was only one hit against its own sequence (100% identity). The rest of the 99 hits were from different bacteria like \textit{Acidobacteria} bacterium, \textit{Ignavibacteriales} bacterium, etc., with 47–91% identity (98–100% protein coverage). |
| NGB42162.1 (184) | Gamma-carboxyl anhydrase family protein \textit{(Staphylococcus aureus)} | \textit{S. aureus} strain UG302, unfinished genome with 167 contigs$^a$ | When this sequence was subjected to BLASTp search in NCBI, there was no hit even against its own sequence or any \textit{S. aureus} proteins. All of the 100 hits were from \textit{Salmonella enterica}, with 99–100% identity. |
| NGG14433.1 (97) | Gamma-carboxyl anhydrase family protein, partial \textit{(Staphylococcus aureus)} | \textit{S. aureus} strain UG271, unfinished genome with 397 contigs$^a$ | When this sequence was subjected to BLASTp search in NCBI, there was only one hit against its own sequence (100% identity). The rest of the 99 hits were all from \textit{Salmonella enterica} with 99% identity and 100% protein coverage. |
| OWU61334.1 | Carboxyl anhydrase, partial \textit{(Staphylococcus aureus)} | \textit{S. aureus} strain W1, unfinished genome with 380 contigs | When this sequence was subjected to BLASTp search in NCBI, there was only one hit with 100% identity but it was annotated as SulP family inorganic anion transporter, partial from \textit{S. aureus}. The rest were almost all from \textit{Mycobacterium tuberculosis} (100% identity). |
| SPZ78435.1 (61) | Carboxyl anhydrase \textit{(Staphylococcus aureus)} | \textit{S. aureus} strain NCTC12981, unfinished genome with 15 contigs | When this sequence was subjected to BLASTp search in NCBI, there was only one hit against its own sequence (100% identity). The rest were multiple hits from other \textit{Staphylococcus} species, with the highest identity from \textit{Staphylococcus coagulans} (98% identity with 86% coverage). |
| SPZ78436.1 (193) | Carboxyl anhydrase \textit{(Staphylococcus aureus)} | \textit{S. aureus} strain NCTC12981, unfinished genome with 15 contigs | When this sequence was subjected to BLASTp search in NCBI, there was only one hit against its own sequence (100% identity). The rest were multiple hits from other \textit{Staphylococcus} species with the highest identity from \textit{Staphylococcus schleiferi} which covers 69% of the protein length with 100% identity. |
| WP_094666538.1 (149) | Gamma-carboxyl anhydrase family protein, partial \textit{(Staphylococcus aureus)} | \textit{S. aureus} strain UV695, unfinished genome with 468 contigs | When this sequence was subjected to BLASTp search in NCBI, there was only one hit against its own sequence (100% identity) and almost all the hits were from \textit{Enterococcus faecium} or \textit{Enterococcus} sp. with 99–100% identity. |

$^a$SulP, sulfate permease.
$^b$Submitted by the same group.
$^c$Submitted by the same group.
| Species            | UniProt ID/length (amino acids) | Ref | S. aureus (NCBI taxid: 1280) | S. camosus (NCBI taxid: 1281) | S. pseudintermedius (NCBI taxid: 283734) | Comment |
|--------------------|---------------------------------|-----|------------------------------|------------------------------|------------------------------------------|---------|
| *Enterococcus faecium* | Q3XYE8 (234) | 33  | No significant similarity found | No significant similarity found | No significant similarity found | When WP_181892146.1 was subjected to BLASTp search in NCBI BLASTp, it showed only one hit against its own sequence and the rest of the 99 hits were from *H. pylori* with 98–100% identity. |
| *Helicobacter pylori*     | A0A0M3KL20 (234) | 34  | No significant similarity found | No significant similarity found | 98 (53/54) (WP_181892146.1) in only one unfinished genome of *S. pseudintermedius* strain ST525 |
| *Neisseria gonorrhoeae*  | Q50940 (252) | 35, 36 | No significant similarity found | No significant similarity found | No significant similarity found |
| *Vibrio cholerae*        | Q9KMP6 (239) | 37  | No significant similarity found | No significant similarity found | 37 (18/49) (WP_181892146.1) in only one unfinished genome of *S. pseudintermedius* strain ST525 |
| Human CA1              | P00915 (261) | 38, 39 | 38 (27/72) (MBO8666615.1) in only one unfinished genome of *S. aureus* strain IHMA56 and another hit of 37 (22/60) (MBO8619751.1) in only one unfinished genome of *S. aureus* strain IHMA68 | No significant similarity found | See Table 2 for comment on this protein |
| Human CA2              | P00918 (260) | 38, 40 | 50 (27/54) (MBO8619751.1) in one unfinished genome of *S. aureus* strain IHMA68 and another hit 37 (24/75) (MBO8666615.1) in one unfinished genome of *S. aureus* strain IHMA56 | No significant similarity found | See Table 2 for comment on this protein |

*Identity refers to shared identical residues with each of the CA proteins (UniProt ID) from selected bacteria and the indicated *Staphylococcus* species using BLASTp.*
diversity (PubMLST) (15). According to PubMLST, the predicted taxa for C0673 are actually 83% Staphylococcus sciuri, now known as Mammaliococcus sciuri. Using the IMG/M database, pairwise average nucleotide identity (ANI) with two finished S. sciuri genomes revealed that C0673 has 97% nucleotide identity with S. sciuri SNUDS-18 and 96% nucleotide identity with S. sciuri FDAARGOS_285. C0673 is wrongly annotated as S. aureus in NCBI database, which gave us false-positive hits in our BLASTp because S. sciuri but not S. aureus has a CA as stated in Table 1 and our previous work (8).

All protein sequences annotated as S. aureus CAs in NCBI are not from S. aureus. Given the observation that not a single strain out of 4,849 S. aureus genomes has a reasonable protein identity with any of the sequences annotated as S. aureus CA, we proceeded to examine the authenticity of these sequences. All of the nine sequences listed in Table 2 originated from unfinished genomes, and most of them contain many contigs, indicating these genomes are of low quality (16). When these sequences were subjected to BLASTp search, they showed only one hit against their own sequences and the rest were from either other staphylococcal species or other microorganisms, or even human. This clearly suggests that these genome assemblies were contaminated or contain sequencing errors and therefore are not accurate and should be corrected.

Deletion of mpsAB in four different backgrounds of S. aureus causes severe growth defect in atmospheric conditions. In our previous study, we demonstrated that deletion of mpsAB in two different S. aureus backgrounds, SA113 and HG001 (both are methicillin-susceptible S. aureus), could not grow under normal atmospheric conditions, indicating that there is no functional CA (4, 17). Here, we deleted mpsAB in two more S. aureus strains, JE2 and MW2, which are methicillin resistant (MRSA). Like with SA113 and HG001, the MRSA deletion mutants could not grow under atmospheric air, indicating that MpsAB is the only DICS system (Fig. S1).

MpsAB-harboring strains are resistant to CA inhibitors. CA inhibitors, especially sulfonamides, are able to effectively inhibit most of the CAs and consequently hinder the bacterial growth (18, 19). With regard to this, we tested eight such inhibitors to provide further evidence that CA does not present in S. aureus. Acetazolamide (AZA),

TABLE 4 Protein sequence similarity search for selected γ-CAs in the genomes of S. aureus, S. carnosus, and S. pseudintermedius using BLASTp

| Species                  | UniProt ID/length (amino acids) | Identity (%) protein coverage (amino acids) | S. aureus (NCBI taxid: 1280) | S. carnosus (NCBI taxid: 1281) | S. pseudintermedius (NCBI taxid: 283734) |
|--------------------------|---------------------------------|---------------------------------------------|-----------------------------|---------------------------------|-------------------------------------------|
| Enterococcus faecium     | Q3XX77 (161)                    | 3 hits in S. aureus annotated as γ-CAs (NGG14433.1, NGB42162.1, and WP_094666538.1) and the rest of the hits are from S. aureus proteins annotated as phenylacetic acid degradation protein PaaY with low identity (39, 32/83) and/or sugar O-acetyltransferase | No significant similarity found | No significant similarity found |
| Escherichia coli         | P0A9W9 (184)                    | 3 hits in S. aureus annotated as γ-CAs (NGG14433.1, NGB42162.1, and WP_094666538.1) and another as phenylacetic acid degradation protein PaaY in S. aureus | No significant similarity found | No significant similarity found |
| Methanosarcina thermophila | P40881 (247)                    | 3 hits in S. aureus annotated as γ-CAs (NGG14433.1, NGB42162.1, and WP_094666538.1) | No significant similarity found | No significant similarity found |
| Halobacterium salinarum  | Q9HR64 (220)                    | 31 (51/163) (MVWS54107.1) in only one unfinished genome of S. aureus strain mecC 165 PE | No significant similarity found | No significant similarity found |

Identity refers to shared identical residues with each of the carbonic anhydrase (CA) protein (UniProt ID) from selected bacteria and the indicated Staphylococcus species using BLASTp.

See Table 5 for comments regarding these protein sequences.
TABLE 5  Homology of protein annotated as *S. aureus* CAs in all finished and permanent genomes sequences of *S. aureus*

| Accession no./protein length (amino acids) | Identity (%)\(^a\)/protein coverage (amino acids) | Comment |
|------------------------------------------|--------------------------------------------------|---------|
| Finished genomes in IMG/M (259 strains)   |                                                 |         |
| MBO8619751.1 (64)                         | No significant similarity found                  |         |
| MBO8666615.1 (77)                         | No significant similarity found                  |         |
| MVW54107.1 (151)                          | No significant similarity found                  |         |
| NGB42162.1 (184)                          | No significant similarity found                  |         |
| NGG14433.1 (97)                           | No significant similarity found                  |         |
| OWU61334.1 (172)                          | No identity in 253 strains, except in 5 strains which showed 27 (46/170) identity in proteins annotated as sulfate permease |         |
| SPZ78435.1 (61)                           | No significant similarity found                  |         |
| SPZ78436.1 (193)                          | No significant similarity found                  |         |
| WP_094666538.1 (149)                      | All strains show 33 (40/122) identity in proteins annotated as acetyltransferase (isoleucine patch superfamily), acetyltransferase-like (isoleucine patch superfamily), galactoside O-acetyltransferase, or hypothetical protein | Annotated as such because these sequences have the related COG, KOG, or Pfam motifs |
| Permanent draft genomes in IMG/M (4,590 strains) |                                                 |         |
| MBO8619751.1 (64)                         | No significant similarity found                  |         |
| MBO8666615.1 (77)                         | No significant similarity found                  |         |
| MVW54107.1 (151)                          | No identity in all 4,586 strains except 4 strains: DEU37 (30 [49/163] as CA, partial gene "no stop"), DEU28 (30 [49/163] as CA), DEU35 (33 [39/117] as CA, partial gene "no start"), DEU41 (33 [39/117] as CA, partial gene "no start") | All the 4 strains listed here were marked as "anomalous assembly: contaminated" by NCBI |
| NGB42162.1 (184)                          | No identity in 4,584 strains except DEU28, DEU35, DEU37, DEU41, and DEU42 (37 [57/156] as CA or acetyltransferase), DEU39 (36% [31/86] as transferase hexapeptide [six repeat-containing protein]), C0673 (39 [67/171] as CA or acetyltransferase encoded by gene V070_00826) | All the 6 strains listed here were marked as "anomalous assembly: contaminated" by NCBI. C0673 is wrongly annotated as *S. aureus* in NCBI database |
| NGG14433.1 (97)                           | No identity in all 4,583 strains except DEU28, DEU35, DEU37, DEU41, and DEU42 (35 [27/77] as CA or acetyltransferase), DEU39 (partial gene "no start"), C0673 (41 [38/92] as CA or acetyltransferase encoded by gene V070_00826) | All the 6 strains listed here were marked as "anomalous assembly: contaminated" by NCBI. C0673 is wrongly annotated as *S. aureus* in NCBI database |
| OWU61334.1 (172)                          | No identity in 4,514 strains except in 76 strains which showed 27 (46/170) identity in proteins annotated as sulfate permease |         |
| SPZ78435.1 (61)                           | No identity in all 4,589 strains except C0673 (66 [35/53] as CA encoded by gene V070_02709) | C0673 is wrongly annotated as *S. aureus* in NCBI database |
| SPZ78436.1 (193)                          | No identity in all 4,589 strains except C0673 (71 [95/133] as CA encoded by gene V070_02709 and another 25 [32/126] as CA encoded by V070_01492) | C0673 is wrongly annotated as *S. aureus* in NCBI database |
| WP_094666538.1 (149)                      | All 4,590 strains showed 33 (40/122) as acetyltransferase (isoleucine patch superfamily), galactoside O-transferase, or hypothetical protein. A few strains have unspecific hits, for example: NRS384 (35 [33/95] as hexapeptide repeat of succinyltransferase), OCM6607 (32 [23/66] as 2,3,4,5-tetrahydropyridine-2-6, dicarboxylate N-acetyltransferase), 65-1322 (33 [40/122] as transferase hexapeptide repeat-containing protein), ATCC BAA-39 (33 [40/122] as galactoside-6-phosphate isomerase LacA subunit), C0673 (50 [72/144] as CA or acetyltransferase encoded by a gene V070_00826, 35 [26/77] as maltose O-acetyltransferase in gene V070_00366, and 25 [26/103] as acetyltransferase [isoleucine patch superfamily] encoded by gene V070_00906). | These strains have the same similarity as all the finished genomes. As there only a few strains out of 4,590 permanent draft sequences with unspecific and low identity, the origins of each of these strains were not examined. C0673 is wrongly annotated as *S. aureus* in NCBI database. |

\(^a\)COG, Clusters of Orthologous Genes; KOG, Eukaryotic Orthologous Groups; IMG/M, Integrated Microbial Genomes and Microbiomes; Pfam, protein families.

\(^b\)Identity refers to shared identical residues with each of the CA proteins (NCBI accession number) against the indicated *S. aureus* genomes in IMG/M database using BLASTp.
ethoxyzolamide (EZA), dorzolamide (DOR), and methazolamide (MEZ) are FDA-approved CA inhibitors used in the treatment of glaucoma, while celecoxib (CEL), chlorthalidone (CL), and famotidine (FAM) are a nonsteroidal anti-inflammatory agent, a thiazide diuretic, and an antulcer drug, respectively (20). S0859 is an N-cyanosulphonamide synthetic compound reported to be a selective inhibitor of sodium-bicarbonate cotransporters (NBC, SLC4) in mammalian heart (23). The chemical structures are provided in Figure S2.

At the highest concentration tested (1,000 μM), all the compounds did not inhibit MpsAB-harboring S. aureus and S. epidermidis as well as strains where CAs were deleted and complemented with MpsAB instead, including S. carnosus carrying plasmid containing mpsABC (Table 6). In CA-harboring strains, only EZA showed an MIC of 64 μM against S. carnosus, which was increased to 250 μM when CA was overexpressed in S. carnosus TM300 (pRB473 can) (Table 6). To verify that activity of EZA is mediated through the inhibition of CA, we repeated the MIC determinations in both normal atmospheric and 5% CO₂ conditions. EZA was inactive against these strains when incubated in the presence of 5% CO₂ compared to atmospheric air, while there was no difference in S. aureus (Fig. 1; Table S3). Vancomycin and oxacillin were used as a control and, as expected, displayed no difference in MIC in both conditions. Collectively, these results suggest that the target for EZA is most likely the intracellular CA, which is not present in S. aureus and S. epidermidis.

**DISCUSSION**

The discrepancies in the current literature regarding the presence of CA in S. aureus are substantial to warrant a comprehensive study to correct them, especially given that S. aureus is a clinically important pathogen. The first publication was in 1990 when Nafi et al. used a protein-binding monospecif antibody prepared against purified *Neisseria sicca* CA by immunoblotting method and also determined CA activity in cell extracts of various bacteria to screen for the presence of CA (18). Although CA activity was not detected in S. aureus, there was a positive reaction in the immunoblot, suggesting a reaction with a CA-like protein. In 1999, Smith et al. reported a molecular mass of 23 kDa in immunoblot with antisera raised against β-CA from *Methanobacterium thermoautotrophicum* ΔH and also some CA activity in S. aureus cells extract (24). Detection of target proteins by immunoreactivity alone is highly questionable in S. aureus because of its two IgG-binding proteins.

In 2015, Capasso and Supuran reported that the genome of S. aureus encodes only for γ CA, but no other information or citation was given to support this statement (9). In the following year, the same authors stated that S. aureus has a γ-CA, referring to protein EVX10196.1, which was used to build a CA phylogenetic tree (10). In NCBI database, EVX10196.1 is annotated as 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase from S. aureus M20916, which is an unfinished genome with 67 contigs. This 239-amino-acid protein is listed as nonessential by AureoWiki and is annotated as dapD.
which is part of an operon consisting of six genes involved in the biosynthesis of lysine (25). In *S. aureus*, lysine is an important amino acid, as it is needed not only as a building block for proteins but also as a component of the cell wall peptidoglycan. Therefore, EVX10196.1 is not a CA. Last year, the same group which reported earlier that *S. aureus* encodes only γ-CA now presented the production, kinetics, and inhibitory characterization of β-CA from the *S. aureus* (11). The CA gene was obtained from UniProt ID EZX15767 and was synthesized to produce a recombinant protein in *Escherichia coli*. A search in UniProt revealed that this protein is encoded by a gene V070_02709 from the *S. aureus* strain C0673. From our results above, C0673 is in fact *S. sciuri* and not *S. aureus*, and hence the CA activity described was actually from *S. sciuri*. A very recent publication from the same group followed up on the study by reporting its inhibition profile of *S. aureus* CA with anions and other small molecules (12). The same recombinant protein described earlier was used in this study, meaning that the CA inhibition referred to *S. sciuri* and not *S. aureus*.

Our bioinformatics analyses have clearly shown that *S. aureus* CAs are wrongly annotated as such, while in fact they are not present in *S. aureus* (Table 1 to 5). The absence of a CA in *S. aureus* is also supported by the deletion of *mpsABC* in four different *S. aureus* backgrounds (4, 17) (Fig. S1) and the fact that *S. aureus* is resistant to CA inhibitor EZA whereas *S. carnosus*, which has been shown to possess only CA, was inhibited by EZA (Fig. 1; Table 6; Table S6). The MIC values also imply that EZA is specific only for CA but not bicarbonate transporters (Table 6), which further complicates the treatment of pathogens such as *S. aureus* and *S. epidermidis*. The MIC values for CA-possessing *S. carnosus* and *S. pseudintermedius* in our study (64 to 250 μM) were comparable to those of *Helicobacter*

FIG 1 Disk diffusion results showing inhibition zones of CA inhibitor ethoxyzolamide (EZA) against selected staphylococcal strains. The Mueller-Hinton agar plates were inoculated with *S. aureus* HG001, *S. carnosus* TM300, and *S. carnosus* TM300 (pRB473-can), in which the CA was overexpressed, and *S. pseudintermedius* ED99. Paper disks impregnated with 10 μl of EZA, oxacillin (OXA), and vancomycin (VAN) as positive controls at concentrations of 1 mM each and appropriate concentration of DMSO as negative control were incubated at 37°C overnight in atmospheric and CO₂ conditions.
pylori (also harboring CA genes), which were in the range of 200 to 300 μM for EZA. For AZA, our MIC values were >1 mM, consistent with those reported for H. pylori at 2 to 8 mM (26). In another study with vancomycin-resistant Enterococcus faecium (VRE), which harbors CA genes, the MIC values were 0.5 μM for AZA and 1 μM for EZA, while S. aureus USA300 (MRSA) showed an MIC of >16 μM for both compounds, which was the upper limit tested (20). We also tested a selective human sodium-bicarbonate cotransporter inhibitor (S0859) (Fig. S2) and found that this inhibitor has no effect on bacterial MpsAB type bicarbonate transporter in S. aureus and S. epidermidis (Table 6), suggesting distinct differences in human and bacterial bicarbonate transporters. Although these MIC data (Table 6) are preliminary and require further research, this could imply that MpsAB can be a novel and promising target for such inhibitors in the treatment of infections. Furthermore, this can also be extended to other clinically relevant pathogens, such as Bacillus anthracis, Bacillus subtilis, Legionella pneumophila, Vibrio cholerae, and Burkholderia multivorans. Based on Pfam motifs, these bacteria possess both MpsAB homologs and CAs (4), thus making them resistant to CA inhibitors.

MATERIALS AND METHODS

Bioinformatic analyses. For the screening of CA based on Pfam motifs, the 259 S. aureus finished genomes in the IMG/M database (accessed 8 June 2021) were searched for the presence of Pfam00484, Pfam00194, and Pfam10563 for prokaryotic-type CAs, eukaryotic-type CAs, and putative CA-like domain, respectively (Table 1). Next, the protein sequence of S. carnosus CA, which is a β-CA (NCBI accession number WP_015900702.1), was subjected to BLASTp (27) search in representative strains of genus Staphylococcus for the protein similarities and the presence of β-CAs (Table 1). To look for the presence of α- and γ-CAs, the sequences from some experimentally confirmed CAs as listed in Tables 3 and 4 were subjected to BLASTp search in S. aureus, S. carnosus, and S. pseudintermedius using NCBI database (https://www.ncbi.nlm.nih.gov) and http://dbis.uni-regensburg.de/frontdoor.php?titel_id=481; accessed 12 June 2021). In order to confirm that there is no CA present, the protein sequences annotated as S. aureus CA in NCBI were subjected to BLASTp search in all 259 finished and 4,590 unfinished genomes of S. aureus found in IMG/M (Table 5). Finally, these proteins annotated as S. aureus CA in NCBI and their origins were examined in NCBI Assembly database to provide the details about the strains’ numbers, assembly levels, and numbers of contigs (Table 2). Each of these protein sequences was also subjected to BLASTp search to check if it has any similarities with S. aureus protein.

Bacteria strains and growth conditions. All the strains used in this study are listed in Table S1. For cloning procedures, the E. coli and S. aureus strains were grown in basic medium (BM) at 37°C with shaking at 150 rpm, unless otherwise specified. The BM consists of 1% soy peptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, and 0.1% K2HPO4 adjusted to pH 7.2. Bacterial cultures were cultivated in 10 ml medium using baffled 100 ml flasks. When necessary, the culture medium was supplemented with the following antibiotics at the indicated concentrations: chloramphenicol at 10 μg/ml and amphotericin B at 100 μg/ml for staphylococcal strains and 100 μg/ml ampicillin for E. coli strains.

Construction of staphylococcal deletion mutants and their complementation. The oligonucleotides used in this study are listed in Table S2. The nucleotide sequences were obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG). The deletion mutant of ΔmpsABC in S. aureus JE2 (KEGG accession numbers SAUSA300_0425, SAUSA300_0426, and SAUSA300_0426) and MW2 (KEGG accession numbers MW0407, MW0408, and MW0409) were constructed as markerless deletions using allelic replacements as described in reference 28. Up- and downstream flanking regions were approximately 2 kb each for both deletions. The recombinant plasmid from our previous study (17) was used for transformation into S. aureus JE2, and the subsequent deletion steps were the same as those for S. aureus MW2. For the construction of S. aureus MW2 ΔmpsABC, the up- and downstream regions of mpsABC were amplified from the chromosomal DNA of S. aureus MW2. The amplified fragments were assembled using linearized plasmid pBAS56 (Smal restriction site) (29) via Gibson assembly (30) using Hi-Fi DNA assembly master mix (New England Biolabs). The resulting plasmid was transformed into chemically competent E. coli DC10B (31). The clones harboring the right genes were then transformed into S. aureus MW2 via electroporation. Deletion of mpsABC in both of the strains was confirmed by PCR and sequence analysis.

Complementation of ΔmpsABC in both the strains was performed with the plasmid pBR473 carrying mpsABC along with its putative native promoter from our previous study (17). The plasmid was transformed into competent S. aureus ΔmpsABC JE2 and MW2, respectively, via electroporation and confirmed with PCR.

For growth visualization on agar, the wild type, ΔmpsABC, and its complemented mutants of S. aureus JE2 and MW2 were streaked on BM agar with inoculum adjusted to an optical density at 578 nm (OD578) of 0.5. The plates were incubated overnight at 37°C in atmospheric air and 5% CO2 conditions.

MIC determination. The CA inhibitors acetazolamide (AZA), ethoxyzolamide (EZA), dorzolamide (DOR), methazolamide (MEZ), celecoxib (CEL), chlorthalidone (CL), and famotidine (FAM) and selective sodium-bicarbonate cotransporters inhibitor S0859 (Table 6; Fig. S2) were purchased from Sigma-Aldrich (Germany). All the CA inhibitors were dissolved in dimethyl sulfoxide (DMSO) as stock solutions except for FAM, which was dissolved in methanol. The MIC values were determined by microdilution method according to the guidelines of Clinical and Laboratory Standards Institute (32). The CA inhibitors were
serially diluted (from the highest concentration of 1 mM to the lowest concentration of 2 μM) with 50 μl of cationic adjusted Muller Hinton broth (MHB) in 96-well microtiter plates. Equal volumes of bacterial inoculum (1 × 10^7) were added and the plates were incubated at 37°C with continuous shaking for 24 h in atmospheric air (Table 6) and, if necessary, in 5% CO₂, conditions (Table S6). The MIC was determined as the lowest concentration that completely inhibited visible growth of the bacteria and also confirmed with a TECAN Reader (Infinite M200). Antibiotics vancomycin and oxacillin were used as standard antibi-otic controls, while positive controls referred to the bacterial cells treated with DMSO or methanol at a concentration equivalent to the highest concentration used to dissolve the CA inhibitors. MHB alone was used as negative control. The MIC determinations were performed in three independent biological replicates with three technical replicates each.

For visual representation of the semiquantitative results on agar, four strains that were inhibited by EZA were used (Fig. 1). MHB agar plates were swabbed with bacterial inoculum adjusted to an OD₆₀₀ of 0.1. Disks made of filter paper were impregnated with 10 μl of 1 mM EZA, vancomycin, and oxacillin (positive controls) and DMSO at appropriate concentration (negative control) before being placed on the agar. The agar plates were incubated overnight at 37°C in atmospheric air and 5% CO₂, conditions.

**Data availability.** The main data supporting the findings of this work are available within the article and in the Supplemental Material or from the corresponding author upon reasonable request.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**

PDF file, 0.4 MB.

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F.G. and S.H.F. conceived the idea and designed the study. S.H.F. performed the bioinformatic analysis and cloning experiments. E.L. carried out the MIC determinations. F.G. and S.H.F. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

We declare no conflicts of interests.

**REFERENCES**

1. Casey JR. 2006. Why bicarbonate? Biochem Cell Biol 84:930–939. https://doi.org/10.1139/o06-184.

2. Berg IA. 2011. Ecological aspects of the distribution of different autotrophic CO₂ fixation pathways. Appl Environ Microbiol 77:1925–1936. https://doi.org/10.1128/AEM.02473-10.

3. Smith KS, Ferry JG. 2000. Prokaryotic carbonic anhydrases. FEMS Microbiol Rev 24:335–366. https://doi.org/10.1111/1574-6976.2000.tb00546.x.

4. Fan S-H, Ebben P, Reichert S, Hertleit T, Zabel S, Lankapalli AK, Nieselt K, Ohslen K, Götz F. 2019. MpsAB is important for Staphylococcus aureus virulence and growth at atmospheric CO₂ levels. Nat Commun 10:3627. https://doi.org/10.1038/s41467-019-11547-5.

5. Badger M. 2003. The roles of carbonic anhydrases in photosynthetic CO₂ concentrating mechanisms. Photosynth Res 77:83–94. https://doi.org/10.1023/A:1025821717777.

6. Mangiapia M, Uf M, Brown TW, Chaput D, Haller E, Hamner TL, Hashemy Z, Klee C, Leonard J, Manca P, Nicholson D, Stevens S, Fan C, Scott KM, MSF MCB404L. 2017. Proteome and mutant analysis of the CO₂ concentrating mechanism of hydrothermal vent chemolithoautotrophic Thiomicrospira crunogena. J Bacteriol 199. https://doi.org/10.1128/JB.00871-16.

7. Desmarais JJ, Flamholz AI, Blikstad C, Dugan EJ, Laughlin TG, Oltrogge LM, Chen AW, Wtmore K, Diamond S, Wang YJ, Savage DF. 2019. DABs are inorganic carbon pumps found throughout prokaryotic phyla. Nat Microbiol 4:2204–2215. https://doi.org/10.1038/s41564-019-0520-8.

8. Fan S-H, Matsuo M, Huang L, Tribelli PM, Friedrich G. 2021. The MpsAB bi-carbonate transporter is superior to carbonic anhydrase in biofilm-forming bacteria with limited CO₂ diffusion. Microbiol Spectr 9:e00305-20. https://doi.org/10.1128/Spectrum.00305-21.

9. Capasso C, Supuran CT. 2015. An overview of the alpha-, beta- and gamma-carbonic anhydrases from bacteria: can bacterial carbonic anhydrase shed new light on evolution of bacteria? J Enzyme Inhib Med Chem 30:325–332. https://doi.org/10.3109/14756366.2014.910202.

10. Supuran CT, Capasso C. 2016. New light on bacterial carbonic anhydrases phylogeny based on the analysis of peptide sequences. J Enzyme Inhib Med Chem 31:1254–1260. https://doi.org/10.1080/14756366.2016.1201479.

11. Urbanski LJ, Bua S, Angeli A, Kuslahtii M, Hytolen VP, Supuran CT, Parkkila S. 2020. Sulphonamide inhibition profile of Staphylococcus aureus beta-carbonic anhydrase. J Enzyme Inhib Med Chem 35:1834–1839. https://doi.org/10.1080/14756366.2020.1826942.

12. Urbanski LJ, Vullo D, Parkkila S, Supuran CT. 2021. An anion and small molecule inhibition study of the beta-carbonic anhydrase from Staphylococcus aureus. J Enzyme Inhib Med Chem 36:1088–1092. https://doi.org/10.1080/14756366.2021.1931863.

13. Chen IA, Chu K, Palaniappan K, Ratner A, Huang J, Huntermann M, Hajek P, Ritter S, Varghese N, Seshadri R, Roux S, Woyke T, Elo-Radfod E, Ivanova NN, Kyripdes NC. 2021. The IMG/M data management and analysis system v.6.0: new tools and advanced capabilities. Nucleic Acids Res 49:D751–D762. https://doi.org/10.1093/nar/gkaa939.

14. Fuchs S, Mehlhan H, Bernhardt J, Henning A, Michalik S, Surmann K, Panne-Farre J, Giese A, Weiss S, Backert L, Herbig A, Nieselt K, Hecker M, Volker U, Mader U. 2018. AureoWiki—the repository of the Staphylococcus aureus research and annotation community. Int J Med Microbiol 308:558–568. https://doi.org/10.1016/j.ijmm.2017.11.011.

15. Jolley KA, Bray JE, Maiden MCJ. 2018. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res 3:124. https://doi.org/10.12688/wellcomeopenres.14826.1.

16. Smits THM. 2019. The importance of genome sequence quality to microbial comparative genomics. BMC Genomics 20:662. https://doi.org/10.1186/s12864-019-6014-5.

17. Mayer S, Steffen W, Steuber J, Götz F. 2015. The Staphylococcus aureus Nuo-like protein MpsA contributes to the generation of membrane potential. J Bacteriol 197:794–806. https://doi.org/10.1128/JB.02127-14.
18. Naft BM, Miles RJ, Butler LO, Carter ND, Kelly C, Jeffery S. 1990. Expression of carbonic anhydrase in neisseriae and other heterotrophic bacteria. J Med Microbiol 32:1–7. https://doi.org/10.1099/00222615-32-1-1.

19. Abutaleb NS, Elkasif A, Flaherty DP, Seleem MN. 2021. In vivo antibacterial activity of acetazolamide. Antimicrob Agents Chemother 65. https://doi.org/10.1128/AAC.01715-20.

20. Younis W, Abdel-Khalak E, Mayhoub AS, Seleem MN. 2017. In vitro screening of an FDA-approved library against ESRAPE pathogens. Curr Pharm Des 23: 2147–2157. https://doi.org/10.2174/1381622823666170209154745.

21. Swenson ER. 2014. Safety of carbonic anhydrase inhibitors. Expert Opin

22. Howard JM, Chremos AN, Collein MJ, Mcarthur KE, Chernier JA, Maton PN, Howarth CM, Capasso C. 2012. DNA cloning, characterization, and inhibition studies of an alpha-carbonic anhydrase from the pathogenic bacterium Neisseria gonorrhoeae. J Med Chem 55:10742–10748. https://doi.org/10.1021/jm301611m.

23. Ch’en FF, Villafuerte FC, Swietach P, Cobden PM, Vaughan-Jones RD. 2008. 50859, an N-cyano-sulphonamide inhibitor of sodium-bicarbonate cotransport in the heart. Br J Pharmacol 153:972–982. https://doi.org/10.1038/sj.bjp.0707667.

24. Smith KS, Jakubzick C, Whittam TS, Ferry JG. 1999. Carbonic anhydrase is an ancient enzyme widespread in prokaryotes. Proc Natl Acad Sci U S A 96:15184–15189. https://doi.org/10.1073/pnas.96.26.15184.

25. Wiltshire MD, Foster SJ. 2001. Identi

26. Modak JK, Tikhomirova A, Gorrell RJ, Rahman MM, Kotsanas D, Korman TM, Matten WT, McGinnis SD, Merezhuk Y, Raytselis Y, Sayers EW, Tao T, Ye J, Zaretskaya I. 2013. BLAST: a more efficient report with usability improvements. Nucleic Acids Res 41:W29–W33. https://doi.org/10.1093/nar/gkt282.

27. Boratyn GM, Camacho C, Cooper PS, Coulouris G, Fong A, Ma N, Madden TL, Matten WT, McGinnis SD, Merezhuk Y, Raytselis Y, Sayers EW, Tao T, Ye J, Zaretskaya I. 2013. BLAST: a more efficient report with usability improvements. Nucleic Acids Res 41:W29–W33. https://doi.org/10.1093/nar/gkt282.

28. Bae T, Schneewind O. 2006. Allelic replacement in Staphylococcus aureus aureus and Staphylococcus epidermidis. mBio 3. https://doi.org/10.1128/mbio.00277-11.

29. Keilin D, Mann T. 1940. Carbonic anhydrase. Puri

30. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilo-

31. Monk IR, Shah IM, Xu M, Tan MW, Foster TJ. 2012. Transforming the untransformable: application of direct transformation to manipulate genetically

32. Cockerill FR, Clinical and Laboratory Standards Institute. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically approved standard, 9th ed. CLSI, Wayne, PA.

33. Kaur J, Cao X, Abutaleb NS, Elkasif A, Graboski AL, Krabill AD, Abdel-Khalak AH, An W, Bhardwaj A, Seleem MN, Flaherty DP. 2020. Optimization of acetazolamide-based scaffold as potent inhibitors of vancomycin-resistant Enterococcus. J Med Chem 63:9540–9562. https://doi.org/10.1021/acs.jmedchem.0c00734.

34. Del Prete S, Iksik S, Vullo D, De Luca V, Caregnale V, Scozafava A, Supuran CT, Capasso C. 2012. DNA cloning, characterization, and inhibition studies of an alpha-carbonic anhydrase from the pathogenic bacterium Vibrio cholerae. J Med Chem 55:10742–10748. https://doi.org/10.1021/jm301611m.

35. Lowe N, Edwards YH, Edwards M, Butterworth PH. 1991. Physical mapping of the human carbonic anhydrase gene cluster on chromosome 8. Genomics 10:882–888. https://doi.org/10.1016/0888-7543(91)90176-f.

36. Muralahi H, Marelch GR, Grubb JH, Kyle JW, Sly WS. 1987. Cloning, expression, and sequence homologies of cDNA for human carbonic anhydrase II. Genomics 1:159–166. https://doi.org/10.1016/0888-7543(87)90008-5.

37. Abutaleb NS, Elkashif A, Flaherty DP, Seleem MN, Butterworth PH. 2012. Structure and sequence homologies of cDNA for human carbonic anhydrase II. Genomics 1:159–166. https://doi.org/10.1016/0888-7543(87)90008-5.

38. Cockerill FR, Clinical and Laboratory Standards Institute. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically approved standard, 9th ed. CLSI, Wayne, PA.

39. Chirica LC, Elleby B, Lindskog S. 2001. Characterization of carbonic anhydrase from Neisseria gonorrhoeae. Eur J Biochem 244: 755–760. https://doi.org/10.1046/j.1432-1327.2001.02031.x.

40. Cockerill FR, Clinical and Laboratory Standards Institute. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically approved standard, 9th ed. CLSI, Wayne, PA.

41. Cockerill FR, Clinical and Laboratory Standards Institute. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically approved standard, 9th ed. CLSI, Wayne, PA.

42. Cockerill FR, Clinical and Laboratory Standards Institute. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically approved standard, 9th ed. CLSI, Wayne, PA.

43. Cockerill FR, Clinical and Laboratory Standards Institute. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically approved standard, 9th ed. CLSI, Wayne, PA.

44. Cockerill FR, Clinical and Laboratory Standards Institute. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically approved standard, 9th ed. CLSI, Wayne, PA.

45. Cockerill FR, Clinical and Laboratory Standards Institute. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically approved standard, 9th ed. CLSI, Wayne, PA.